Universidade de Lisboa

Faculdade de Medicina de Lisboa

TOWARDS THE THERAPEUTIC USE OF REGULATORY T CELLS FOR THE TREATMENT OF HUMAN AUTOIMMUNE DISEASES

Maria Catarina Mota da Silva

Tese orientada pela Doutora Íris Maria Ferreira Caramalho

e co-orientada pelo Professor Doutor Rui Manuel Martins Victorino

Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, especialidade de Imunologia Clínica

2015

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Para o Guilherme, luz da minha vida.

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CHAPTER 1. INTRODUCTION

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ABREVIATIONS

ADAM: metalloproteinase domain-containing protein family metalloproteinases

ADP: adenosine diphosphate

Ag: antigen

AID: autoimmune diseases

ANCA: anti-neutrophil cytoplasmic antibodies

APC: antigen-presenting cells

ATP: adenosine triphosphate

cAMP: cyclic adenosine monophosphate

CD4ISP: CD4 Immature Single Positive cells

cDC: classical dendritic cells

CDP: common dendritic cell progenitors

CLP: common myeloid progenitors

CNS1: conserved non-coding sequence 1

CNS2: conserved non-coding sequence 2

CMP: common myeloid progenitors

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAPT: N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester

DC: dendritic cells

DL: delta-like

DN: double negative

Dnmt: DNA methyltransferases

DP: double positive

EAE: experimental autoimmune encephalomyelitis

ESAM: endothelial cell-selective adhesion molecule

ETP: early thymic progenitors

Foxp3/FOXP3: forkhead box P3

GATA3: GATA binding protein 3

GFP: green fluorescent protein

GITR: glucocorticoid-induced TNFR-related protein

GVHD: graft-versus-host disease

γc: Common-gamma chain

HCV: hepatitis C virus

HIF-1: hypoxia-inducible factor 1

HIV: human immunodeficiency virus

HLA: human leucocyte antigen

HSC: hematopoietic stem cell

HSCT: hematopoietic stem cell transplantation

ICAM-1: intercellular adhesion molecule-1

ICOS: inducible T-cell costimulator

ICOSL: inducible T-cell costimulator ligand

IDO: indoleamine 2,3-dioxygenase

IgA: immunoglobulin A

IgG: immunoglobulin G

IgE: immunoglobulin E

ILC: innate lymphoid cell

IL: interleukin

IL-2R: interleukin 2 receptor

IL-2Rα: interleukin 2 receptor alpha chain

IL-2Rβ: interleukin 2 receptor beta chain

IL-6R: interleukin 6 receptor

IL-7R: interleukin 7 receptor

IL-12Rβ2: interleukin 12 receptor beta 2 subunit

ILC: innate lymphoid cells

IFN-γ: interferon gamma

IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked

IRF: interferon regulatory factor

iTreg: *in vitro* induced regulatory T cells

JAG: jagged

JAK3: janus kinase 3

LAG3: lymphocyte-activation gene 3

LFA-1: lymphocyte function-associated antigen 1

LES: lúpus eritematoso sistémico

mAb: monoclonal antibody

MAML: mastermind-like

mDC: myeloid dendritic cells

MDP: macrophage-DC progenitors

MHC: major histocompatibility complex

miRNA: microRNA

MPP: multipotent progenitors

MS: multiple sclerosis

mTEC: medullary thymic epithelial cells

MZB: marginal zone B

mTOR: mammalian target of rapamycin

NF-κB: nuclear factor-κB

NICD: notch intracellular domain

NIH: national institute of health

NK: natural killer

NOD: non-obese diabetic mice

Nrp1: neuropilin 1

OVA: ovalbumin

pDC: plasmocytoid dendritic cells

PD-1: programmed cell death protein 1

PSGL-1: P-selectin glycoprotein ligand-1

PTEN: phosphatase and tensin homolog

PTPN: protein tyrosine phosphatase, non-receptor type

pTreg: peripheral regulatory T cells

RAG: recombination activating gene

RBPJ: recombination signal binding protein for immunoglobulin κJ region

rh: recombinant human

RORγt: RAR-related orphan receptor gamma

SLE: systemic lupus erythematosus

SOCS: suppressor of cytokine signaling protein

SP: single positive

STAT: signal transducer and activator of transcription

T-bet: T-box transcription factor

TCR: T cell receptor

Teff: effector T cells

TEC: thymic epithelial cell

TET: ten eleven translocation

Tfh: follicular helper T cells

TGF-β: transforming growth factor β

Th: T helper

TLR: toll-like receptor

TN: triple negative

TNF- α: tumor necrosis factor alpha

TOC: thymic organ cultures

Treg: regulatory T cells

T Regs: células T reguladoras

tTreg: thymus-derived regulatory T cells

Tr1: type 1 regulatory T cells

Tr3: type 3 regulatory T cells

TSDR: Treg-specific demethylated region

T1D: type 1 diabetes

UCB: umbilical cord blood

US: United States

VEGF: vascular endothelial growth factor

WT: wild type

YFP: yellow fluorescent protein

SUMMARY

Regulatory T Cells (Treg), constitutively expressing the transcription factor Foxp3/FOXP3, play a crucial role in maintaining self-tolerance, assuming particular relevance in the context of autoimmunity. Adoptive transfer of Treg has been shown to be highly efficient in the prevention and treatment of autoimmunity in rodents and clinical trials exploring Treg-based adoptive therapy in Type I Diabetes (T1D) are currently ongoing. These therapies require large numbers of Treg, stressing the importance of a better knowledge of the molecular and cellular requirements for human thymic and peripheral Treg development. Moreover, widespread application of Treg-based therapy dealt with several limitations regarding the stability and function of *in vitro* expanded populations for *in vivo* use. The creation of efficient protocols enabling stable FOXP3 acquisition by human non-regulatory cells could overcome the limited availability of thymus-derived (t)Treg and would facilitate the generation of antigen-specific Treg, an ideal candidate in autoimmune diseases (AID) setting.

The overall objective of this work was to provide new insights into the principles dictating human thymic and peripheral Treg development and homeostasis, thus facilitating the progress of Treg-based immunotherapy.

First, we proposed to investigate the capacity of human non-regulatory memory $CD4^+$ T cells to differentiate in *vitro* into *bona-fide* FOXP3-expressing cells and to assess the role of the Notch signaling pathway in modulating this conversion. We showed that stable and functional bona-fide Treg can be generated from memory $CD4^+$ T cells and that Delta like (DL)1-mediated Notch signaling activation enhanced this conversion. We additionally showed that DL1 increased Treg proliferation, reinforcing the possible role of Notch in the homeostasis of the human peripheral Treg compartment. Importantly, we also demonstrated that DL1 enhanced the expression of function-related molecules within these cells, contributing to the maintenance of their regulatory phenotype.

In order to better clarify the principles governing Treg development in the human thymus, we investigated the role of common gamma-chain (γc) cytokines in human tTreg differentiation. We identified interleukin (IL)-2 and IL-15 as key molecular determinants in this process and excluded a major function for IL-4, IL-7 and IL-21. Moreover, we

revealed that IL-2 and IL-15 are expressed in a non-overlapping pattern in the human thymus, with the former produced mainly by mature αβ and γδ thymocytes and the latter by monocyte/macrophages and B lymphocytes.

Overall, this work has provided a better understading of the core mechanisms governing human Treg differentiation and homeostasis that should facilitate the further establishment of Treg-based therapies.

Key Words: Regulatory T Cells; Self-tolerance; Autoimmune diseases; Common gamma-chain cytokines; Regulatory T Cell-based immunotherapy

SUMÁRIO

As Células T Reguladoras (T Regs), expressando constitutivamente o factor de transcrição Foxp3/ FOXP3, desempenham um papel crucial na manutenção da auto-tolerância, assumindo particular relevância no contexto de auto-imunidade. A transferência adoptiva de T Regs demonstrou ser altamente eficaz na prevenção e no tratamento de auto-imunidade em roedores e ensaios clínicos explorando terapêuticas baseadas em T Regs na Diabetes Tipo I estão actualmente em curso. Estas terapias exigem um grande número de células, pelo que é essencial um melhor conhecimento dos requisitos moleculares e celulares para o desenvolvimento no timo humano e na periferia de T Regs. Além disso, a aplicação generalizada desta terapêutica tem ainda várias limitações no que respeita à estabilidade e função das populações expandidas *in vitro* para utilização *in vivo*. A criação de protocolos eficientes que permitam a aquisição estável de FOXP3 por células não-reguladoras poderá ultrapassar a disponibilidade limitada de T Regs de origem tímica e facilitar a geração de T Regs com especificidade antigénica, potencialmente ideais no contexto de doenças auto-imunes.

O objetivo global deste trabalho foi investigar os princípios que ditam o desenvolvimento tímico e periférico e a homeostasia das T Regs humanas, facilitando assim o progresso da imunoterapia utilizando T Regs.

Em primeiro lugar, propusemo-nos investigar a capacidade das células T $CD4^+$ de memória não-reguladoras para se diferenciarem *in vitro* em células que expressam FOXP3, avaliando o papel da via de sinalização Notch na modulação desta conversão. Revelámos que T Regs estáveis e funcionais podem ser geradas a partir de células T $CD4^+$ de memória isoladas do sangue periférico de indivíduos saudáveis e que a activação de Notch mediada por DL1 aumenta esta conversão. Adicionalmente, demonstrámos que DL1 aumenta a proliferação de T Regs circulantes, reforçando o possível papel de Notch na homeostasia do compartimento periférico de T Regs. Mostrámos também que DL1 aumenta a expressão de moléculas relacionadas com a função de T Regs circulantes, contribuindo para a manutenção do fenótipo das T Regs.

Com a finalidade de clarificar os princípios que regem o desenvolvimento de T Regs no timo humano, investigámos o papel das citocinas que utilizam um receptor com cadeia

gama comum na diferenciação de T Regs humanas. Identificámos as interleucinas IL-2 e IL-15 como determinantes moleculares chave neste processo, tendo sido excluída uma função de relevo para IL-4, IL-7 e IL-21. Mais ainda, revelámos que IL-2 e IL-15 são expressas num padrão não sobreposto no timo humano, sendo a primeira produzida principalmente por timócitos maduros αβ e γδ e a última por monócitos/ macrófagos e linfócitos B.

Em conclusão, este trabalho proporcionou um melhor conhecimento dos mecanismos fundamentais que regem o desenvolvimento, a homeostasia e manipulação *in vitro* das T Regs em humanos, contribuindo para o estabelecimento das terapêuticas baseadas em T Regs.

Palavras-Chave: Células T Reguladoras; Auto-tolerância; Doenças Auto-Imunes; Citocinas que utilizam um receptor com cadeia gama comum; Imunoterapia utilizando células T reguladoras

SUMÁRIO EXTENSO

Um dos grandes desafios da Imunologia e da Medicina é, ainda actualmente, compreender os mecanismos subjacentes à manutenção da auto-tolerância imunológica. O controlo rigoroso da dimensão das respostas imunes adaptativas a antigénios próprios e não-próprios encerra um potencial terapêutico inquestionável em diferentes cenários clínicos, entre eles a patologia auto-imune.

As Células T Reguladoras (T Regs), expressando constitutivamente o factor de transcrição Foxp3/ FOXP3, desempenham um papel fundamental na manutenção da tolerância periférica. O compartimento periférico de T Regs compreende uma população de origem tímica e uma população convertida na periferia a partir de células T $CD4^+$ não-reguladoras. Em humanos, o estabelecimento da contribuição exacta das duas populações para o compartimento periférico de T Regs tem sido dificultado pela inexistência de um marcador fiável que as distinga com precisão *in vivo*. As T Regs assumem reconhecidamente particular relevância na prevenção da auto-imunidade tanto em modelos animais como em humanos. Têm sido descritas deficiências qualitativas e/ou quantitativas de T Regs em diferentes doenças imuno-mediadas, com um interesse crescente na manipulação desta população celular especialmente no contexto da auto-imunidade e da transplantação. Assim, vários ensaios clínicos com terapêutica adoptiva de T Regs estão actualmente em curso com resultados promissores. Dada a necessidade de um grande número de T Regs para atingir eficácia clínica, tem sido feito um grande esforço para desenvolver protocolos clínicos adequados que permitam a expansão eficaz *ex vivo* de T Regs humanas.

Os protocolos visando a expansão eficiente de T Regs para aplicação clínica têm enfrentado grandes desafios, em particular no que respeita a estabilidade e função das populações expandidas *in vitro*. Mais ainda, esta estratégia pode não ser ideal quando a população de T Reg apresenta defeitos intrínsecos, como previamente descrito no contexto de patologia auto-imune. A grande maioria dos estudos de indução tem utilizado células T $CD4^+$ não-reguladoras naive como população original. Ainda assim, alguns grupos induziram FOXP3 em células T $CD4^+$ não-reguladoras de memória *in vitro*, com resultados controversos no que respeita ao fenótipo e função da população obtida. No contexto particular da auto-imunidade, pensa-se que as células não-reguladoras de

memória possam ser enriquecidas em células T auto-reactivas, tornando esta população especialmente atractiva para indução de T Reg com especificidades antigénicas relevantes.

Como tal, o desenvolvimento e optimização de estratégias para gerar T Regs a partir de células T não-reguladoras são essenciais para o estabelecimento de imunoterapia adoptiva com T Regs. Concomitantemente, um conhecimento mais profundo e detalhado do desenvolvimento, fisiologia e homeostasia das T Regs humanas proporcionará instrumentos fundamentais para a manipulação de T Regs em contexto clínico.

Várias vias de sinalização têm sido implicadas na geração de células T reguladoras. Entre estas, Notch é apontado como tendo um papel crucial no desenvolvimento, expansão e diferenciação destas células.

O objetivo global deste trabalho foi investigar os princípios que ditam o desenvolvimento tímico e periférico assim como a homeostasia das T Regs humanas, facilitando assim o establecimento da terapêutica adoptiva celular com T Regs, em particular no contexto de auto-imunidade.

Como tal, propusemo-nos investigar a capacidade das células T $CD4^+$ não-reguladoras de memória de se diferenciarem *in vitro* em células que expressam FOXP3, avaliando o papel da via Notch na modulação desta conversão. Revelámos que T Regs estáveis e funcionais podem ser geradas a partir de células T $CD4^+$ de memória e que a activação de Notch mediada por DL1 aumenta esta conversão. Os efeitos benéficos de DL1 foram também demonstrados na conversão de células T $CD4^+$ "naïve" convencionais.

Mostrámos ainda que T Regs convertidas a partir da células T $CD4^+$ não reguladoras, na presença de DL1, apresentam um fenótipo estável em culturas de longo prazo e na presença de citocinas pró-inflamatórias, o que é particularmente relevante em cenários reconhecidamente inflamatórios, como no contexto de patologia auto-imune.

Notch desempenha, como tal, um importante papel na conversão *in vitro* de células T $CD4^+$ não reguladoras em T Regs, efeito que se estende provavelmente a cenários *in vivo* onde a geração de Treg a partir de precursores não-reguladores é fundamental para um melhor controlo da resposta inflamatória.

Avaliámos também o impacto da DL1 sobre a proliferação homeostática e fenótipo de T

Regs circulantes estimuladas via receptor de antigénio de células T. Os nossos resultados mostram que a activação de Notch via DL1 aumenta a proliferação de T Reg humanas assim como a expressão de moléculas relacionadas com função reguladora, reforçando o possível papel de Notch na homeostasia e conservação do fenótipo das T Reg periféricas.

Investigámos adicionalmente os mecanismos responsáveis pelo efeito de DL1 na diferenciação de T Reg a partir de células $CD4^+$ não-reguladoras de memória. Os nossos dados sugerem que o impacto de DL1 na aquisição de FOXP3 por células T não reguladoras de memória envolve vários mecanismos, tais como a interacção cooperativa com via a sinalização TGF-β e a modulação transcripcional de Foxp3.

O Lúpus Eritematoso Sistémico (LES) é uma doença auto-imune multissistémica com um amplo espectro de manifestações clínicas e significativo impacto na qualidade de vida dos doentes. A imunossupressão farmacológica neste contexto acarreta actualmente grande morbilidade, sendo crucial o desenvolvimento de terapias dirigidas, com potencial intervenção na patogénese da doença, maior eficácia clínica e menores efeitos adversos. Demonstrámos que a indução de T Regs a partir de células T não-reguladoras de memória de um doente com LES foi eficaz e potenciada na presença de DL1, validando num contexto auto-imune a eficácia *in vitro* dos protocolos previamente estabelecidos.

O processo pelo qual as T Regs se desenvolvem no timo humano está ainda mal esclarecido, sendo importante compreendê-lo detalhadamente para manipular esta população celular de forma adequada em contextos clínicos. Com o objectivo de clarificar os princípios que regulam o desenvolvimento de T Regs no timo humano, investigámos o papel das citocinas que utilizam o receptor de cadeia gama comum na diferenciação de T Regs humanas. Revelámos uma importante contribuição das interleucinas IL-2 e IL-15 para o desenvolvimento tímico das T Regs humanas, clarificando aspectos fundamentais do seu mecanismo de acção, padrão de expressão e populações envolvidas na sua produção. Revelámos que IL-2 e IL-15 (mas não IL-4, IL-7 e IL-21) aumentam a frequência e número de T Regs em culturas de timo humano, assim como os seu níveis de expressão de FOXP3 e de moléculas associadas a função reguladora. IL-2 e IL-15 exibiram, adicionalmente, um evidente impacto sobre a sobrevivência e proliferação de T Regs já diferenciadas. IL-2 e IL-15 demonstaram também a capacidade de consignar precursores de T Regs de origem tímica à linhagem reguladora.

Mostrámos ainda que IL-2 e IL-15 são expressas no timo humano em nichos não sobreponíveis, sendo a IL-2 produzida por timócitos maduros αβ e γδ e estando a produção de IL-15 essencialmente confinada a macrófagos e células B.

Assim sendo, os nossos dados suportam um modelo de duas etapas para o desenvolvimento no timo humano de T Regs. Neste modelo hipotético, sinais via receptor de antigénio de células T nos timócitos em desenvolvimento podem ser suficientes para induzir a expressão de CD25, permitindo que estas células respondam a IL-2 e/ ou IL-15, com a consequente activação de STAT5 e transcrição subsequente de FOXP3.

Em conclusão, este trabalho proporcionou um conhecimento aprofundado do desenvolvimento, fisiologia e homeostasia *in vivo* e *in vitro* das T Regs humanas, contribuindo para o progresso da terapêutica adoptiva celular com T Regs.

CHAPTER 1. INTRODUCTION

1.1. Regulatory T Cells

1.1.1. Regulatory T cells

Over the last two decades, the different mechanisms by which mammalian organisms acquire tolerance to self have been scrutinized[1]. Self-tolerance is acquired through two types of mechanisms, "recessive" and "dominant". The deletion of immature thymocytes before acquiring functional maturity in the thymic medulla and migration to the periphery, proposed by the studies of Burnet[2], Lederberg[3] and Medawar[4], is recognized as a crucial mechanism. Concomitantly, other processes have been described that permit ectopic expression of peripheral tissue antigens in thymic epithelial cells (TEC) as well as immigration of antigen-presenting cells (APC) from peripheral tissue to the thymus[5]. These so-called "recessive mechanisms of central tolerance" are, yet, imperfect and allow the escape of some self-reactive cells that probably have T cell receptors (TCR) of relatively low affinity for self. Further "recessive peripheral tolerance mechanisms" in secondary lymphoid organs, such as deletion and/or anergy, have been additionally described[6]. Nevertheless, it became clear that these passive mechanisms were insufficient to account for self-non-self-discrimination. Thereafter, "dominant peripheral tolerance" accounted by "suppressor/ regulatory" cells emerged as a fundamental piece in the establishment of immunological tolerance. In the mid 1990's, seminal papers by Sakaguchi et al., using a combination of cell depletion and adoptive cell transfers, demonstrated the crucial role of a minor population of $CD4^+$ T cells that coexpresses the interleukin-2 receptor alpha chain (IL-2Rα, CD25) for preventing multi-organ autoimmunity as well as lethal systemic inflammatory and wasting disease[7, 8]. These findings opened the challenging field of Regulatory T Cells (Treg).

Treg are a thymus-derived independent T cell lineage that plays a fundamental role in tightly controlling peripheral immune responses, promoting the maintenance of self-tolerance and immune homeostasis[9]. Expression of the transcription factor box P3 (Foxp3/FOXP3) in mice and humans, respectively, is the best available marker to identify Treg and is also indispensable for their development, stability and effector function[10, 11]. Loss-of-function mutations in this gene lead to Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans and to the Scurfy

phenotype in mice, characterized by early and fatal autoimmunity[11]. Notwithstanding the essential role of Foxp3/FOXP3 in Treg differentiation, maintenance and function, it has been shown that, unlike mice, TCR-driven activation of human $CD4^+$ T cells leads to FOXP3 expression, that is not necessarily associated with a regulatory phenotype[12, 13, 14].

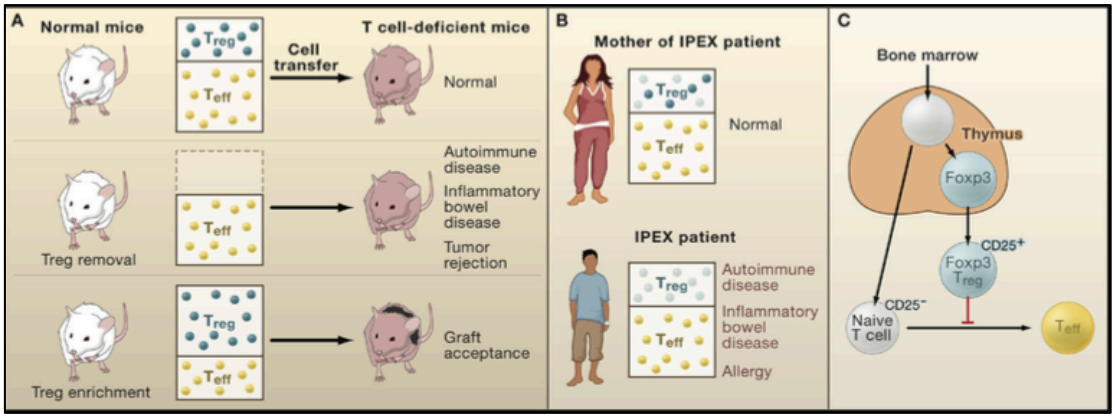

Figure 1. Consequence of Treg deficiency in mice and humans. Adapted from Sakaguchi. *Cell*. 2008.[15]

The peripheral Treg compartment consists both of thymus-derived (t)Treg and a peripheral population converted from $CD4^+CD25^-Foxp3^-$ precursors (peripheral (p)Treg)[16]. pTreg differentiation occurs particularly in the gut mucosa and inflammatory tissue sites, likely via a transforming growth factor β (TGF-β)-dependent mechanism[16]. Helios, a member of the Ikaros transcription factor family, has been proposed as a specific mouse and human tTreg marker[17]. However, recent findings have shown that Helios expression can be induced during T cell activation and proliferation and hence neuropilin 1 (Nrp-1) expression was suggested as a more adequate marker to distinguish tTreg from peripherally-generated pTreg in mice[19]. The lack of a reliable marker in humans to accurately distinguish these subsets *in vivo* hampers the establishment of the exact contribution of tTreg and pTreg subsets to the peripheral Treg pool.

Additional regulatory T cell populations have been identified, including interleukin (IL)-10-secreting type 1 regulatory T cells (Tr1), TGF-β-secreting type 3 regulatory T cells (Tr3) and $CD8^+CD28^{neg}$ regulatory T cells, all of them lacking the Foxp3/FOXP3 expression. However, some constraints regarding the phenotypic characterization of these subsets have made their contribution to self-tolerance and immune homeostasis

maintenance less well defined[20, 21].

1.1.2. Thymic development of Regulatory T Cells

Human T cell development involves different intrathymic events that progress through a series of sequential stages defined by the surface expression of CD4, CD8 and CD3 (Figure 2). T cell progenitors contained within the early $CD3^{neg}CD4^{neg}CD8^{neg}$ Triple Negative (TN) subset initially acquire CD4 (becoming CD4 Immature Single Positive cells, CD4ISP) and subsequently CD8 expression, giving rise to Double Positive (DP) thymocytes in the cortex. A progressive increase in surface CD3 expression occurs in parallel with surface TCRαβ in DP cells, followed by final differentiation into CD4 Single-Positive (SP) and CD8SP thymocytes that mature in the medulla[22].

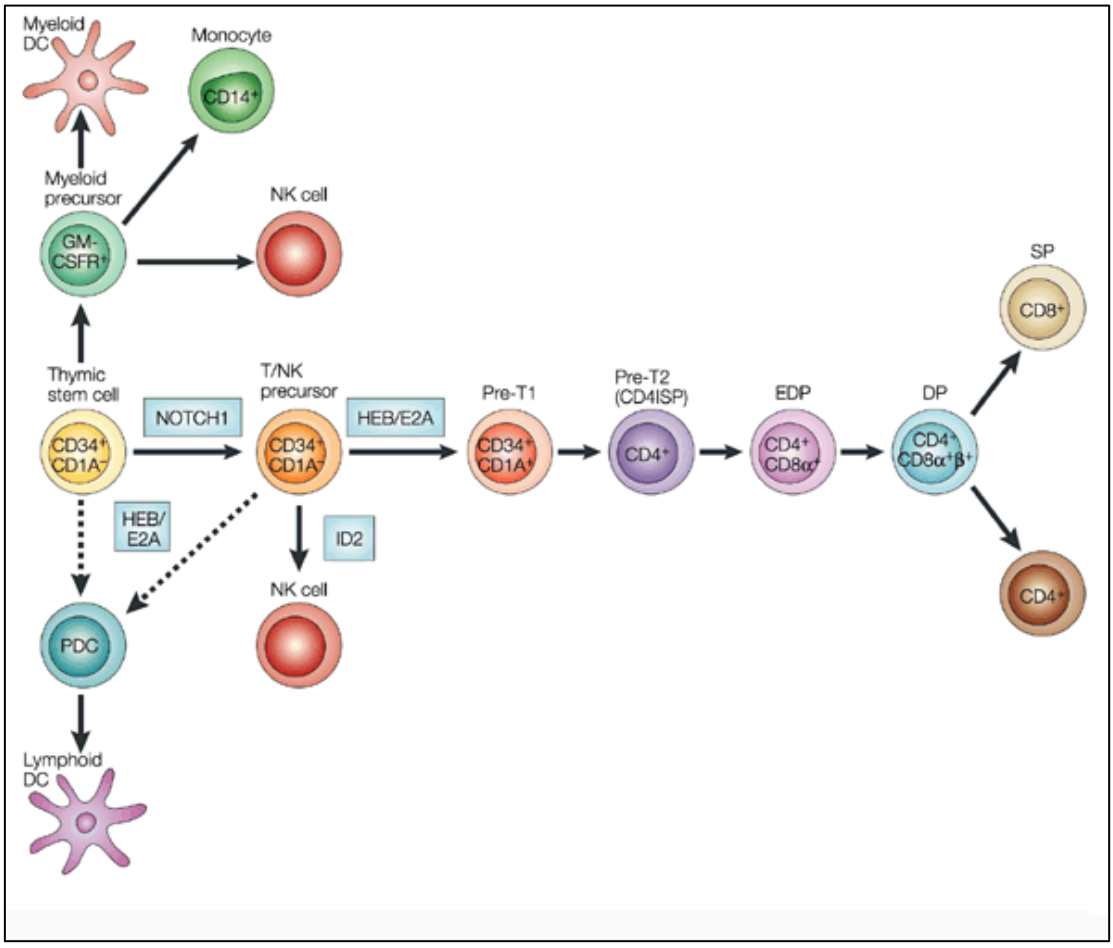

Figure 2. Schematic representation of the development of αβ T cells in the human thymus. Adapted from Spits H. *Nat Rev Immunol*. 2002.[23]

In secondary lymphoid organs, naive $CD4^+$ and $CD8^+$ T cells encounter antigens presented by professional APC, with subsequent activation and differentiation into effector lymphocytes and some into Treg. It has been shown that a significant proportion of Treg differentiate and acquire functional capacity already in the thymus.

In the last two decades, thymic Treg development has been widely scrutinized in murine models. Still, little is known about the developmental program ruling tTreg differentiation in the human thymus. Regarding this gap, the knowledge provided by genetic human disorders characterized by thymus-related disturbances has been of utmost importance. DiGeorge syndrome, caused by the deletion of a small piece of chromosome 22, presents in most cases with thymic hypoplasia, mild to moderate T-cell lymphopenia and increased infection and autoimmunity[24], along with significant alterations in Treg compartment[25, 26, 27]. Omenn Syndrome, associated with hypomorphic missense mutations in the recombination activating genes (RAG) 1 and 2, is characterized by a limited pool of T lymphocytes with a restricted repertoire and activated phenotype[28]. Autoimmune regulator gene (AIRE) controls the promiscuous expression of tisse-specific antigens in medullary thymic epithelial cells (mTEC), displaying a crucial role as a regulator of central tolerance[29]. In 2 patients with Omenn Syndrome, loss of cortico-medullary junction and Hassall's bodies with depletion of AIRE-expressing mTEC and thymic dendritic cells (DC) has been described, concomitantly with a dramatic decrease of thymic Treg[30], stressing the putative role of AIRE-expressing mTEC and/or thymic DC in their differentiation. Accordingly, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), resulting from loss-of-function mutations in the AIRE gene, has been associated with a defective circulating Treg compartment[31, 32, 33].

At the time that mature T cells are observed in the human thymus, around the 12^{th} to 13^{th} gestational weeks, human tTreg can already be found intrathymically[34, 35]. Notwithstanding, in humans expression of FOXP3 is clearly detected in post-selection DP thymocytes[36, 37, 38], which has been shown to significantly contribute to the mature $FOXP3^+$ cell pool that is largely composed of CD4SP and some CD8SP thymocytes[38]. $FOXP3^+$DP thymocytes also express other Treg function-associated markers, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), CD39 and glucocorticoid-induced TNFR-related protein (GITR), and display supressive function[39, 38]. It has been also demonstrated that human tTreg can be selected by both myeloid (mDC) and plasmocytoid

dendritic cells (pDC) [40, 41].

Previous data from murine studies have shown that tTreg generation is TCR instructive, depending on the recognition of self-antigens in the thymus[36, 37, 42, 40, 38]. In humans, technical constraints foreclose the direct assessment of TCR role in tTreg development. Notwithstanding, it has been shown that human tTreg differentiation is associated with markers of positive selection, such as CD69 and CD27[36, 37, 38, 43]. Additionally, binding sites for the TCR downstream targets nuclear factor of activated T-cells (NFAT) and activator protein (AP1), directly activated by TCR stimulation, are present within the human *FOXP3* gene promoter[44]. Importantly, human zeta-chain-associated protein kinase 70 (ZAP70)-deficient patients display a substantial decrease in the frequency and number of tTreg[45]. Interestingly, CD4SP $CD25^+$ tTreg were shown to oftentimes express two functional TCR, in association with enhanced FOXP3 expression, suggesting that dual TCR expression may underly tTreg lineage commitment in humans[46]. Therefore, the existing data support that TCR signaling strength drives thymocyte commitment into the Treg lineage in humans.

Several studies have shown other relevant factors and signaling pathways for tTreg differentiation and/or proliferation in the human thymus, including Jakus kinase (JAK3)/ signal transducer and activator of transcription (STAT)5, Notch, inducible T-cell costimulator (ICOS)/ inducible T-cell costimulator ligand (ICOSL), CD28:B7 costimulation CD40:CD154 and thymic stromal lymphopoietin (TSLP)[41, 40, 47, 48, 49].

In mice, signaling through the common γ-chain (γc) receptor triggered by IL-2, IL-7 and IL-15, has been claimed as participating in tTreg generation[50]. Accordingly, by following the $CD25^+Foxp3^-$ tTreg precursor population early in ontogeny, and denoting the critical roles IL-2 and TCR signaling strenght had in tTreg differentiation, Lio and Hsieh proposed a two-step model of tTreg differentiation[51]. The model suggests that functional high avidity TCR signals lead to the upregulation of CD25 with consequent increase in the responsiveness of tTreg precursor cells to IL-2 signals that facilitate the induction of Foxp3 (Figure 3)[51]. A likely candidate transcription factor for direct regulation of Foxp3 expression is, in this scenario, STAT5 since it is activated downstream of IL-2 and other γc cytokine receptors[51]. Even though, how IL-2 signaling in these immediate tTreg precursors instructs Foxp3 induction and tTreg differentiation is not clear[37].

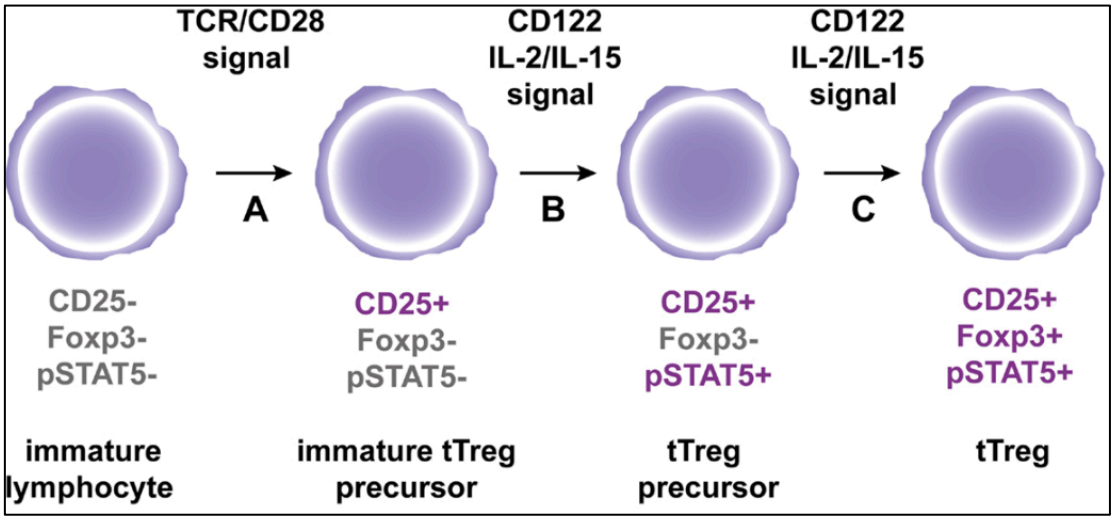

Figure 3: Two-step model for tTreg differentiation. Schematic representation of a hypothetical model for tTreg differentiation in the murine thymus. Adapted from Goldstein et al. *Front Immunol*. 2013.[52]

In humans, it is well known that Treg homeostasis and function in the periphery depends on IL-2[53]. Human tTreg in fact express CD25 as well as the interleukin-2 receptor beta chain (IL-2Rβ, CD122) that are utilised by both IL-2 and IL-15[36, 38]. Previous data also support a role for IL-2 and IL-7 in human tTreg development[38]. Accordingly, it has been shown that human FOXP3^{+} thymocytes, although featuring reduced levels of the α-chain of the IL-7 receptor (IL-7R) compared to their FOXP3^{neg} counterparts, phosphorylate STAT5 in response to IL-7[38]. Moreover, IL-2 increases CD25 and FOXP3 expression levels within FOXP3^{+}DP thymocytes[38]. Importantly, polymorphisms in IL-2, CD25 or IL-2R downstream signaling molecules are associated with impaired Treg number and/or function as well as increased risk of autoimmunity in humans[53], which may be due to defective tTreg generation in addition to reduced peripheral Treg survival. Accordingly, indirect evidence supports a thymic involvement in patients undergoing IL-2 therapy, based on the observed expansion of Treg expressing CD45RA and the recent thymic emigrant marker CD31[54, 55, 56].

Despite the suggested role for these interveners, particularly γc cytokines, in tTreg generation, the principles governing human tTreg ontogeny and the precise contibution of these and aditional factors remains to be elucidated.

Among additional signals beyond TCR-stimulus suggested to be enrolled in tTreg specification, the contribution of TGF-β remained for long debatable, with studies in mice showing that it is both vital[57] or redundant[58]. A recent reevaluation of this issue has demonstrated that intrathymically produced TGF-β derived from thymocyte apoptosis is crucial for murine tTreg development[59], establishing an important apoptosis-TGF-β-Foxp3 axis in the development of these cells.

1.1.3. Peripheral Induction of Regulatory T Cells

It was initially thought that Treg could only arise from the thymus through cognate interaction with major histocompatibility complex (MHC)/ self-peptide complexes. However, it has become clear that Foxp3$^+$ Treg can also develop in the periphery from mature conventional CD4$^+$ T cells under particular conditions, pTreg[60, 61, 62].

Historically, data from murine studies have suggested that extrathymic generation of Treg is favored in two particular contexts. The first scenario is characterized by T cell activation in the absence of inflammation (the so-called "subimmunogenic context"), exemplified by the use of non-depleting anti-CD4 antibodies, which weaken coreceptor engagement[63]; "clean" antigen (Ag) delivery by osmotic pumps[64]; Ag presentation by APC in the absence of maturation signals[61]; and Ag presentation in tolerogenic microenvironments, such as the small intestine mucosa[60]. One illustrative experiment of this last category was carried out with chicken ovalbumin (OVA) administration to OVA-specific TCR-transgenic mice crossed with RAG- deficient and Foxp3-deficient mice[65]. In these mice, oral administration of Ag does not induce Foxp3$^+$ pTreg as mice are Foxp3 deficient. However, T cells in these mice did not become effector T cells (Teff), even though they upregulated CD69 which is indicative of Ag exposure[62]. Thus, under these noninflammatory conditions, T cell stimulation can generate pTreg but not Teff. In a second context, inflammatory conditions allow the quasi-simultaneous development of both pTreg and Teff in inflammatory sites, with evidence of a lower rate of pTreg generation[66]. Also in humans, it is now increasingly clear that pTreg can arise in several different conditions, constituting an important part of peripheral Treg compartment[67, 68].

The lack of reliable markers and the constraints of *ex vivo* studies result in scarce data in human pTreg, with most of the knowledge resulting from murine studies. The requirements for the induction of pTreg are quite different from those required for thymic generation of tTreg. As above-mentioned, it has been postulated that murine tTreg development in the thymus is associated with high avidity TCR/ MHC-peptide interactions, while pTreg differentiation in the periphery is induced under subimmunogenic conditions[61, 64, 69]. It has also been proposed that a low dose of high affinity agonist peptides supports pTreg induction while low affinity peptide agonists poorly generates pTreg[70]. The clear knowledge of the signaling pathways that promote the development of tTreg in the thymus and that elicit conversion into pTreg in the periphery has not yet been completely clarified.

Besides TCR engagement and IL-2 signaling, indispensable for the generation of all Treg, pTreg seem to require additional factors such as TGF-β and retinoic acid[71, 72]. Accordingly, blockade of TGF-β *in vivo* inhibits the differentiation of Ag-specific pTreg[60]. In mice lacking Smad3 (a TGF-β pathway downstream target) binding sites in the conserved non-coding sequence 1 (CNS1) of the Foxp3 enhancer region, there is a lack of pTreg development[73]. Upon transfer of congenically marked Wild Type (WT) or CNS1$^{-/-}$ CD4^{+} Foxp3 T cells into *RAG1*$^{-/-}$ recipient mice, the induction of Foxp3 was observed only in WT and not in the CNS1$^{-/-}$ cells. *In vitro* assays also demonstrated a significant reduction in the induction of Foxp3 in naïve T cells deficient in CNS1[73] suggesting a dominant role for TGF-β signaling in extrathymic pTreg generation.

In what concerns costimulation, it has also been claimed that tTreg and pTreg have different requirements. CTLA-4 has been shown to be upregulated within TGF-β *in vitro* induced (i)Treg, whereas its role in tTreg generation is still controversial[74, 75]. On the other hand, the CD28-mediated co-stimulation role in tTreg generation is well documented in murine studies. CD28-deficient mice indeed show markedly lower number of Foxp3^{+} in thymus and the periphery[76] and it has been suggested that CD28 promotes tTreg generation through alteration of avidity of T cell-APC interaction, stimulation of IL-2 production or directly through T cell signaling and survival[76].

The role of APC in pTreg generation has also been scrutinized. In mice, APC such as lung resident macrophages have the ability to induce pTreg[77]. DC are also known to be highly

tolerogenic in certain circumstances and their depletion can lead to decreased Foxp3$^+$ Treg numbers and increased Teff responses, suggesting a major role for DC-mediated Ag presentation in maintaining/ converting Treg in the periphery[78, 19]. In the thymus, recent studies in mice have established that CD70 expression on mTEC and on DC enhances the positive selection of tTreg and promotes tTreg survival, suggesting an important role for CD27-CD70 in thymic Treg development promoted by DC and mTEC[79]. In sum, the conjuction of soluble factors in the microenvironment, namely TGF-β and IL-2, and antigen presentation by specialized APC seem to play play a critical role in pTreg generation. Illustrating the relevance of these interactions, for instance in the gut mucosa pTreg are generated with precise antigen specificities, resulting in a specialized pTreg subset able to control local inflammatory responses[80].

A large effort has been made towards the characterization of the Treg peripheral pool using recent identified markers. The comprehensive gene-expression analysis performed by Feuerer et al. to characterize murine Foxp3$^+$ Treg generated under different conditions *in vivo* showed a remarkable heterogeneity between different populations, highlightening the true adaptive nature of pTreg[81]. Helios, an ikaros family transcription factor, was recently described as a specific marker for tTreg. According to Thornton et al., Helios is highly expressed on tTreg, responsible for approximately 70% of the peripheral Treg compartment[17], and the authors suggested that Helios could be used to distinguish between tTreg and pTreg. Others have shown, however, that Helios is also upregulated in iTreg and pTreg as a result of T cell activation and proliferation[18]. Recently, Nrp1, a receptor for semaphorins and vascular endothelial growth factor (VEGF), has been suggested as a genuine marker for tTreg in mice[69, 82]. Functionally, the semaphorin-Nrp1 axis seems to play a role in maintaining Treg stability after TCR activation, by restraining Akt signaling via recruitment of phosphatase and tensin homolog (PTEN)[83]. Disappointingly, in contrast to murine Treg and regardless of their origin (blood, thymus, spleen, lymph node or tonsil), human Treg do not seem to specifically express Nrp-1[84].

pTreg may have different functions from tTreg although some of them may be overlapping, and the features that differentiate tTreg from pTreg are not universally accepted. It has been previously postulated that tTreg are crucial in preventing autoimmunity and exaggerated immune responses whereas pTreg play a limited role in

these contexts. However, studies aimed directly at analyzing pTreg function *in vivo* have been few, owing to the lack of appropriate animal models. The functional analysis of pTreg has mostly been limited to mucosal tolerance, inflammatory responses to foreign antigens and animal models that may not reflect appropriate physiological conditions. In fact, most functional studies have utilized TGF-β-induced iTreg and have demonstated them to be protective[85, 86]. TGF-β-induced Ag-specific iTreg are highly efficient in controlling the onset of autoimmunity in murine model of autoimmune gastritis, through inhibition of DC functions and modulation of T cell trafficking[87, 88]. Interestingly, Haribhai et al. recently showed that murine tTreg were unable to suppress chronic inflammation and autoimmunity in the absence of pTreg[89]. In this model, tTreg alone were not sufficient to maintain tolerance when transferred into Foxp3-deficient mice. However, when conventional T cells were co-injected with tTreg, peripherally generated pTreg represented 15% of Treg pool and acted in concert with tTreg to restore tolerance. Moreover, one of the most prominent functions of pTreg has been reported in the maintenance of fetal tolerance during pregnancy in murine studies. During pregnancy, pTreg are generated against a paternal alloantigen in a CNS1 dependent manner and enforce maternal-fetal tolerance. CNS1-deficient females exhibit increased embryo resorption accompanied by increased immune cell infiltration during allogeneic but not syngeneic pregnancy, which are features observed in human preeclampsia[90]. A similar phenomenon has been observed in human pregnancy, where Helios$^+$Foxp3$^+$ Treg are increased in the peripheral blood of healthy pregnant women when compared to non-pregnant controls or preeclamptic patients[91]. These results argue that pTreg serve as the predominant subset in suppressing the fetal-specific immune response and defects in pTreg may be central to the pathogenesis of preeclampsia[92, 93]. Recently, murine data have shown that Nrp1 deficiency impairs Treg stability under certain inflammatory conditions, but does not lead to spontaneous development of autoimmune disease[94]. Interestingly, it has been shown that Nrp1 is expressed by approximately 50% of Treg in the colon and 65% in the small intestinal lamina propria, suggesting a similar contribution of pTreg and tTreg to the intestinal Treg pool[69]. These findings support a more recent, conciliatory and interesting paradigm: pTreg are possibly generated to complement tTreg. Both populations seem to cooperate due to a mutual complementation of TCR repertoires, and contributions by both subsets are necessary to protect from disease development and maintain tolerance[94].

The different conditions in which pTreg can be generated additionally complicate the

characterization of pTreg as a sole entity as well as the distinction between tTreg and pTreg, since heterogeneity of Treg populations may not only reflect the origin (thymus *versus* periphery) but also the context of the milieu[94]. Recently it has been shown that Treg can in fact undergo stimulus-specific differentiation regulated by transcription factors typically associated with the conventional $CD4^+$ T cells differentiation. Initially interpreted as the result of lineage instability, these Treg seem to display unique migratory and functional properties, expressing transcription factors involved in regulation of the corresponding type of effector immune responses[95]. The first evidence of these specialized Treg came from findings showing that the expression of interferon regulatory factor (IRF) 4, required for the differentiation of T helper (Th)2 and Th17 cells, is necessary for the control of Th2-driven autoimmunity[96]. Subsequently, different studies showed that (T-box transcription factor) T-bet and STAT3 expression in Treg control their migration and suppressive functions during Th1 and Th17 immune responses, respectively[97, 98]. This suggests the interesting possibility that the pTreg pool includes different subsets with unique properties, better equipped to deal with different effector immune responses.

Finally, tissue-resident Treg, the so-called "tissular Treg", are thought to display a unique phenotype that is reminiscent of the tissue microenvironment[99]. In the course of inflammation, Treg significantly increase in the relevant tissue, locally up to 50% of $CD4^+$ T cells count. Tissular Tregs control inflammation locally and exhibit unique functions, such as limiting inflammation in the intestine and controlling insulin sensitivity in the fat[100, 101].

At the end, a broader and accurate understanding of cellular heterogeneity and mechanisms of lineage stability and plasticity in human Treg may provide new insights into the role of Treg imbalance in disease pathogenesis and improve the development of optimized Treg-based therapies.

1.1.4. Molecular basis of Regulatory T Cells development and function

There is substantial evidence that Foxp3, considered a master regulator of Treg function, unequivocally plays a critical role in the development and function of Treg[10, 11]. Notwithstanding, not all of the $Foxp3^+$ T cells are functional suppressive Treg and Treg

signature molecules can be expressed, at least to a certain extent, in the absence of Foxp3. This suggests that Foxp3 expression *per se* is not the sole responsible for delineating stable functional Treg[102]. In agreement, proteomic analysis in Treg revealed that Foxp3 forms complexes with a number of co-factors, with a cooperative functional interaction between them[103]. Furthermore, combination of Foxp3 with several other transcription factors induces a common Treg-type gene expression pattern, which is not achieved solely by Foxp3 expression[102]. Recent data additionally suggests that the generation of functional and stable Treg requires complementary molecular events along with Foxp3 and its transcriptional partners expression.

Epigenetic modifications, which include histone modifications, DNA methylation, microRNA (miRNA), nucleosome positioning, chromatin interaction and chromosome conformational changes, have been shown to play an important role in cell differentiation, particularly in cell-lineage stabilization[104]. Particularly relevant, DNA methylation and histone modifications, heritable through cell divisions, importantly contribute to cell-lineage determination and maintenance. Regarding the process of methylation, genomic DNA is methylated by DNA methyltransferases (Dnmt) family members and it can be demethylated in different steps, such as methylcytosine hydroxylation mediated by ten eleven translocation (TET) family members [105]. Histones modifications enabling gene activation or repression include acetylation or deacetylation, methylation or demethylation, and phosphorylation or dephosphorylation[106]. Although modifiable in the early stages of development, epigenetic features, particularly the DNA methylation status, become stably maintained throughout subsequent differentiation processes. Accordingly, particular specific loci epigenetic changes are also stably sustained in specific cell lineages, such as Treg[107].

Recently scrutinized in murine studies, Treg-specific epigenetic changes seem to cooperate with Foxp3 expression in lineage specification and functional stability of Treg. Work from several groups has established that epigenetic changes do take place in the course of Treg differentiation. For instance, DNA hypomethylation at the *Foxp3* conserved non-coding sequence 2 (CNS2) has been suggested to be required for stable expression of Foxp3[108]. DNA demethylation also occurs within the genes accepted as composing the "Treg signature," namely *Foxp3*, *Ctal4*, *Ikzf2* (Helios), *Ikzf4* (Eos), and *Tnfrsf18* (GITR)[108]. Epigenetic components of Treg seem to participate in the definition of Treg-type gene

expression pattern, either independently of Foxp3 or cooperatively with Foxp3.

Based on murine studies, it has been suggested that epigenetic changes specific of Treg development are not induced in response to TCR or TGF-β stimulation[102]. Accordingly, *in vitro* generated iTreg show the lack of Treg-specific DNA hypomethylation, which has been associated with the lack of a significant part of Treg-type gene expression and stability of Treg signature molecule expression[102]. Nevertheless, in humans it has been shown that iTreg with a partially methylated Treg-specific demethylated region (TSDR) displayed stability and suppressive function consistent with a Treg phenotype[109]. The role of epigenetic imprinting in stabilizing the Treg phenotype is, therefore, controversial. Accordingly, in a mouse model of autoimmunity, experimental autoimmune encephalomyelitis (EAE), it has been recently shown that, although the majority of polyclonal and Ag-specific Treg stably expressed Foxp3 during the autoimmune response, a substantial fraction of antigen-specific Treg with "signature" features ($Foxp3^{hi}$, $CD25^{hi}$, demethylated TSDR) downregulated Foxp3 transcription, lost Foxp3 expression and acquired effector T cell characteristics, such as interferon gamma (IFN-γ) production and pathogenic potential *in vivo* [110]. These recent findings suggest that, although Treg-specific epigenetic changes contribute to Treg stability, probably they are not the sole determinants and even absolutely required for a stable Treg phenotype. Possibly, additional extrinsic signals are also important for controlling Foxp3 expression and maintaining stable and functional Treg, an issue requiring further clarification.

Moving on from epigenetic intrinsic modifications to molecular extrinsic inputs, previous findings indicate that metabolic-signal-dependent transcriptional regulation would be important for lineage choices. For instance, hypoxia-inducible factor 1 (HIF-1), a key metabolic sensor, has been shown to participate in the balance between Treg and Th17 cell differentiation in mice and humans[111],[112]. Similarly, murine data have shown that complement fragments also affect the balance between Th17 and Treg[113]. In fact, signaling through the G-protein-coupled receptors for the complement fragments C3a and C5a in DC and $CD4^+$ T cells enhances Th17 cell induction[113]. Conversely, when signals from C3aR and C5aR are inhibited in $CD4^+$ T cells, signal reduction in the PI3K-Akt-mTOR pathway and an increase in autocrine TGF-β signaling enhance $Foxp3^+$ iTreg generation[113]. Additionally, Foxo transcription factors, which integrate extrinsic signals to regulate cell division, differentiation, and survival, have a pivotal role in the development of both

thymic and induced Treg[114]. Hence, extracellular stimulation seems to play an additionaly important role in the stability and plasticity of Foxp3$^+$ Treg.

Interestingly, in mice it has been demonstrated that T-bet$^+$ Treg, a Treg subpopulation with particular functions, is induced in a STAT1-dependent manner upon exposure to either IFN-γ or IL-27 and that each cytokine has distinct roles in driving T-bet$^+$ Treg at inflammation sites [115]. Some authors have investigated the molecular pathway leading to the upregulation of T-bet within Foxp3$^+$ Treg and the possible mechanisms by which T-bet$^+$ Treg function is maintained under Th1 cell-polarizing inflammatory environments in mice. It has been demonstrated that activation of STAT1 by IFN-γ derived from activated Th1 cells induced T-bet expression in Treg; notwithstanding, and despite similar induction of CXCR3 expression in both Foxp3$^+$ and Foxp3$^-$ T cells via T-bet activation, the levels of IL-12 receptor expression were different between the two populations[116]. The expression of interleukin 12 receptor beta subunit (IL-12Rβ2), which is induced in naive T cells upon STAT1 and T-bet activation, was refractory to T-bet expression in Foxp3$^+$ Treg in part because of altered epigenetic status of the IL12rb2 locus in Treg. This prevents T-bet$^+$ Treg from completing IL-12-STAT4-dependent Th1 cell differentiation and thereby ensuring the maintenance of stable Treg suppressive function[116]. Besides stressing the importance of cytokine-induced Treg differentiation in the periphery, these findings reinforce the importance of environmental cues on Treg differentiation through epigenetic modifications. Hence, changes in environmental stimuli and similar *in vitro* manipulation of non-regulatory T cells may alter the gene expression and histone modification and render highly differentiated Treg adaptive to the environment[102].

1.1.5. Homeostasis and Stability of Regulatory T Cells

Immune tolerance depends on the adequate homeostasis of immunosuppressive Treg. The stability of tTreg and pTreg has crucial importance for maintaining immune homeostasis in a dynamic environment and has been the subject of deep controversy[117, 118, 119]. Recent work has shown that Treg numbers and function are controlled by unique signals in different tissue environments, suggesting a homeostatic subdivision in Treg populations based on their localization[120].

A number of extrinsic factors have been identified as affecting Treg stability, some of them discussed in the previous chapter. The role of different cytokines in this phenotypic and functional balance has been widely discussed. The IL-2 axis is a key determinant for Treg stability, as stressed in several murine studies[121]. IL-2 expression is for instance altered in the Non-obese Diabetic (NOD) mouse model, which might contribute to Treg instability and autoimmune diabetes triggering[121]. IL-2 is known to be crucial for continued, stable expression of Foxp3 in murine Treg[122] and polymorphisms in loci containing IL-2 responsive genes are highly associated with Type 1 Diabetes (T1D) incidence, which may be a consequence of the Treg instability in the inflamed pancreas[123]. In humans, IL-2 has been also demonstrated to play a major role in Treg homeostasis. For instance, the presence of an IL2Rα haplotype associated with T1D correlates with diminished IL-2 responsiveness in Ag-stimulated $CD4^+$ T cells and is associated with lower levels of FOXP3 expression by Treg and a reduced suppressive capacity[124]. Recently, it has been also shown that reduced Treg numbers in patients with primary sclerosing cholangitis are associated with polymorphisms in the IL2Rα gene[125].

IL-6 is implicated in the abrogation of Treg-mediated Teff control *in vitro*[126], by destabilizing Foxp3 expression within these cells[127] with consequent reprogramming of $Foxp3^+$ Treg into IL-17-producing cells *in vivo*[128]. The opposite effect has been reported for the anti-inflammatory cytokine IL-10, which is important in maintaining high Foxp3 expression in Treg during colonic inflammation[129]. These are interesting observations since both IL-6 and IL-10 signaling depend on the activation of STAT3. In the absence of STAT3, Treg function is abrogated: Treg-specific STAT3 deficiency results in loss of immune homeostasis and the selective alteration of genes implicated in Treg suppressor function, such as IL-10, Ebi3 and TGF-β[97]. The resultant opposing effects are partially explained by the fact that IL-10 also induces suppressor of cytokine signaling protein (SOCS) 3 expression that suppresses the IL-6 signaling pathway, and is important for maintaining a regulatory phenotype. The relative level of expression of transcription factors, modulated by extracellular signals, will ultimately determine the maintenance of the Treg phenotype. Interestingly, germline STAT3 gain-of-function mutations in humans have been recently associated with secondary defects in STAT5 and STAT1 phosphorylation and Treg compartment, resulting in lymphoproliferation and early-onset multiorgan autoimmunity[130].

Other controversial issue regarding Treg homeostasis, as discussed above, is the "lineage" stability and maintenance of function. This issue of cell lineage stability is obviously critical for Treg because they readily undergo robust cell expansion upon activation in lymphopenic and lymphoreplete hosts, and mediate potent regulatory activity in lymphoid and nonlymphoid sites under a variety of inflammatory and metabolic changes. Human data is obviously scarce owing to *in vivo* studies methodological constraints. Murine studies have, nevertheless, provided some important insights into this controversial issue.

Whereas some studies have observed long-lasting Treg function, recent studies suggest that Treg adapt to microenvironmental changes and consequently manifest functional plasticity by reprogramming into inflammatory T cells[131, 132, 109]. The potential to visualize in murine models cells that had previously expressed Foxp3 but lost it, the so called "exFoxp3" cells, was described recently[133]. Zhou et al., using two yellow fluorescent protein (YFP) reporter mouse strains, either in mixed or NOD background, showed that YFP^{+}green fluorescent protein $(GFP)^{-}$exFoxp3 cells could be detected in the spleen and lymph nodes. More importantly, the number of Treg that down-regulate GFP in the YFP^{+} exFoxp3 population was significantly increased during inflammation[133]. It was in fact reported that 10-15% of $Foxp3^{+}$ cells lose Foxp3 protein expression and that a subset of these acquired the capacity to secrete IFNγ or IL-17. Furthermore, the YFP^{+} $Foxp3^{-}$ GFP^{-} exFoxp3 cells are enriched in the inflamed pancreas of NOD mice at the onset of autoimmune inflammation[133]. Therefore, some studies have challenged the concept of a stable and committed Treg lineage and have suggested that Treg lineage commitment is not irreversible[119]. Still, it remains unclear whether Treg reprogramming is imprinted during normal T cell development or is an adaptation mechanism of subsets of cells responding to changes in homeostatic or inflammatory conditions in the extracellular environment.

The notion that Treg can become unstable or be reprogrammed was however recently challenged in a study using a different Foxp3 lineage tracer mice[134]. In this system, GFP-Cre-mutated human estrogen receptor fusion protein was knocked-in to the endogenous *Foxp3* locus ($Foxp3^{GFP\text{-}Cre\text{-}ERT2}$). Foxp3-driven Cre translocated to the nucleus only after ligand binding by tamoxifen. Thus, Cre-recombinase functioned in a temporally controlled manner. As a consequence of $Foxp3^{GFP\text{-}Cre\text{-}ERT2}$ activation, a labeled subset

of Treg could be followed in terms of stability. In this setting, the investigators found >96% of the labeled Treg remained Foxp3-GFP^{+} under normal conditions and observed a decrease in Foxp3 expression only under a lymphopenic setting. They studied the labeled polyclonal Treg in an infectious model and TCR transgenic Treg in NOD mice, concluding that there was minimal Foxp3 loss and no inflammatory cytokine production in Treg or exFoxp3 cells. Therefore, and probably due to methodological constraints, the issue of Treg (in)stability remains quite debatable and controversial.

It has been also proposed that Treg are a heterogeneous pool and only a minor uncommitted population retains the capacity to be reprogrammed. Supporting this hypothesis, the loss of Foxp3 expression within Treg in lymphoreplete recipients was suggested to be restricted to a Foxp3^{+} Treg subset expressing low levels of CD25 (i.e., Foxp3^{+}CD25^{Low}), in contrast to the CD25^{high} fraction, which displayed a stable phenotype[117]. Despite reports supporting this so-called "heterogeneity model", a more recent study demonstrated that Treg that maintain Foxp3 expression and resist reprogramming in a lymphopenic host, and therefore represent a stable population, will subsequently lose Foxp3 expression to the same extent as Foxp3^{+} *ex vivo*, if reintroduced into another lymphopenic recipient[117]. Future lineage fate-maping studies aimed at reassessing Treg reprogramming potential in different environments, are needed in order to further clearify this issue.

Along with the question of Treg stability and reprogramming, the functional fate of reprogrammed Treg is still unclear and may depend on the conditions triggering the loss of Foxp3 expression. Some studies propose that such cells lose the capacity to suppress Teff proliferation *in vitro*, lose the expression of Treg markers and become effector-like (with the potencial to produce IL-17, IFN-γ and IL-2), contributing to pathology in different inflammatory settings[117]. This is supported by the fact that tTreg bear TCR with high afinity to self-antigens and therefore with the potential to become autoreactive upon loss of Foxp3 expression[117]. Contrary, other reports propose a beneficial role for newly emerged Foxp3$^{low/-}$ cells. For instance, in the gut environment and in response to environmental cues in Peyer's patches, reprogrammed Treg reprogram into follicular helper T cells (Tfh), participating in germinal center reactions and promoting immunoglobulin A (IgA) production in the gut[135]. Moreover, as stated above, recent studies have demonstrated that Treg use canonical Th cell-associated transcription programs in order to maintain or

restore immune homeostasis during polarized Th1-, Th2- and Th17-driven immune responses. In response to IFN-γ Treg upregulate T-bet, the Th1 lineage transcription and T-bet$^+$ Treg accumulate at sites of Th1-type inflammation factor[136, 137]. Similarly, IRF4 expression by Treg is involved in controling IL-4 production by CD4$^+$ T cells and is required for Treg mediated-control of Th2-type immune responses[96]. Additionally, deletion of the transcription factor STAT3 in Treg results in development of spontaneous fatal intestinal inflammation due to a selective dysregulation of Th17 responses and excessive IL-17 production[97].

Although the mechanisms by which T-bet, IRF4 and STAT3 control Treg performance during Th1, Th2 and Th17 responses are still unclear, it has been suggested that they probably impact on Treg migration, function and homeostasis[136]. In fact, Treg deficient in T-bet, IRF4 or STAT3 display reduced expression of chemokine receptors implicated in Treg migration during Th1- (CXCR3), Th2- (CCR8) or Th17- (CCR6) mediated responses, enfasizing the important of these effector transcriptional Treg features[98, 96, 97]. Moreover, Treg lacking T-bet, IRF4 or STAT3 show reduced expression of IL-10, suggesting that loss of these transcription factors may also impact on the functional properties of Treg[98, 96, 97]. Finally, loss of T-bet expression resulted in impaired proliferation and accumulation of Treg cells during Th1-type inflammatory responses, suggesting a possible additional impairment in Treg survival and proliferation in a highly polarized Th1-type setting[98].

Although the teleological reason for Treg reprogramming is still under discussion, recent findings suggest that it possibly enables the modulation of Treg response to the different inflammatory contexts as well as promoting a balanced response to pathogens. It seems indeed that reprogrammed Treg may hence in particular contexts acquire the transcriptional armament of Teff not only to better control the specific effector response but also to produce inflammatory cytokines that will contribute to pathogen clearance (Figure 4).

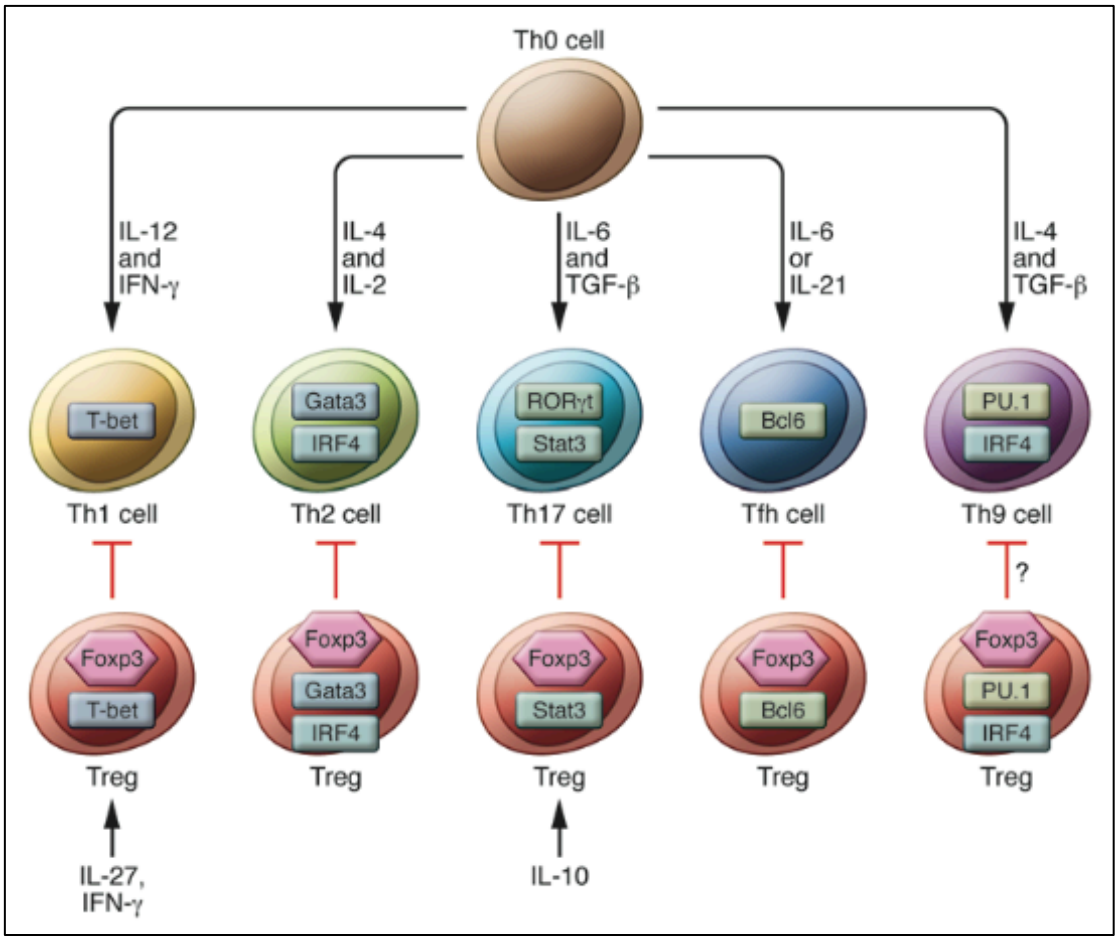

Figure 4: Peripheral naïve $CD4^+$ T cell differentiation is controlled by cytokine signaling. Schematic representation of the Teff transcriptional program coopted by Treg for a tailored suppression of immune responses. Chaudhry A. and Rudensky A. *The Journal of Clinical Investigation.* 2013.[95]

1.1.6. Effector Mechanisms of Regulatory T Cells

Treg are known to employ manifold contact-dependent and soluble effector mechanisms within the tissues and draining lymph nodes in order to inhibit both the innate and adaptive arms of the immune response[138]. Their supressive function involves several distinct mechanisms, such as metabolic disruption of the target cells (e.g. IL-2 and cyclic adenosine monophosphate (cAMP) expression), modulation of the cytokine microenvironment (via production of e.g. IL-10, TGF-β and IL-35), direct cytolysis of antigen-presenting or effector cells, or direct alteration of dendritic cell maturation and

activating capacity (e.g. CTLA-4 and Lymphocyte-activation gene 3 (LAG3))[138, 139] (Figure 5). These mechanisms will be further discussed below.

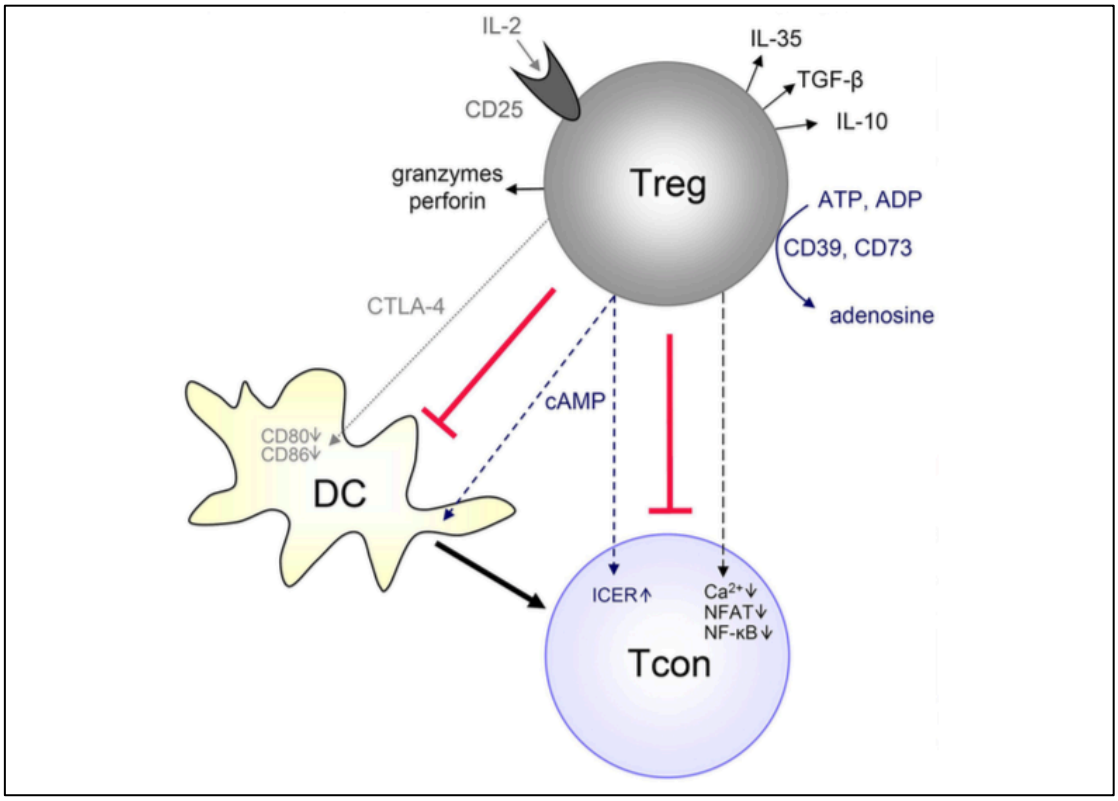

Figure 5: Mechanisms of Treg-mediated suppression. Schematic representation of the different mechanisms employed by Treg to restrain effector T cell responses. Schmidt et al. *Front Immunol.* 2012.[140]

Most of the knowledge regarding Treg effector mechanisms derives from *in vitro* studies. These mechanisms may also operate *in vivo* depending on the target cell type and activation status as well as the location, cytokine and microorganism milieu of the immune reaction. The relevance of each mechanism seems therefore to be context dependent and distinct suppressor mechanisms may predominate in particular tissue and inflammatory settings.

IL-2 consumption by Treg, due to their high CD25 expression, has been firstly proposed as a major suppressive mechanism used by Treg[141]. Contrary to Teff, Treg don't produce IL-

2. However, IL-2 has been clearly implicated in Treg generation and homeostasis[141]. Lenardo et al. proposed that Treg induce IL-2 deprivation-mediated apoptosis in mouse Teff, relying on close proximity between the cells[142]. In contrast to these results, Vignali et al. data showed no cell death induction by Treg[143]. Szymczak-Workman et al., using responder Teff resistant to cytokine withdrawal-induced apoptosis due to either Bim deficiency, Bim/Puma double deficiency or Bcl-2 overexpression, showed that Treg suppression is independent of apoptosis induction *in vitro* and *in vivo* assays. Thornton et al., among others, have shown that exogenous IL-2 could arrest Treg-mediated suppression of proliferation and/ or of IL-2 production *in vitro*, which corroborates the importance of Treg IL-2 consumption by Treg in the suppression of effector responses[144]. Notwithstanding, Tran et al. have shown that IL-2 consumption does not result in suppression of proliferation during inhibition of murine Teff by human Treg[145]. Additionally, human CD25 blockage did not abrogate suppression under optimal stimulation conditions by human Treg[145]. Oberle et al. found that, although exogenous IL-2 partially abrogated suppression of Teff proliferation by human Treg, rapid suppression of IL-2 transcription by human pre-activated Treg was not affected, suggesting different suppression mechanisms controlling proliferation and cytokine transcription. These discrepancies in the effect of IL-2 on IL-2 expression may depend on time, species, IL-2 amount and cellular activation status. Overall, the role of IL-2 consumption in Treg-mediated supression remains controversial, and probably depends on the particular scenario and stimulation conditions of the cells.

In what concerns other supression mechanisms through metabolic disruption of target cells, additional cell-surface molecules such as CD39 and CD73, two ectoenzymes highly expressed on murine Treg, have also recently been implicated in Treg function. They mediate the generation of adenosine and the extrusion of cAMP, therefore contributing to the metabolic disruption of target cells[146]. The ectoenzyme CD39, expressed by all murine Treg and by about 50% of human Treg, uses the hydrolysis of extracellular adenosine triphosphate (ATP) to adenosine diphosphate (ADP) or cAMP as another Treg-mediated anti-inflammatory mechanism[147]. CD39 knockout Treg showed reduced suppressive capacities *in vitro* and *in vivo*[148]. In human Treg, CD39 expression was suggested to identify a highly suppressive Treg subset[149] and suppression of Teff proliferation by this subset could be partially abrogated by blockage of ectonucleotidase activity[149]. CD73,

expressed by murine but not human Treg, further degrades AMP to adenosine[150]. Adenosine signaling initiated by Treg directly inhibits the proliferation of Teff and additionally negatively impacts the function of DC. Therefore, generation of adenosine seems to play an important role in the suppressive function of particular Treg subsets.

Several secreted molecules identified by gene expression studies have been implicated in Treg suppressive function, including IL-10, IL-35, granzyme B, IL-9, and TGF-β[146, 138]. IL-10 is tipically associated with the imunossupressive function of other regulatory subset, Tr1[151]. Nevertheless, it is also an important effector cytokine essential for proper Treg function. IL-10 plays an important role in restraining overactivation of APC for instance in the gut[129]. The importance of IL-10 in Treg function *in vivo* has been extended to infection and EAE models[152]. Although different cell types are capable of producing IL-10, Treg represent an important source in the control of intestinal inflammation. Indeed, a high proportion of Treg in the intestine (30–50%) produce IL-10 under steady-state conditions[153]. More recent studies suggest IL-10 may also function in an autocrine manner to preserve Treg function[154]. The precise mechanism underlying the IL-10 receptor signaling-mediated promotion of Treg function is still not known; it may either preserve Foxp3 expression and thereby lineage stability under inflammatory conditions or it might be necessary to sustain IL-10 expression by Treg, both required for Treg-mediated control of pathogenic inflammatory responses, particularly in the gut[155].

IL-35 constitutes a recently discovered member of the anti-inflammatory cytokines secreted by murine Treg[140]. Although not constitutively expressed by human Treg, IL-35 may also play a role in human immunosuppression, as treatment of naïve human or mouse T cells with IL-35 induced a regulatory population that mediated suppression via IL-35 but did not required IL-10, TGF-β or Foxp3[156]. Moreover, these iTreg were strongly suppressive in several *in vivo* mouse models[156].

One important mechanism through which Treg contribute to immune homeostasis is their capacity to secrete and activate TGF-β. TGF-β knock-out, similarly to Foxp3 deficient mice, develop a fatal wasting syndrome leading to early death at around 20 days of age[157]. Expression of TGF-β by $CD4^+$ T cells is especially important at mucosal sites as mice with a T cell-specific deletion of the TGF-β1 gene showed a more attenuated disease phenotype, with later onset (6 months of age) and confinement of pathology to the colon, lung and liver[158]. Bioavailability of TGF-β is tightly regulated by secretion, cleavage and activation

of the TGF-β inactive precursor molecule[159]. Importantly, Treg themselves are capable of cleaving TGF-β[160]. Human and mouse Treg express high amounts of the integrin αvβ8, which enables them to activate latent TGF-β[161]. Particularly in the intestinal lamina propria, DC are capable of activating pro-TGF-β via their expression of integrin αvβ8, a crucial mechanism to preserve intestinal homeostasis[162]. Recently, it has been shown in murine studies that Treg-cell-specific deletion of integrin αvβ8 results in uneffective suppression of pathogenic T cell responses during active inflammation[161].

Cytolysis of target cells as a mechanism of suppression by Treg was first proposed because of the finding that, in human Treg, granzyme A can be induced by a combination of CD3 and CD46 stimulation, resulting in the induction of apoptosis of activated target cells[163]. Later, several groups reported that granzyme B (but not granzyme A) is highly upregulated in mouse Treg. Upon activation, murine Treg can kill either responder T cells or APC in a granzyme B-dependent manner *in vitro*[164]. These findings have been further confirmed by *in vivo* studies in which granzyme B has been shown to be critical in maintaining Treg–dependent long-lived skin graft tolerance as well as in Treg–mediated suppression of tumor clearance[165, 166].

Regarding direct alteration of DC maturation and activation capacity, several cell-surface molecules were proposed to play a role in Treg–mediated suppression. CTLA-4, a Treg–specific molecule, is enrolled in Treg cell–mediated suppression function, additionally to its important cell-intrinsic role in limiting activated T cell responses[146]. CTLA-4 is constitutively expressed in murine and human Treg and exposed on the cell surface upon activation[167, 75]. A role for CTLA-4 in suppression *in vivo* has been suggested, since CTLA-4 deficiency or blockade in mice results in spontaneous autoimmunity, mitigated by Treg transfer[168]. In addition, CTLA-4 blockade abrogates the protective effects of Treg in murine colitis models[169]. Nonetheless, CTLA-4 deficient Treg are still able to suppress through compensatory mechanisms, involving TGF-β and IL-10 *in vitro* and *in vivo*[170]. Similarly, data in human Treg are not consentual: in some *in vitro* studies, CTLA-4 is not involved in Treg-mediated suppression[171], contrary to others showing partial abrogation of suppression by CTLA-4 blockage[172]. These discordant data may rely on the possible

involvement of CTLA-4 in some, but not all aspects of suppression. Schmidt et al. showed that rapid Treg-mediated suppression of cytokine transcription in human Teff was unaffected by CTLA-4 blockage, irrespective of the presence of APC, while suppression of proliferation in the presence of APC was partially dependent on CTLA-4[140]. Recently, the molecular mechanism of Treg-mediated suppression via CTLA-4 was elucidated in more detail in murine models. In BALB/c mice, known for intrinsic susceptibility to various immune-mediated disorders, it has been suggested that the reduced suppression capacity of CTLA-4-deficient Treg is due to their inability to downregulate CD80 and CD86 via trans-endocytosis[173]. The enzyme indoleamine 2,3-dioxygenase (IDO), responsible for catalyzing degradation of tryptophan to kynurenine, is expressed by some human and murine DC subsets, resulting in Teff starvation and direct cell cycle arrest. Concomitantly, IDO leads to iTreg generation[174]. Additionally, Treg themselves can increase IDO expression in DC through CTLA-4-induced signaling. The importance of CTLA-4 in Treg-mediated suppression is therefore undisputed, although requiring cooperation with other suppressive mechanisms and probably different between species. In fact, it has been recently shown that CTLA-4 expression on Treg from Rheumatoid Arthritis (RA) patients is significantly reduced and result in an abnormal Treg function[175]. Importantly, CTLA-4 blockage has already shown promising outcomes in clinical trials for metastatic melanoma, presumably due to its effects on both Teff and Treg[176].

LAG3, a CD4 homolog that exhibits high binding affinity for MHC class II, is an additional molecule suggested to be required for maximal suppressive activity of both thymic and peripheral-derived Treg in murine studies[177]. Engagement of a MHC molecule on immature DC through LAG3 led to the inhibition of their maturation and co-stimulatory capacity[177]. The long-lasting Treg interactions with DC were also shown to be facilitated by Nrp1, a molecule highly expressed by most murine Treg. Moreover, blockade or ablation of Nrp1 alters Treg suppression function[146, 178].

Recently, it has been shown that murine Treg lacking P-selectin glycoprotein ligand-1 (PSGL-1) expression were unable to suppress EAE and failed to inhibit T cell proliferation *in vivo* in lymph nodes[179]. PSGL-1-deficient Treg lost the ability to modulate T cell movement and failed to inhibit the T cell-dendritic cell contacts and T cell clustering, essential for sustained T cell activation during the late phase of the immune response[179]. However, PSGL-1–deficient Treg were still able to suppress early T cell priming soon

after Ag challenge, possibly meaning that Treg use phase-specific mechanisms to control immune responses[179]. Thus, PSGL-1 has been suggested as a novel phase-specific mechanism for Treg-mediated suppression[179].

Finally, plasticity between $CD4^+$ T cell subsets might be higher than originally anticipated and the stability of the Treg lineage *in vivo* is highly controversial[134]. As above mentioned, recent studies have suggested that Treg may be specialized in the suppression of a particular $CD4^+$ T cell subset by expressing its hallmark transcription factor. In this regard, it was shown that a Treg subset upregulated T-bet in response to IFN-γ, which was essential for the control of Th1-mediated inflammation[98]. Similarly, IRF-4 expression in Treg was required for suppression of Th2 responses[96]. Moreover, expression of STAT3 in Treg was crucial for the control of Th17-mediated intestinal immune responses[97]. However, the mechanism through which Treg supposedly suppress through these effector transcription factors is still unclear, and may involve competition for limiting factors.

1.1.7. Regulatory T Cells in Autoimmune Settings

Since long time ago, T cell-mediated suppression was suggested to be involved in the mechanisms that maintain immunologic tolerance and self-/ non-self-discrimination[1]. This raised the hypothesis that in autoimmune diseases autoreactive Teff responses overwhelm the capacity of a weakened Treg compartment, placing the regulatory subset as a crucial player in autoimmune pathology. A number of genetic and mechanistic defects have in fact been suggested to lead to defective regulation by Treg, with resulting imbalance in the immune system (Table 1).

Potential Treg defect
Defects in positive or negative selection in the thymus lead to peripheral imbalance of Treg:Teff
Genetic susceptability leads to abnormal Treg development and function
Tregs not capable of suppressing Teffs can lead to autoimmunity – effector T cells (both $CD4^+$ and $CD8^+$) develop and become increasingly refractory to suppression
Epitope spreading increases the demand on Tregs to suppress autoimmunity
Deficient IL-2 production by Teffs leads to decreased Treg numbers or function
Low surface CD25 expression delivers deficient IL-2 signals resulting in impaired survival and function of resident Tregs
Deficiencies in TGF-β and IL-10 prohibit induction of adaptive Tregs from naive T cells (defective conversion)
APC maturation defects may lead to altered T-cell activation
Increased costimulation (CD40 and ICOSL) by the APC leads to refractory/pathogenic T cells
Pro-inflammatory cytokine milieu blocks suppression by Tregs and leads to resistant effector T-cell responses
Tregs, regulatory T cells; Teffs, effector T cells.

Table 1: Potential defects leading to autoimmune disease. Adapted from Brusko. *Immunol Rev.* 2008.[180]

Several models of systemic autoimmunity have shown that Treg impairment contributes to the development of autoimmune disease[10]. Similarly, numerous studies of phenotypic and/or functional characterization of Treg in several human AID (e.g. RA, Systemic Lupus Erythematosus (SLE), Primary Sjogren's syndrome, anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitidies, Inflammatory myopathies, Systemic sclerosis, T1D, Multiple Sclerosis (MS), Autoimmune Hepatitis, Psoriasis) have shown decreased Treg numbers or compromised function[180]. Studies in a variety of autoimmune scenarios have also shown that, besides defective Treg number and/or function, pro-inflammatory cytokines present at the site of inflammation may abrogate the suppressive activity of Treg[181] or cause Teff to become resistant to suppression[182]. In agreement, it has been recently shown that Treg from MS patients expressed higher levels of toll-like receptor (TLR) 2 and that stimulation with the synthetic lipopeptide Pam3Cys, an agonist of TLR1/2, reduced Treg function and induced Th17 skewing[183].

Treg have also the capacity to halt or reverse autoimmunity in a large number of experimental settings[184], and the first clinical trials consisting in the adoptive transfer of human Treg in T1D are currently ongoing, with promising preliminary results[185].

Reinforcing the central importance of Treg in immune homeostasis and their critical role in regulating autoimmunity, several immune-related gene variants and pathways that increase the risk for autoimmunity are shared among several AID, constituting a general "autoimmune signature"[186]. Strikingly, many of these genes encode molecules involved in peripheral tolerance and in many cases, proteins implicated in Treg function. These include the IL-2/ CD25 axis, CTLA-4, and protein tyrosine phosphatase, non-receptor type (PTPN) 22[187, 188, 189]. These results strongly reinforce the potential use of drug therapies that promote Treg generation as well as the adoptive immunotherapy of autologous Treg in patients with autoimmunity to correct the established immunological imbalance.

In the spectrum of systemic autoimmune pathologies, diseases such as SLE are interesting potential targets for Treg intervention, evolving through periods of relapse and remission and making it possible to intervene at different time-points of disease severity. SLE is a multisystemic chronic inflammatory/ autoimmune disease in which organs and cells undergo damage mediated by tissue-binding autoantibodies and immune complexes. It is characterized by inflammation and damage of various tissues including the joints, skin, kidneys, heart, lungs, blood vessels and brain. Animal models of SLE have suggested that reduced numbers of Treg may underly the physiopathology of SLE. In humans, the majority of studies showed a decrease in the number of $FOXP3^+$ T cells or a disturbancy of their function[190, 191, 192], giving great therapeutic potential to Treg-based cellular therapies in SLE. Some studies have, however, described unaltered or even increased proportions of Treg in SLE patients. Possible reasons for these controversial observations have been suggested: among them, the lack of a reliable and specific Treg marker at least in humans and in particular under conditions of T cell activation, as well as the different methods used for the isolation of Treg[190].

In SLE, the interaction between environmental, transcriptional and epigenetic modulation targeting Treg function is also evident. Several miRNAs have been linked to the abnormal development and function of Treg[146]. Interestingly, miRNA-155 is upregulated in Treg from MRL/ lpr mice as compared to non-autoimmune mice, resulting in a reversible phenotypic alteration and deficient suppressive capacity[193]. Moreover, miRNA-31 that negatively regulates FOXP3 expression by directly binding to the 3′ UTR of *FOXP3* mRNA is upregulated in SLE-prone mice[194]. Additionally, it is known that cAMP is critical for the suppressive capacity of Treg. Notably, miRNA-142-3p downregulates

adenylyl cyclase 9 mRNA and its elevated expression leads to decreased cAMP levels in Treg and consequently to their abnormal function[195]. Hence, miRNA-31, miRNA-155 and miRNA-142-3p contribute to SLE pathogenesis by cooperatively affecting the development and function of Treg.

Thus, in autoimmune scenarios, where imbalance between autoreactive Teff and protective Treg has been clearly established, the adoptive use of expanded/ induced Treg can supersede the classical Teff blocking approach with immunosuppression and its inherent physiologic risks and toxicity.

1.1.8. Therapeutic potential of Treg-based cellular therapies

The potential of harnessing Treg as immunotherapy in distinct clinical settings is compelling. Treg application for immunotherapy is supported by solid pre-clinical data, with the recent emergence of safe and efficient protocols in different clinical scenarios requiring induction of clinical tolerance. Strategies involving the adoptive transfer of *ex vivo* expanded Treg and the *in vivo* manipulation to expand circulating Treg are currently promising approaches in order to treat inflammatory, autoimmune and alloimmune conditions.

The concept of adoptive cell transfer arose in the mid- 1950s in order to manipulate immune responses in the field of cancer therapy[196]. Adoptive cellular therapies have been used since then in different clinical scenarios, such as human immunodeficiency virus (HIV) infection[197] and autologous/ allogeneic stem cell transplantation[198]. A similar approach specifically involving transfer of Treg has been further employed to modify the immune response in autoimmune scenarios. Adoptive transfer of autologous Treg appears to provide most of the benefits of efficient immunossupression, without the adverse effects of standard imunossupressive drugs and it is, in fact, considered a method for so-called "intelligent imunossupression"[185].

Atopy is an intricate immune disorder characterized by Th2-predominant inflammation, production of allergen-specific IgE, attraction of pro-inflammatory cells and degranulation of effector cells (e.g., mast cells)[199]. Recent literature supports an important role for Treg in maintaining allergen tolerance in healthy individuals and an imbalance between Treg

and Th2 cells has been shown to drive an atopic phenotype[199]. In this context, the E3 ligase Itch has been recently pointed out as a critical protein in the control Th2 inflammation by Treg[200] and may hence constitute a therapeutic target in atopic disease. Allergen-specific desensitization immunotherapy decreases allergen-specific T-cell proliferation, Th2-type cytokine production and inflammatory cell activity[201] and it has been shown to involve the *in vivo* induction/ expansion of $FOXP3^+$ T cells[202]. Although clinical trials involving the adoptive transfer of Treg in allergic settings are not yet in course, protocols aiming the expansion of allergen-specific Treg may potentially improve clinical outcomes in atopic patients, particularly in the ones with severe asthma non-responding to current available therapy.

Graft-versus-host disease (GVHD) is the consequence of donor T cell-mediated systemic inflammation that prevails over immune regulatory mechanisms in the setting of allogeneic hematopoietic stem cell transplantation (HSCT)[203]. When donor (i.e., graft) cells recognize host cells as foreign and trigger a systemic inflammatory reaction, overt clinical disease emerges. Notwithstanding the routine use of post-HSCT immunosuppressive pharmacotherapy with the purpose of silencing T cell alloreactivity, tissue damage often occurs. Treg from HSCT donors are easily available, making Treg immunotherapy protocols particularly achievable. Accordingly, *ex vivo* expanded human Treg have been shown to prevent rejection of skin alografts, transplant arteriosclerosis and GVHD in humanized mouse models[204]. Beyond preclinical studies, Trzonkowski et al. reported the first two cases of *ex vivo*-expanded donor-derived Treg successfully treating post-HSCT GVHD[205]. Brunstein et al. demonstrated the safety profile and efficacy of human umbilical cord blood (UCB)-derived partially human leucocyte antigen (HLA)-matched *ex vivo*-expanded Treg in reducing the incidence of grades II–IV GVHD in 23 patients compared with 108 controls in a phase I dose-escalation trial[206]. These investigators isolated Treg with anti-CD25 magnetic beads, expanded them with anti-CD3/ CD28 microbeads and recombinant human (rh)IL-2 and infused the expanded Treg at the time of HSCT (Figure 6). Di Ianni et al. used adult expanded Treg isolated from the same HLA-haploidentical donor to address the efficacy and safety of this strategy in the prevention of chronic GVHD in 28 patients undergoing HLA-haploidentical HSCT for high-risk acute leukemia, with enthusiastic results[207]. In fact, only 2 of 28 patients developed chronic GVHD. Several other clinical trials exploring human Treg adoptive therapy for prevention and/ or

treatment of GVHD are currently ongoing. For instance, it is currently ongoing a phase 1/2 clinical trial registered at the United States (US) National Institute of Health (NIH) for the treatment of steroid-refractory GVHD after an allogeneic transplant of hematopoietic progenitors with donor CliniMACS-selected Treg (João F. Lacerda, Instituto de Medicina Molecular, Lisboa). At Nanjing Medical University (Ling Lu), it is in course a trial which involves the generation of donor alloantigen-specific Treg from peripheral blood of pre-transplant patients and the administration of Treg at several time-points (for graft-specific tolerance induction), among others.

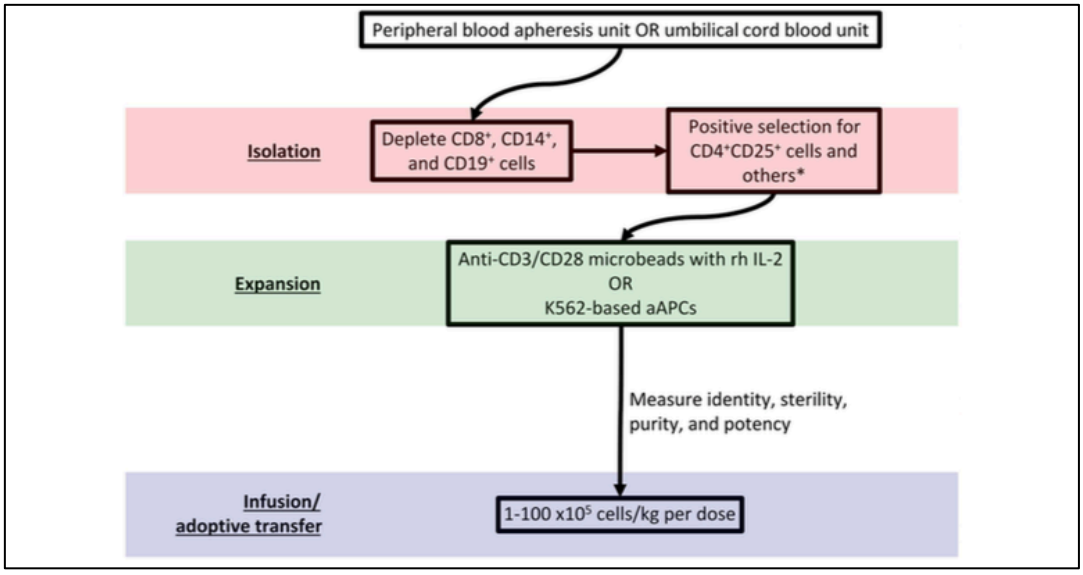

Figure 6: Adoptive transfer of Treg. Schematic representation of a strategy to isolate, expand and infuse Treg. Singer et al. *Front Immunol*. 2014.[208]

Supporting the potential of targeting Treg in autoimmune pathology, this subset was demonstrated to be either decreased in proportion and/or dysfunctional in several Autoimmune Diseases (AID)[10, 209]. Numerous studies have demonstrated diminished numbers of peripheral blood Treg in patients with autoimmune conditions and an association of Treg deficit with disease development[210]. Moreover, redistribution of the Treg population to the tissue compartment does not completely explain the association between peripheral blood Treg deficiency and disease development[192]. Intensely scrutinized, failure to control islet-specific conventional T cells results in T1D. The risk of

T1D increases with the loss of FOXP3-expressing Treg and Treg adoptive transfer to NOD mice can prevent the development of T1D[211]. The first clinical trials with the adoptive transfer of human Treg in T1D are currently ongoing, with promising preliminary results[185]. In this regard, Trzonkowski et al. have revealed the first results of their work. They have infused *ex vivo* expanded Treg in 10 children with recent-onset T1D and found higher C-peptide levels and lower insulin requirements in treated children, with 2 individuals remaining independent of exogenous insulin eleven months after diagnosis[185]. These results reinforce the promising expectations regarding the therapeutic use of Treg in autoimmune scenarios.

In the context of acute inflammation, Treg adoptive transfer has been shown to limit fibroproliferation[212]. Treg also promote repair from ischemic acute kidney injury[213] and demonstrated protective immunomodulatory effects following acute stroke[214]. These findings emphasize the importance of Treg in tissue injury repair and stress the possibility of Treg immunotherapy application in other acute inflammatory conditions.

1.1.9. *In vitro* generation of Regulatory T Cells

Recently, there has been enormous progress in developing novel immunotherapies to treat autoimmune diseases. However, relatively non-specific therapies (such as anti-CD3 monoclonal antibody (mAb), thymoglobulin, cytokines, and anti-cytokines, etc.) can be associated with significant side effects and "off-target" effects. These studies have nevertheless enabled two substantial findings: first, short-term immune regulation of T cells can have a long- term effect on disease progression; second, many of the recognized immunomodulatory drugs induce Treg subsets that are likely to be responsible for the long-lived efficacy[180]. The establishment of the importance of Treg in the effective regulation of the basic processes that maintain tolerance has opened an important new weapon of therapeutic intervention in immunology and particularly on autoimmunity– Treg adoptive immunotherapy[180].

Adoptive transfer of autologous or donor-derived Treg represents nowadays an exciting and promising immunotherapeutic approach[215]. Despite the unquestionable therapeutic potential of Treg-based cellular therapies in humans, therapeutic efficacy of this approach requires large numbers of Treg, which impose an additional challenge since human Treg constitute a minor subset of peripheral $CD4^+$ T cells. The establishment of efficient protocols to expand Treg or to convert non-regulatory T cells into $FOXP3^+$ T cells is, currently, a promising strategy towards the treatment of human diseases with described Treg defects and has been the subject of intense research. In what concerns *ex vivo* Treg expansion, protocols for adoptive transfer require isolation of Treg from the host or a donor, its enrichment, its expansion, and Treg re-infusion. Indeed, Treg *ex vivo* expansion strategy enables a careful cellular phenotyping and control over the dose of administered cells[216]. Notwithstanding, there are a number of clear challenges that need to be overcome to employ Treg in clinical settings, including: the need for isolation of pure populations of Treg from peripheral blood in patients; improved technologies to expand and test Treg function *ex vivo*; determining the survival and long term stability of Treg in the adoptively transferred host; issues related to safety, especially as it relates to potential pan-immunosuppression with polyclonal Treg populations; and the challenges related to generating Ag-specific Treg for adoptive immunotherapy[217].

Despite positive results in murine models of T1D and myasthenia gravis, adoptive transfer of tTreg has not found full success. Adoptive transfer of tTreg had only a nominal effect on controlling disease progression in a collagen-induced arthritis model[218] and was unavailing in suppressing glomerulonephritis and sialadenitis in mice with lupus[219]. In other Th17-mediated autoimmune settings, tTreg showed variable performance[208]. The inefectiveness of tTreg in many autoimmune disorders may relate to pro-inflammatory cytokines that suppress their function[126] or convert them to pathogenic T cells upon adoptive transfer. Additionally, activated Th17 cells possibly resist many suppressive mechanisms used by tTreg. In this regard, iTreg have been suggested to constitute a more appropriate subset for use in autoimmune immunotherapy, as data suggest that iTreg exhibit increased stability in highly inflammatory environments[220].

The establishment of efficient protocols enabling the *in vitro* generation of *bona-fide* induced iTreg could overcome the problems raised with the previous approaches, particularly in the specific setting of AID. Although both populations can exhibit

considerable heterogeneity in their genetic signatures[65], iTreg induced in the presence of IL-2 and TGF-β share many additional characteristics with tTregs, including the expression of CD25, CTLA-4 and GITR, and the secretion of TGF-β and IL-10, which contribute to their regulatory functions[65]. Functionally, iTreg also display similar features to tTreg: they have been shown to inhibit naïve T cell proliferation *in vitro* and *in vivo*, to inhibit the differentiation of other helper T cell subsets as well as inhibiting effector functions such as IFN-γ production[65]. Since TGF-β-induced iTreg can be easily grown *in vitro* from abundant precursors, they are becoming attractive candidates for Treg adoptive therapies.

In protocols of *in vitro* conversion, naive $CD4^+$ T cells are the usual target population and in fact many reports, both in mice and in humans, show these cells can acquire high levels of Foxp3 expression associated with regulatory properties. Studies from many groups showed that iTreg could be induced from naïve $CD4^+$ T cells by TCR stimulation in the presence of IL-2 and TGF-β[221, 220, 19, 222, 223]. Accordingly, previous data on models of T1D showed that adoptively transferred TGF-β-induced iTreg distributed throughout the lymphoid compartment and in the pancreas of recipient NOD mice, and were in fact able to prevent the localization of pathogenic Th1 cells in the pancreas. Moreover, similarly to tTreg, they proliferated within the islets where they mediated local control of inflammation, in a TGF-β-dependent maner[224].

The potential of human conventional memory $CD4^+$ T cells to acquire *in vitro* a stable Treg phenotype remains poorly explored, although a significant proportion of the circulating Treg compartment is thought to result from the conversion of memory $CD4^+$ T cells into Treg in response to antigenic stimulation *in vivo*[225]. Accordingly, varicella zoster virus-specific memory T cells and $FOXP3^+$ Treg specific for the same antigen accummulate in parallel at the site of specific antigen challenge *in vivo*, suggesting that a significant proportion of Treg may be derived from memory T cells in the course of a localized immune response *in vivo*[226]. Treg found in the mucosa of ulcerative colitis and colon carcinoma patients are derived from memory conventional T cells[227]. Moreover, human Treg and memory $CD4^+$ T cells have been shown to display, on average, 80% homology in their TCR Vβ usage[225]. Importantly, conventional memory T cells in autoimmune patients are likely enriched in self-reactive T-cells, making them particularly

relevant substrates for the induction of iTreg with relevant specificities in the setting of autoimmunity.

Along with the several transcriptional regulators of Foxp3 expression downstream from TCR and IL-2R, additional signaling pathways have been involved in Treg differentiation and maintenance[146]. Among them, the Notch signaling pathway has been incrementally implicated in Treg homeostasis[146]. Thus, it is currently a promising target in order to better understand Treg physiology and further manipulate this subset in clinical settings.

1.2. Notch signaling pathway

1.2.1. Notch Signaling Pathway in T cell development and differentiation

Notch is an evolutionary conserved signaling pathway involved in cell differentiation processes in several organs and at distinct developmental stages. Signaling is mediated by Notch ligand–receptor interactions between neighbouring cells. Mammals possess four receptors (Notch 1–4) that are bound by five ligands of the Jagged family and Delta-like family (Jagged (JAG)1 and JAG 2, and Delta-like (DL)1, DL3 and DL4)[228]. Newly synthesized receptors are proteolytically processed in the Golgi during their transport to the cell surface by a furin-like convertase, generating heterodimeric receptors present at the cell surface. Signaling is initiated with ligand binding to the receptors, which subsequently undergo two successive proteolytic cleavages (Figure 7). The first cleavage is mediated by disintegrin and metalloproteinase domain-containing protein (ADAM) family metalloproteinases at the extracellular S2 cleavage site close to the transmembrane domain, resulting in the shedding of the extracellular part of the receptors and their endocytosis by the ligand-expressing cell[228]. The second cleavage within the transmembrane domain is triggered by the γ-secretase activity of a presenilin multi-protein complex, resulting in the liberation of the Notch intracellular domain (NICD). This last proteolytic reaction is a rate-limiting step during Notch activation, susceptible to pharmacological blockage by small-molecule γ-secretase inhibitors[229]. After the liberation of NICD, it translocates to the nucleus and binds to the transcription factors of the recombination signal binding protein for immunoglobulin κJ region (RBPJ) family (also known as CSL in humans). After binding to RBPJ, the NICD recruits additional co-activators, including mastermind-like (MAML) proteins 1–3 and p300 in order to induce transcriptional expression of downstream target genes. Notch signaling is regulated at multiple levels: for instance, Notch receptors undergo post-translational modifications by Fringe family glycosyltransferases, which results in different efficiencies or signaling strength of Notch receptors[230, 231].

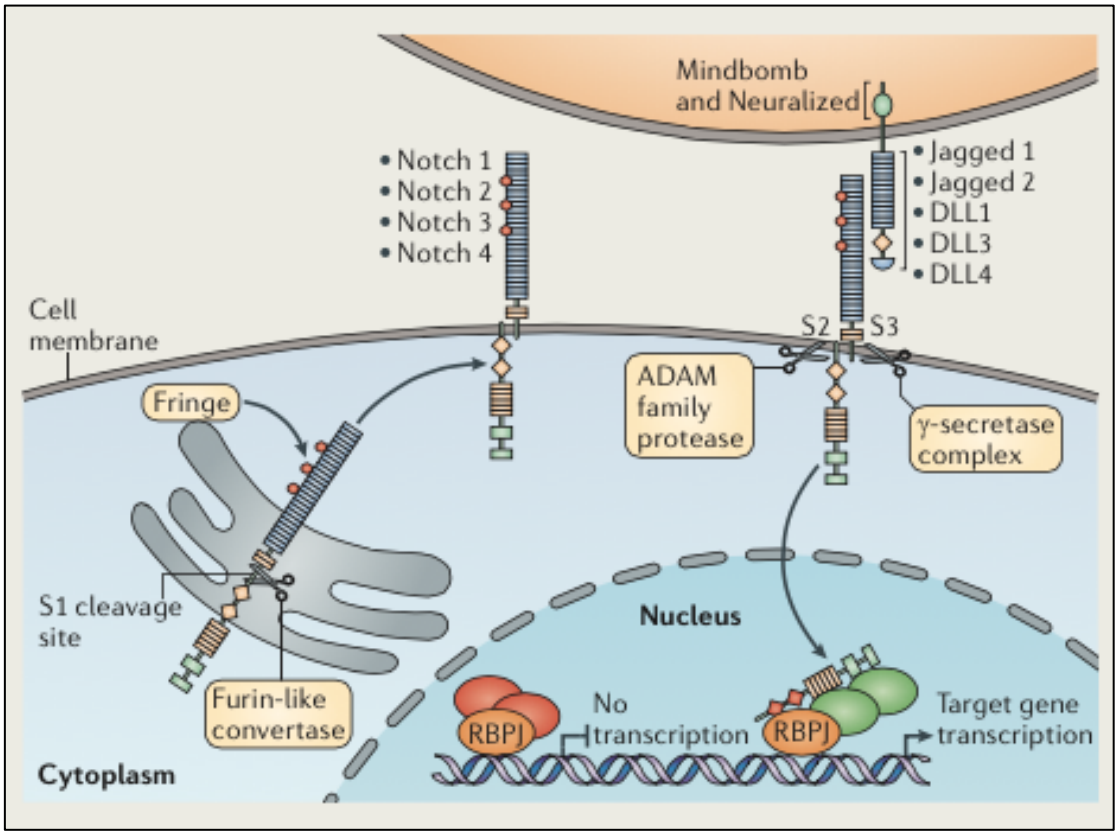

Figure 7: A brief overview of Notch signaling. Schematic representation of the Notch signaling pathway. Radtke et al. *Nat Rev Immunol*. 2013.[228]

More recently, evidence suggested a non-canonical Notch signaling that does not require the RBPJ transcriptional mediator complex[232, 233]. These non-canonical signal transduction pathways may occur in the absence of receptor cleavage or through crosstalk with other signaling pathways (including the nuclear factor-κB (NF-κB), TGF-β and hypoxia-induced signaling pathways)[232, 233].

The Notch signaling cascade has been pointed-out as an important regulator of multiple cell fate decisions and differentiation processes during the development and function of the haematopoietic system. Among the most well established functions of Notch are its essential role in the specification and maturation of T cells, as well as of marginal zone B (MZB) cells. These processes use canonical Notch signaling and have their receptor–ligand pairs identified by conditional genetic loss-of-function approaches: T cell lineage

commitment and maturation is mediated by DL4–Notch 1 interactions, whereas MZB cell development is mediated by DL1–Notch 2 interactions[228]. Bone marrow haematopoietic stem cells (HSC) give rise to multipotent progenitors (MPP) before differentiating into common myeloid progenitors (CLP). CLP migrate from the bone marrow to the thymus, where thymic epithelial cells (TEC) that express DL4 trigger canonical Notch 1 signaling in early thymic progenitors (ETP)[234]. This Notch 1 signal is essential for T cell lineage commitment and is further required during early phases of thymocyte differentiation up to the double-negative (DN)3 stage[234]. Active Notch signaling during these early stages of T cell development inhibits other lineage potentials, such as B cell and myeloid cell potential[234]. During β-selection, Notch signaling is turned off as a consequence of pre-T cell receptor signaling[234]. Thus, subsequent stages of T cell development exhibit very low levels of Notch signaling.

In bone marrow-residing CLP, Notch signaling must be switched off to allow proper B cell development. After migration of immature B cells to the spleen, interaction of DL1 with Notch 2 (mediated by RBPJ) induces Notch signaling in transitional B (T2) cells to specify MZB cells, as opposed to follicular B cells[234]. The vast majority of DC is derived from common myeloid progenitors (CMP) in the bone marrow, which give rise to macrophage–DC progenitors (MDP). Subsequently, common DC progenitors (CDP) develop into pre-DC, seeding lymphoid and non-lymphoid organs via the bloodstream. In the spleen these pre-DC are specified into multiple DC subsets, including classical DC (cDC) and plasmacytoid DC (pDC). Splenic $CD8^-$ endothelial cell-selective adhesion molecule $(ESAM)^+$ DC and $CD103^+CD11b^+$ DC in the lamina propria of the intestine also require Notch signaling mediated by the Notch 2 receptor[228].

Notch has been also enrolled in innate lymphoid cells (ILC) development and/or expansion[228]. ILC, that do not express antigen receptors, fulfil important functions in innate immune responses through their ability to generate and secrete different cytokines and/or to exhibit cytotoxic activity. They can be grouped into three major classes: group 1, group 2 and group 3, depending on whether they express Th1-type, Th2-type or Th2-type cytokines, respectively. Notch signaling can influence the development and/or expansion of the different subsets of ILC, which is probably microenvironment dependent[228].

Genetic loss-of-function experiments also show an important role of Notch signaling in both Th2 cell differentiation and Th1 cell function. Experimentally, JAG or DL ligand expression on APC have been associated with Th2 cell and Th1 cell differentiation, respectively. Th1 cell-promoting signals induce the expression of DL ligands and the release of the NICD, which can bind to the NF-κB family proteins p50 and p65[228]. In addition, the NICD can control the release of IFNγ either directly or indirectly[228]. Th2 cell-promoting signals induce the expression of JAG ligands and the release of the NICD, which interacts with RBPJ, converting it to a transcriptional activator. RBPJ recruits co-activators and the complex binds and transactivates the promoter of GATA binding protein 3 (GATA3) transcribing exon 1a. IL-4 can also initiate Th2 cell differentiation by triggering STAT6, which induces the transcription of *Gata3*. *Gata3* and *Il4* expression reinforce GATA3 expression. GATA3 modifies the conformation of the *Il4*, *Il5* and *Il13* loci, allowing their transcription. Therefore, DL-mediated Notch signaling play a role in Th1 cell differentiation and function in a canonical RBPJ-mediated signaling-independent way and JAG-mediated Notch signaling participates in Th2 cell differentiation through canonical RBPJ-mediated pathway[228]. In what concerns Th17 cells, recent data has shown that Notch signaling is activated in both mouse and human *in-vitro* polarized Th17 and that blockade of Notch signaling downregulates the production of Th17-associated cytokines, suggesting a role for Notch signaling during Th17 differentiation[235]. Recently, it has been also shown that T cell-specific gene ablation of Notch1 and Notch2 impaired differentiation of Tfh cells in draining lymph nodes of mice immunized with T-dependent antigens or infected with parasites, impacting in germinal center development, and establishing Notch signaling as a major player in the development and function of Tfh cells[236].

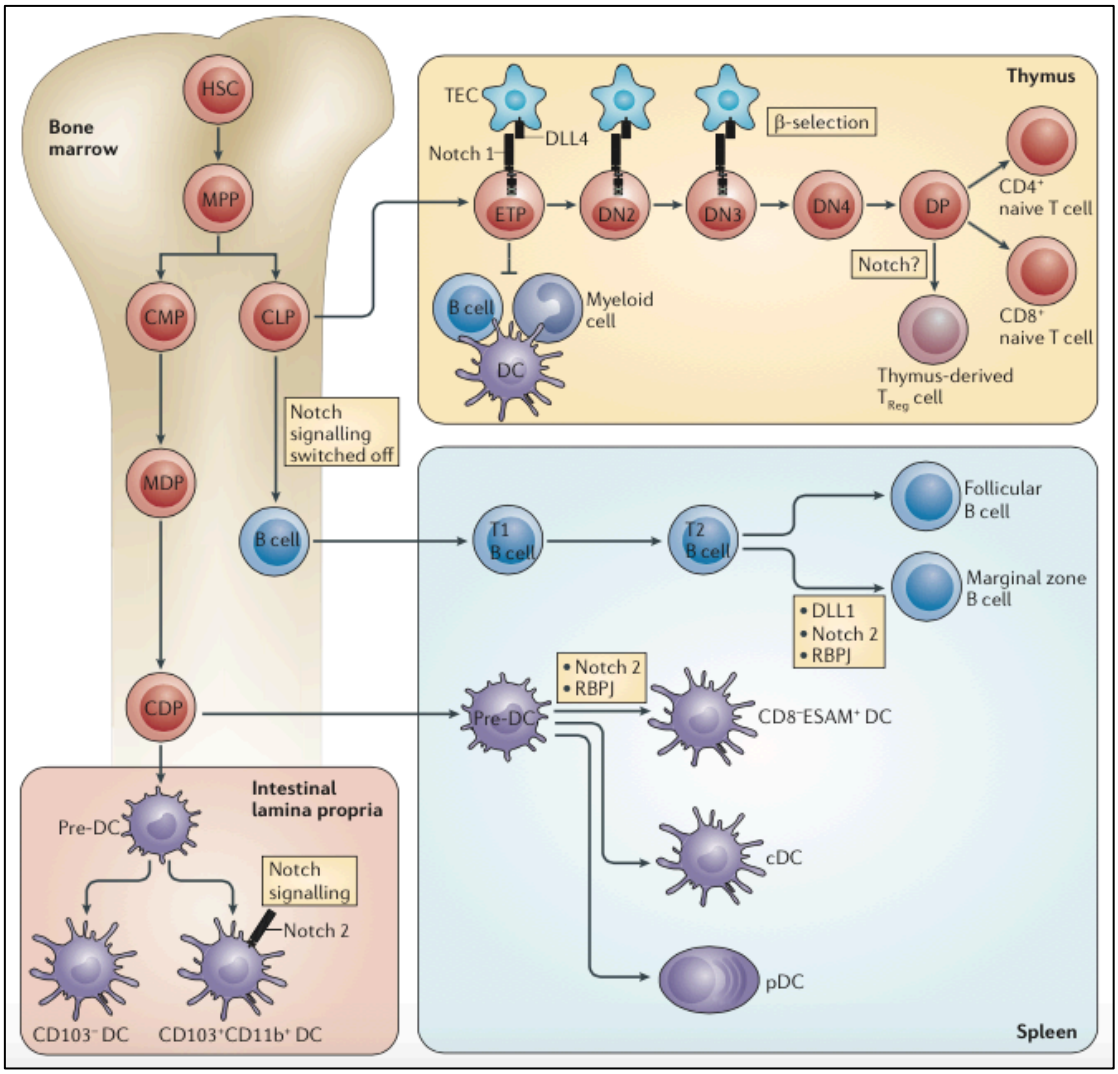

Figure 8: Notch signaling in immune cell development. Schematic representation of the role of Notch signaling pathway in immune cell development. Radtke et al. *Nat Rev Immunol*. 2013.[228]

1.2.2. Notch Signaling Pathway and Regulatory T Cell development

In mice, Notch has been shown to influence the thymic generation of Treg, their peripheral expansion and the *in vitro* conversion of non-regulatory T cells into Treg. The first indications that Notch signaling could be involved in Treg function resulted from studies showing that splenic $CD4^+CD25^+$ T cells expressed higher levels of Notch3 receptor than their counterpart $CD4^+CD25^-$ population. Moreover, transgenic expression of

constitutively active intracellular domain of Notch3 in T cells induced enriched thymic and peripheral Treg compartments, that afforded protection against experimentally induced autoimmune diabetes in mice[237]. Further studies by the same group reported that Notch3 promoted the development of Treg and improved their suppressive activity by upregulating FOXP3 expression[238]. In a more physiological setting, JAG2-expressing hematopoietic precursors isolated from mouse spleen upon appropriate mobilization, triggered Treg expansion through Notch3 engagement, with prevention of disease onset in an experimental TID model[239]. APC that overexpress human JAG1 promoted the expansion of $CD4^+$ cells mediating active antigen-specific tolerance in mice[240]. Previous studies in mice also indicated that the Notch and TGF-β signaling pathways cooperatively regulate Foxp3 expression and Treg maintenance both *in vitro* and *in vivo*[241]. Recently, it has been also described a TCR-dependent, non-nuclear distribution and function of the processed receptor Notch, which was associated with the improved survival of murine Treg *in vitro* and *in vivo*, suggesting a new mechanism of Notch regulation by spatial localization[242].

In humans, there's evidence suggesting an important role of Notch signaling in Treg differentiation and peripheral expansion. In fact, human cord-blood $CD34^+$ cells can differentiate into mature Treg upon co-culture with OP9-DL1 cells[49], suggesting Notch may be involved in human thymic development. Moreover, APC that overexpress human JAG1 promoted the expansion of alloantigen-specific cells with regulatory properties in humans[243]. Importantly, the Notch putative role in Treg differentiation is also supported by the presence of Notch-responsive elements in the mouse and human Foxp3 promoter[244].

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CHAPTER 2. AIMS AND WORKPLAN

Regulatory T Cells (Treg) that constitutively express the transcription factor Foxp3/ FOXP3 are essential to the maintenance of immune homeostasis, playing a fundamental role in the prevention of autoimmunity. This is directly supported by the observation that Foxp3 null mutations, both in humans and mice, lead to early and fatal spontaneous autoimmunity[1]. Similarly, diminished frequency and/or dysfunction of Treg have been reported in several human and murine autoimmune diseases[2, 3]. Adoptive transfer of Treg has proved to be highly efficient both in the prevention and treatment of autoimmunity in rodents[4], and the first clinical trials employing the adoptive transfer of Treg are currently ongoing, with encouraging preliminary results[5]. These studies have however established the need for large numbers of Treg to achieve therapeutic efficacy, especially in the presence of established autoimmunity. Since human Treg constitute only a minor *subset* of peripheral $CD4^+$ T cells and given the underlying technical challenges of the *in vitro* expansion of this population, the establishment of novel protocols leading to the efficient and stable *in vitro* conversion of non-regulatory T cells into Treg is vital. Likewise, the process by which human Treg develop within the thymus is still poorly understood, a knowlegde that may contribute to the adequate manipulation of this subset in clinical settings.

Several signaling pathways have been implicated in Treg generation. The Notch signaling pathway is known to be important at several stages of T cell development and differentiation[6, 7, 8]. In mice, Notch signaling enhances the thymic generation of Treg, their peripheral expansion and the *in vitro* conversion of conventional T cells into the Treg phenotype[9, 10, 11, 12, 13]. Compelling evidence suggest an important role for Notch signaling in human Treg differentiation and peripheral expansion[14, 15, 16], although this has never been thoroughly investigated.

The **overall objective** of this work was to provide knowledge on the principles dictating human thymic and peripheral Treg development as well as their homeostasis, to facilitate the establishment of Treg-based immunotherapy, particularly in the context of autoimmunity.

To accomplish this broad objective, the **specific aims** of this work were the following:

1) to investigate the capacity of human non-regulatory memory $CD4^+$ T cells to differentiate in *vitro* into *bona-fide* FOXP3-expressing cells, and assess the role of the Notch signaling pathway in modulating this conversion.

2) to evaluate the role of common-gamma chain (γc) cytokine signaling in human tTreg development.

I. In *vitro* differentation of human non-regulatory memory CD4 T cells into *bona-fide* FOXP3-expressing cells and role of the Notch signaling pathway in modulating this conversion

Despite the recent development of Treg-based cellular therapies using expanded Treg, *in vitro* induced (i)Treg seem to be more stable in inflammatory environments[17]. Moreover, the generation of iTreg from non-regulatory $CD4^+$ T cells could be the ideal target in patients harboring Treg with impaired effector function. In the particular context of autoimmune diseases (AID), the differentiation of Treg specifically from memory non-regulatory $CD4^+$ T cells could be also particularly interesting because of the expressed self-reactivities within this pool, which may facilitate the generation of antigen (Ag)-specific Treg with relevant specificities.

The Notch pathway is an important signaling cascade in thymocyte development. In mice, Notch signaling influences the thymic generation of Treg, their peripheral expansion and *in vitro* conversion of conventional T cells into Foxp3-expressing cells[9, 10, 11, 12, 13]. Strong evidence implies an important role of Notch in human Treg generation and peripheral expansion[14, 15, 16]. Therefore, we hypothesized that memory $CD4^+$ T cells can be efficiently converted into *bona-fide* iTreg and that Notch constitutes a major signaling pathway in the acquisition of FOXP3 expression by human non-regulatory cells as well as in human Treg homeostatic expansion.

To assess the involvement of Notch signaling in human Treg *in vitro* differentiation, we mainly used two approaches: “gain of function” experiments- via co-culture of peripheral non-regulatory $CD4^+$ T cells with OP9 stroma cells expressing the Notch ligand Delta-like (DL)1, under optimal FOXP3-induction conditions (TCR stimulation, plus costimulation and TGF-β, in the presence of IL-2); and “loss of function” experiments- by inhibition of Notch signaling by addition of the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) in the established protocols of in *vitro* conversion (optimal FOXP3-induction conditions).

In both experimental settings, we evaluated:

- Impact of the established protocols in the differentiation of memory conventional $CD4^+$ T cells into FOXP3- expressing cells
- Phenotype and function of the iTreg

In the particular setting of "gain of function" experiments, we also assessed:

- Stability of FOXP3 expression on the iTreg
- Mechanisms mediating the impact of Notch signaling on the *in vitro* Treg differentiation

We additionally evaluated the role of DL1 in modulating iTreg generation from non-regulatory naïve $CD4^+$ T cells and human thymocytes in the established *in vitro* conversion protocols.

To further elucidate the putative role of Notch signaling in human Treg homeostasis, we assessed the impact of DL1-mediated Notch signaling on the homeostatic proliferation and phenotype of TCR-stimulated human circulating Treg.

In order to further dissect the role of Notch signaling in Treg homeostasis and *in vitro* differentiation, we investigated Notch receptor expression on sort-purified circulating Treg and conventional T cells.

As a proof of principle, we evaluated whether the DL1-mediated Notch signaling impact could be extended to an AID context, by targeting memory $CD4^+$ T cells isolated from a Systemic Lupus Erythematosus (SLE) patient in stable remission in the established *in vitro* conversion protocols. Aiming the future profiteering of immunotherapy with Ag-specific Treg in autoimmune settings, we further assessed the self-reactivities present in conventional CD4 pool of an SLE patient.

The results obtained are shown in the section 1 of the Results.

II. Role of common-gamma chain (γc) cytokine signaling in human Treg development

The principles dictating Treg development in the human thymus remain to be fully clarified, a gap that may hinder their proper manipulation for immunotherapy purposes. γc cytokine family, that shares the use of γc to generate signaling receptor complexes, comprises cytokines with a crucial role in the development and differentiation of immune cells, namely IL-4, IL-7, IL-9, IL-15, and IL-21, in addition to IL-2[18, 19]. It has been clearly shown that Treg homeostasis and function in the periphery depends on IL-2[18, 19]. Importantly, human tTreg express the α- (CD25) and the β- chain (CD122) of the IL-2R that are utilised by IL-2 and IL-15[20, 21]. It has been also shown that human $FOXP3^{+}$ thymocytes, despite showing reduced levels of the α-chain of the IL-7R compared to their $FOXP3^{neg}$ counterparts, phosphorylate STAT-5 in response to IL-7[21]. Additonally, IL-2 increased CD25 and FOXP3 expression levels within $FOXP3^{+}$DP thymocytes[21]. Notably, polymorphisms in IL-2, CD25 or IL-2R downstream signaling molecules result in impaired Treg number and/or function and carry a higher risk of autoimmunity in humans[18], possible owing to defective tTreg generation, besides reduced peripheral Treg survival. Accordingly, in patients undergoing IL-2 therapy it has been shown expansion of Treg expressing CD45RA and the recent thymic emigrant marker CD31, supporting thymic involvement[22, 23]. Overall, available data suggest that γc cytokines may also be involved in human thymic Treg development.

To assess the role of common-γ chain cytokine signaling in human Treg development, we performed gain- and loss-of-function experiments in 3D and 2D postnatal thymic cultures, using recombinant cytokines, specific blocking antibodies and pharmacological inhibitors of their signaling pathway.

The results generated are shown in the section 2 of the Results.

In agreement with the Decreto-Lei 388/70, art 8º, parágrafo 2, the results presented here were published:

Mota C, Nunes-Silva V, Pires AR, Matoso P, Victorino RM, Sousa AE and Caramalho I. Delta-like 1-Mediated Notch Signaling Enhances the In Vitro Conversion of Human Memory CD4 T Cells into FOXP3-Expressing Regulatory T Cells. *J Immunol* 2014 193:5854-62.

Caramalho I, Nunes-Silva V, Pires AR, **Mota C**, Pinto AI, Nunes-Cabaço H, Foxall RB and Sousa AE. Human regulatory T-cell development is dictated by Interleukin-2 and -15 expressed in a non-overlapping pattern in the thymus. *J Autoimmun* 2015; 56: 98-110.

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CHAPTER 3. RESULTS

3.1. Harnessing DL-1 for human memory CD4 conversion into iTreg

Delta like-1 mediated Notch signaling enhances the *in vitro* conversion of human memory CD4 T cells into FOXP3-expressing Regulatory T Cells

Delta like-1 mediated Notch signaling enhances the *in vitro* conversion of human memory CD4 T cells into FOXP3-expressing Regulatory T Cells

Catarina Mota[*,†], Vânia Nunes-Silva[*], Ana R. Pires[*], Paula Matoso[*], Rui MM Victorino[*,†], Ana E. Sousa[*] and Iris Caramalho[*]

[*]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

†Hospital Universitário de Santa Maria, Centro Hospitalar Lisboa Norte, Lisboa, Portugal

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Abstract

FOXP3-expressing Regulatory T cells (Treg) are essential for the prevention of autoimmunity and were shown to be reduced and/or dysfunctional in several autoimmune diseases (AID). Although Treg-based adoptive transfer represents a promising therapy, the large cell number required to achieve clinical efficacy constitutes an important limitation. Therefore, novel strategies to generate *bona-fide in vitro* induced (i)Treg are critical. Here we report that human memory CD4 T cells can be efficiently converted into iTreg, and that Delta-like 1 (DL1)-mediated Notch signaling significantly enhances this process. The iTreg generated in the presence of DL1 featured higher levels of Treg function-associated molecules and were efficient suppressors. Importantly, these iTreg displayed a stable phenotype in long-term cultures, even in the presence of pro-inflammatory cytokines. Additionally, DL1 potentiated FOXP3 acquisition by memory CD4 cells through the modulation of the TGF-β signaling pathway and of *Foxp3* transcription.

Our data demonstrate that iTreg can be efficiently induced from memory CD4 cells, a subset enriched in relevant specificities for targeting in AID, and that DL1 enhances this process. DL1 also enhanced the proliferation and Treg function-associated marker expression of *ex vivo* stimulated human circulating $FOXP3^{+}$ cells. Manipulation of the Notch signaling pathway constitutes a promising approach to boost the *in vitro* generation of iTreg and *ex vivo* Treg expansion, thus facilitating the establishment of effective Treg-based adoptive therapy in AID.

Introduction

Regulatory T cells (Treg) are crucial in the induction of self-tolerance and prevention of autoimmunity. These cells were shown to be decreased in number and/or to display compromised function in several human autoimmune diseases (AID) (1), in agreement with experimental animal models of autoimmunity, demonstrating that defects in the Treg compartment contribute to disease development (2). The potential of harnessing Treg for immunotherapeutic purposes in distinct clinical settings is compelling and adoptive transfer of Treg is considered a method for so-called "intelligent imunossupression" (3). Adoptive transfer of autologous Treg appears, in fact, to provide most of the benefits of efficient immunossupression, without the adverse effects of standard imunossupressive drugs (3). The adoptive transfer of Treg in experimental mouse models of autoimmunity lead to disease prevention and remission (2, 4) and the first clinical trials, involving the adoptive transfer of human Treg in Type I Diabetes, are currently ongoing, with promising preliminary results (3).

However, since large numbers of Treg are required for clinical efficacy, and as they constitute a minor subset of peripheral CD4 T cells, a large amount of effort has gone into developing clinical protocols for the *ex vivo* expansion of human Treg. The current approaches have suffered some setbacks, namely: the weak proliferative potential of Treg, the risk of altering Treg phenotype and decreasing their immunosuppressive activity associated with repeated expansion, and the inadequacy of this strategy when the Treg pool features intrinsic defects (3, 5).

The establishment of efficient protocols enabling the *in vitro* generation of *bona-fide* induced (i)Treg could overcome these problems. In protocols of *in vitro* conversion, naive CD4 T cells are the usual target population and in fact many reports, both in mice and in humans, show these cells can acquire high levels of FOXP3 expression associated with regulatory properties (6-11). FOXP3 expression is currently the best available marker to identify Treg and is required for their development and function (1, 2). The potential of human conventional memory CD4 cells to acquire *in vitro* a stable Treg phenotype remains poorly explored, although a significant proportion of the circulating Treg compartment is thought to result from the conversion of memory CD4 cells into Treg in response to antigenic stimulation *in vivo* (12-14). Moreover, human Treg and memory CD4 T cells have been shown to display, on average, 80% homology in their TCR Vβ usage (12).

Importantly, conventional memory T cells in autoimmune patients are likely enriched in self-reactive T-cells, making them particularly relevant substrates for the induction of iTreg with relevant specificities in the setting of autoimmunity.

The Notch signaling pathway is an evolutionarily conserved signaling cascade involved in cell differentiation processes at distinct developmental stages. There is solid experimental data in mice demonstrating a crucial role for Notch signaling in the thymic generation of Treg, their peripheral expansion and function, as well as in the *in vitro* conversion of conventional T cells into iTreg (15-19). In humans, there is evidence suggesting a role of Notch signaling in thymic Treg development as well as in peripheral Treg expansion (20-23), although its putative role in iTreg conversion has never been formally assessed.

In the present study, we investigated the capacity of human non-regulatory memory CD4 T cells to differentiate in *vitro* into *bona-fide* FOXP3-expressing cells and the role of the Notch signaling pathway in modulating this conversion. Our results showed that Notch enhanced the conversion of memory CD4 T cells into stable and efficient iTreg, supporting a role, particularly for DL1, in human Treg conversion. DL1 also enhanced the proliferation of *ex vivo* stimulated human circulating $FOXP3^{+}$ cells as well as their expression levels of molecules associated with effector function. Manipulation of the Notch signaling pathway may therefore constitute a promising approach to facilitate the establishment of Treg-based therapies.

Material and Methods

Flow Cytometry

The following anti-human Abs, clones in brackets, were used: CD4-alexa fluor 450 (RPA-T4), CD39-APC (eBioA1), FOXP3-PerCP Cy5.5 (PCH101), CD8-FITC (RPA-T8), CD14-FITC (61D3), CD19-FITC (HIB19), CD45RO-PE (UCHL1), CD127-APC-efluor 780 (eBioRDR5), CD45RA-PercCP Cy5.5 (HI100), CD4-APC (RPA-T4), S6 (pS235/pS236)-alexa fluor 488 (N7-548) and Akt (pS477)-alexa fluor 488 (H89-61), all from eBioscience, San Diego, USA; Ki67-PE (B56); CTLA-4-PE (BNI3) and CD25-PE-CY7 (clone 2A3), from BD Biosciences, New Jersey, USA; purified Smad2 (pSer465/467)/Smad3 (pSer423/425) (D6G10), from Cell Signaling Technology, Danvers, USA); Bcl2-FITC (124), from DAKO, Glostrup, Denmark; and anti-rabbit IgG-alexa fluor 546, from Invitrogen. After surface staining for 30 min at 4ºC with optimal dilutions of each mAb, cells were fixed, permeabilized and stained for intracellular molecules using the Foxp3 staining kit from eBioscience, according to manufacturer's instructions. 8 to 10-parameter acquisition was performed on a Fortessa Flow Cytometer (BD Biosciences). For phosphorylation level assessment, cells were fixed and permeabilized using the BD Phosflow staining kit (BD Biosciences), stained for 30 min at 4ºC with optimal dilutions of each mAb, and acquired with a FACSCalibur (BD Biosciences). Data were analyzed using the FlowJo (TreeStar, Ashland, USA), after exclusion of dead cells using LiveDead Fixable Viability Dye (Molecular Probes, Eugene, USA) and doublets using a plot of forward scatter height versus amplitude. The $FOXP3^{bright}$ population gate was always defined in each experiment, using the TCR stimulation and TGF-β culture condition and this gate subsequently used to identify this population in all additional culture conditions performed.

Cell Purification

A negative selection kit was used to obtain CD4 T cells (RosetteSep™ Human $CD4^+$ T cell Enrichment Cocktail, Stemcell Technologies, Grenoble, France) from buffy coats of healthy subjects, according to the manufacturer's instructions. After surface staining, the following populations were sort-purified on a FACSAria cell sorter (BD Biosciences) with >95% purity: memory $CD4^+CD25^-CD45RA^-CD127^{hi}$, naive $CD4^+CD25^-$

$CD45RA^{+}CD127^{hi}$ and Treg $CD4^{+}CD25^{hi}CD127^{lo}$. The study was approved by the Ethical Board of the Faculdade de Medicina da Universidade de Lisboa.

OP9 Stromal Cell Line

OP9 stromal cells expressing the human Notch ligand DL1, DL4 or Jag1 and control OP9 cells transduced with the corresponding empty GFP vector (OP9 Empty) were cultured in DMEM medium (Sigma-Aldrich, St. Louis, USA) supplemented with 15% FBS, 2mM L-glutamine, 100U/ml penicillin-streptomycin and 50µg/ml gentamycin (all from Invitrogen, Carlsbad, California). OP9 monolayers were prepared 24h before their use in co-culture experiments by plating 10G irradiated stromal cells in 48-well culture flat-bottomed plates in complete DMEM medium.

Cell Culture

Human T cells were cultured in IMDM medium (Invitrogen) supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 100U/ml penicillin-streptomycin, 50µg/ml gentamycin, 10mM HEPES, 1mM sodium pyruvate, 50µM 2-mercaptoethanol and 1% non-essential amino acids (IMDM complete medium). Sort-purified memory, naive and Treg CD4 cells were TCR-stimulated with either 1µg or 2µg/ml plate-bound anti-CD3 mAb (clone OKT3, Ebioscience), 1µg/ml soluble anti-CD28 mAb (clone CD28.2, Ebioscience) and 50IU/ml IL-2 (AIDS Research and reference Program, Division of AIDS, NIAID, NIH), at a density of $2.5x10^5$ cells/well in complete IMDM, for 5 days, in the presence or absence of 5ng/ml TGF-β (Peprotech, Rochy Hill, USA). Similar culture conditions were used in the presence of OP9 stromal cells, except that memory cells and Treg were TCR-stimulated with 2 and 1µg/ml soluble anti-CD3 mAb, respectively, plus soluble anti-CD28 mAb, whereas naïve CD4 cells were stimulated with CD3/CD28 Dynabeads (Invitrogen) at 1:16, bead:T cell ratio.

For assessment of iTreg phenotypic stability, memory cells stimulated for 5 days under FOXP3 inducing conditions, were washed, re-plated and cultured for 9 more days in complete IMDM supplemented with 50IU/ml IL-2 and 100nM Rapamycin (Sigma-Aldrich), or restimulated with CD3/CD28 Dynabeads (1:2 ratio; beads:T cells) for 3 more days in medium supplemented with IL-2 12.5IU/ml only or with IL-2 12.5IU/ml, 20ng/ml

IL-6 (PeproTech) and 20ng/ml IL1-β (PeproTech). FOXP3 expression levels on recovered iTreg were evaluated by FACS.

A C2 cell differentiation assay in the presence of OP9 cells was performed as described (24).

***In vitro* Suppression Assay**

Memory CD4 cells stimulated for 5 days under FOXP3-favoring conditions, in the presence of control OP9 or OP9 DL1 cells, were washed and replated in medium supplemented with 50IU/ml IL-2 and 100nM Rapamycin and cultured for 5 more days, before use in the *in vitro* suppression assay. Sort-purified $CD25^{neg}CD4$ cells (targets) were re-suspended at $2.5x10^6$/ml in serum free RPMI, stained with 2.5μM CFSE (Invitrogen) for 5 min at room temperature (reaction stopped by the addition of 20% FCS), washed once in RPMI medium (Invitrogen) supplemented with 10% FCS and stimulated either alone (1:0 ratio) or in the presence of the differentiated iTreg population at the ratios of 1:1, 2:1 and 4:1 (Target:iTreg). Their CFSE-labeling intensity was assessed at day 4 of culture by flow cytometry. As controls, sort-purified Treg from the same healthy donor as the target cells were used. The inhibition index was calculated as follows: [(% proliferating target cells when plated alone - %proliferating target cells when co-cultured with Treg)/ % proliferating targets cells when plated alone]x100.

Signaling Experiments

For assessment of PI3K/Akt/mTOR signaling pathway modulation, sort-purified memory cells were stimulated at a density of $1x10^6$ cells/ml in complete RPMI medium for 14 hours with CD3/CD28 Dynabeads, at a 1:2 ratio (beads:T cells), in the presence of OP9 empty or OP9 DL1 cells. As a control, cells were left in medium alone or stimulated with beads and 100nM Rapamycin in the presence of OP9 control stroma. For assessment of TGF-β signaling pathway modulation, memory cells were cultured at $2.5x10^5$ cells/well in complete RPMI medium without FBS for 3 hours. Cells were then stimulated with or without TGF-β (5ng/ml, PeproTech), in the presence of control OP9 or OP9 DL1 for 2 more hours.

qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the Superscript III Reverse Transcriptase Kit (Invitrogen). The mRNA levels of FOXP3 and GAPDH were quantified in duplicates with TaqMan gene expression kits, according to the manufacturer's instructions, using a 7500 Fast Real-Time PCR System (all from Applied Biosystems, Foster City, USA). *Smad-3* (**forward:** 5'-TGCCTTTCACTCCCCCGAT-3' and **reverse:** 5'- TGCCCCGTCTTCTTGAGTTTC-3'); *Notch1* (**forward:** 5'- CGGGTCCACCAGTTTGAATG-3' and **reverse:** 5'-GTTGTATTGGTTCGGCACCAT-3'); *Notch2* **(forward:** 5'-TTGAGAGTTATACTTGCTTGTGTGC-3' and **reverse:** 5'-GATACACTCGTCAATGTCAATGG-3'); *Notch3* **(forward:** 5'-ATGCAGGATAGCAAGGAGGA-3' and **reverse:** 5'- AAGTGGTCCAACAGCAGCTT-3') and *Notch4* **(forward:** 5'- ACCTGCTCAACGGCTTCCA-3' and **reverse:** 5'-AGCTTCTGCACTCATCGATATCCTC-3') gene expression were quantified with SYBR® Green PCR Master Mix (Applied Biosystems). The relative expression of *FOXP3*, *Notch1* to *Notch4* and *Smad-3* were normalized to GAPDH expression, and the fold change in transcription calculated using the $2^{-\Delta Ct}$ comparative method.

Immunofluorescence

Cells were adhered to poly-L-lysine coated slides, fixed with 4% paraformaldehyde, permeabilized with either 0.1% saponin (Notch ligand and receptor stainings) or 0.5%Triton X-100 (Troponin T staining). The following primary antibodies were used (clones in brackets): anti-Notch1 mAb (MHN1-519; BioLegend, San Diego, USA); anti-Notch2 (C651.6DbHN) and anti-Troponin T (CT3) mAbs (Developmental Studies Hybridoma Bank, USA); anti-DL1 (MHD1-314) and anti-DL4 (MHD4-46) mAbs (kindly provided by Dr. Hideo Yagita, Juntendo University, Japan); anti-Jag1 Ab (H-114, Santa Cruz Biotechnology, USA).
Appropriate fluorochrome-conjugated secondary antibodies were obtained from Invitrogen, Carlsbad, California. DAPI was used as for nuclear counterstaining and slides were mounted in mowiol. Confocal images were acquired with a Zeiss 710 Confocal point-scanning microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v5.01 (GraphPad Software Ic., San Diego, CA, USA), using Wilcoxon matched-pairs signed rank test. Results are expressed as mean±SEM.

Results

DL1-mediated Notch signaling potentiates iTreg conversion from human memory CD4 T cells.

Our first aim was to assess whether human memory CD4 T cells are a reliable source of Treg precursors. With this in mind, naïve ($CD4^+CD25^-CD127^{hi}CD45RA^+$) and memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 T cells were sort-purified from healthy subjects and stimulated *in vitro* with increasing concentrations of plate-bound anti-CD3 mAb, in the presence of anti-CD28 mAb, TGF-β and IL-2. The inclusion of CD127 in the sorting strategy guaranteed an effective exclusion of contaminating FOXP3-expressing cells from the starting populations (25, 26) (Supplemental Figure 1A). A considerable proportion of memory as well as naïve CD4 T cells acquired FOXP3 expression (Supplemental Figure 1A). Only $FOXP3^{bright}$ cells were considered for the analysis in order to ensure their regulatory phenotype. Of note, the anti-TCR Ab requirements for conversion of naïve and memory CD4 T cells were different with the lowest concentration of anti-CD3 mAb tested (1μg/ml) the best suited for *in vitro* conversion protocols using memory CD4 T cells (Supplemental Figure 1A). Importantly, the levels of FOXP3 expression in memory CD4 T cells after induction were equal if not higher to those observed in sort-purified Treg activated with anti-CD3 and anti-CD28 mAbs for the same length of time (Supplemental Figure 1B). Our induction protocol consistently resulted in the acquisition of FOXP3 expression by a significant proportion of memory CD4 cells in all individuals tested (30±3.3% of cells acquiring FOXP3 expression, n=10, as compared to 40±9.2%, n=10, when starting with naïve CD4 T cells).

To investigate the potential role of Notch in the *in vitro* conversion of human conventional CD4 into iTreg, we first performed loss-of-function experiments using the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT), a well-known pharmacological inhibitor of the Notch signaling pathway. Addition of 10μM DAPT to sorted memory CD4 T cells cultured with TCR stimulation, TGF-β and IL-2, led to a consistent decrease in the frequency and number of $FOXP3^+CD25^+$ cells recovered at day 5 (Figure 1A), as well as in their *Foxp3* mRNA expression levels (22.3±4.4%, n=2). These results support the hypothesis that Notch signaling plays a role in the *in vitro* conversion of memory CD4 into iTreg, leading us to evaluate the impact of distinct human

Notch ligands, namely Delta-like 1 (DL1), Delta-like 4 (DL4) and Jagged 1 (Jag1), on the induction of FOXP3 expression, after confirming Notch receptor expression on T cells (Supplemental Figure 1C). Purified memory CD4 cells were co-cultured with either control OP9 cells (transduced with an empty vector) or stromal cells separately expressing functional DL1, DL4 and Jag1 (see Supplemental Figure 1E and 1F), in conditions favoring FOXP3 induction. From the ligands we tested, only DL1 systematically increased the efficiency of FOXP3 acquisition by conventional memory CD4 T cells, excluding a major role for DL4 and Jag1 (Figure 1B). Co-culture of sort-purified memory CD4 cells with OP9 DL1 in FOXP3-inducing conditions significantly increased both the frequency and number of $FOXP3^{+}CD25^{+}$ cells in the 23 individuals tested (Figure 1B). Of note, the comparison of OP9 empty versus OP9 DL1 revealed that DL1 neither modulated the survival of $FOXP3^{neg}$ cells (Bcl-2 MFI: 14403±1474 versus 13509±1385, n=7; cell recovery: $21.1x10^4$±$5x10^4$ versus $19.6x10^4$±$3.6x10^4$, n=23), nor their differentiation profile as assessed by CCR6/CXCR3 expression (data not shown). Moreover, addition of DAPT significantly diminished the frequency of converted FOXP3-expressing cells (27.2±2.5 in empty versus 34.2±3.5 in DL1 versus 24.0±5.3 in DL1+DAPT, n=6), suggesting that Notch receptor cleavage is required for the DL1 effect and reinforcing its role in human iTreg conversion using the OP9 system.

DL1 also potentiated the iTreg generation from human naive CD4 T cells. In fact, DAPT-mediated inhibition of Notch signaling resulted in decreased conversion efficiency (Figure 1C), and the presence of DL1 led to an increased frequency of iTreg generated from naïve CD4 T cells (Figure 1D).

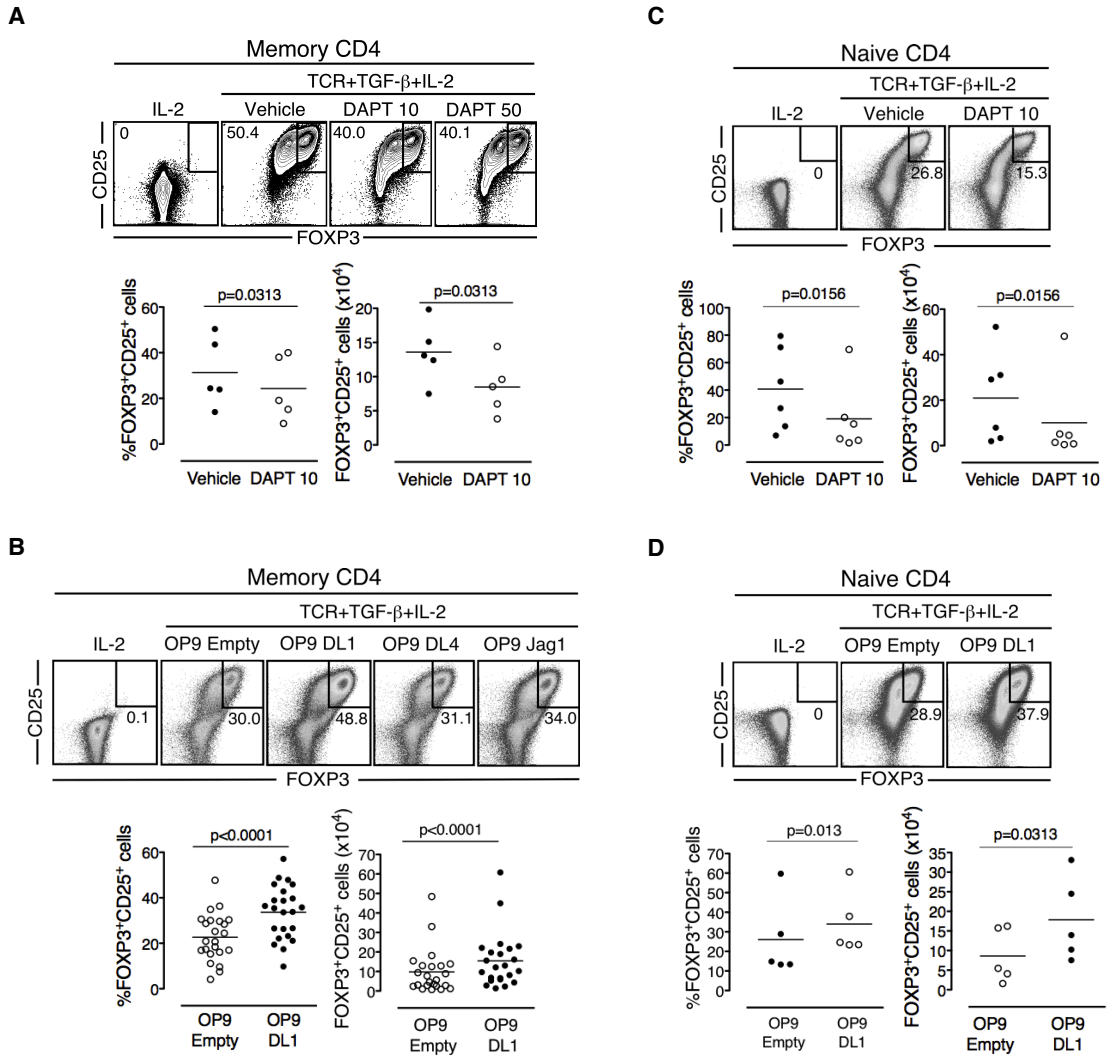


Figure 1. DL1 mediated enhancement of the *in vitro* conversion of memory and naïve CD4 cells into iTreg. Sort-purified memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) and naïve ($CD4^+CD25^-CD127^{hi}CD45RA^+$) CD4 cells were TCR-stimulated for 5 days in medium supplemented with TGF-β and IL-2 (TCR+TGF-β+IL-2). Cells left in medium with IL-2 alone (IL-2) were used as controls. **(A, C)** Dot-plots illustrate FOXP3 and CD25 expression in the presence or absence (vehicle, DMSO) of the γ-secretase inhibitor DAPT (at the indicated concentration in μM) within recovered live cells in memory (A) and naïve (C) CD4 cultures and graphs show the frequency and number of generated $FOXP3^+CD25^+$ cells (n=5 and n=6, memory and naive CD4 cultures, respectively). **(B, D)** Representative dot-plots of CD25 and FOXP3 expression on memory (B) and naive (D) CD4 cells stimulated in the presence of control OP9 stroma cells (Empty) or OP9 stroma expressing the human ligand Delta-like 1 (DL1). Co-cultures of memory CD4 cells with Delta-like 4 (DL4) or Jagged 1 (Jag1) stroma cells are also depicted in (B). Graphs show the frequency and number of $FOXP3^+CD25^+$ cells generated in the presence of OP9 DL1 or OP9 Empty (n=23 and n=5, memory and naive CD4 cultures, respectively).

Overall, these results support the feasibility of targeting human memory and naïve CD4 T cells in protocols of *in vitro* iTreg conversion and show that DL1 increases the efficiency of this process.

DL1 enhances the expression levels of Treg function-associated molecules.

Having established that DL1 enhances the *in vitro* conversion of memory CD4 cells into FOXP3-expressing cells, we next evaluated the phenotype and function of these iTreg. Induction in the presence of DL1 led to significantly higher levels of expression of FOXP3 (Figure 2A), as well as of other Treg lineage-associated markers, namely CTLA-4 (Cytotoxic T-Lymphocyte Antigen-4, Figure 2B) and CD39 (Figure 2C).

Standard *in vitro* suppression assays were used to evaluate the suppressive function of iTreg (27), using as control population freshly-isolated circulating Treg ($CD4^+CD25^{hi}CD127^{lo}$, 80-90% of them expressing FOXP3). The iTreg generated from memory CD4 cells, both in the absence and presence of DL1, efficiently inhibited the proliferation of target cells, in a dose dependent manner (Figure 2D).

Similar results were obtained when targeting human naive CD4 cells, with iTreg generated in the presence of DL1 displaying higher levels of FOXP3 and CD39 expression (Figures 2E and 2F, respectively) and efficient suppressive activity *in vitro* (data not shown).

We also found that DL1 impacted on iTreg induction even in the absence of exogenous TGF-β, significantly increasing both the frequency and number of $FOXP3^+CD25^+$ cells, and enhancing the expression levels of FOXP3 and CD39 as well as of *Foxp3* mRNA (Supplemental Figure 2). Importantly, DL1's effects on iTreg conversion and phenotype were nevertheless more marked in the presence of exogenous TGF-β.

We also evaluated the impact of the Notch signaling pathway on sort-purified circulating Treg, whose expression of Notch receptors we confirmed by qRT-PCR and immunofluoresence (Supplemental Figure 1D). First, we assessed the effect of Notch signaling pathway inhibition using DAPT, and found that it systematically decreased FOXP3 expression levels in TCR-stimulated sort-purified Treg ($CD4^+CD25^{hi}CD127^{lo}$) from healthy subjects (Supplemental Figure 3A). Notably, DL1 also consistently enhanced the expression of FOXP3, CTLA-4 and CD39 on these cells (Supplemental Figures 3B-D).

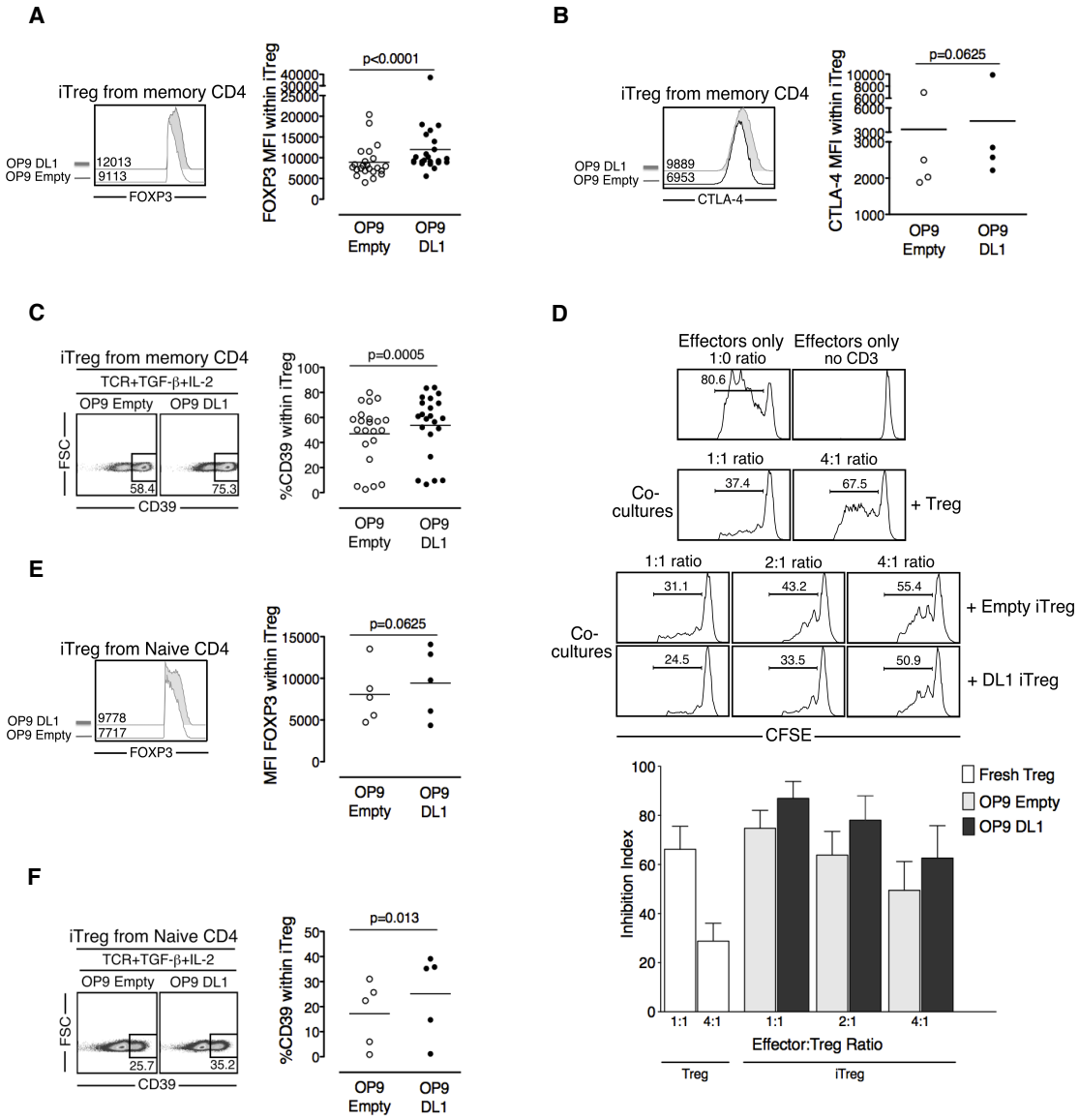


Figure 2. Levels of Treg function-associated molecules and suppressive function of iTreg generated from memory and naïve CD4 cells in the presence of Delta-like 1. Sort-purified memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) and naïve ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 cells were TCR-stimulated for 5 days in medium supplemented with TGF-β and IL-2 (TCR+TGF-β+IL-2), in the presence of OP9 cells expressing Delta-like 1 (DL1) or control OP9 stromal cells (Empty). **(A, E)** Representative histogram of FOXP3 expression and graph of FOXP3 MFI within generated $FOXP3^+$ cells from memory (A) and naive (E) CD4 cultures (n=23 and n=5, memory and naive CD4 cultures, respectively). **(B)** Representative histogram of CTLA-4 expression and graph of CTLA-4 MFI within converted $FOXP3^+$ cells from memory CD4 cells (n=4). **(C, F)** Illustrative dot-plots of the expression of CD39 and graph of the frequency of $CD39^+$ cells within differentiated $FOXP3^+$ iTreg from memory (C) and naive (F) CD4 cells (n=22 and n=5, memory and naive CD4 cultures, respectively). **(D)** Suppressive function of iTreg differentiated from memory CD4 cells, assessed after an additional 5 days resting period in medium supplemented with IL-2 and Rapamycin, using allogeneic target cells ($CD4^+CD25^-$ cells isolated from the peripheral blood of a healthy subject). Representative histograms show the CFSE intensity of target cells when stimulated alone (1:0 ratio) or at the indicated ratios of iTreg or freshly-isolated Treg from the same donor of target cells. Graph shows the inhibition index of fresh Treg and iTreg (n=3), calculated as described in methods.

Overall, our data demonstrated that DL1, in addition to increasing the efficiency of conversion, also enhanced the expression of FOXP3 and other Treg-associated molecules related to effector function, both in the absence and presence of exogenous TGF-β. Moreover, they further support the hypothesis that *bona-fide* iTreg can be differentiated *in vitro* from human memory CD4 cells.

Treg induced in the presence of DL1 display a stable phenotype.

Next we evaluated the stability of the FOXP3-expressing phenotype of iTreg generated from memory CD4 cells, specifically in a pro-inflammatory context, which is of utmost importance for their therapeutic use. After 5 days stimulation in conditions favoring FOXP3 induction (induction period), differentiated cells were left for a further 9 days in medium supplemented with IL-2 and Rapamycin only (resting period), and the expression of FOXP3 was assessed at two separate time-points (days 5 and 9). FOXP3 expression was maintained in iTreg at both time-points (D5 induction: 14.9±3.8 versus 32.7±8.4; D5 resting: 31.6±10.1 versus 38.4±6.3; D9 resting: 35.6±12.0 versus 40.2±8.7; empty versus DL1, n=3), suggesting a stable phenotype in long-term cultures, even in the absence of TCR stimulation and exogenous TGF-β (Figure 3A). Of note, the increased FOXP3 frequency we observed in long-term cultures was not associated with the preferential survival of $FOXP3^+$ cells (data not shown). In addition, we evaluated the behavior of iTreg in the presence of pro-inflammatory cytokines. Cells recovered after the 5-day induction period, were TCR-restimulated in the presence or absence of IL1-β and IL-6 for 3 more days. We observed that $FOXP3^+$ expression was maintained in both culture conditions (D5 induction: 43.4±15.4 versus 55.8±13.0; D3 restimulation, no IL1-β and IL-6: 53.9±18.6 versus 52.2±16.1; D3 restimulation, in presence of IL1-β and IL-6: 52.7±16.2 versus 57.3±13.5; empty versus DL1, n=3) (Figure 3B).

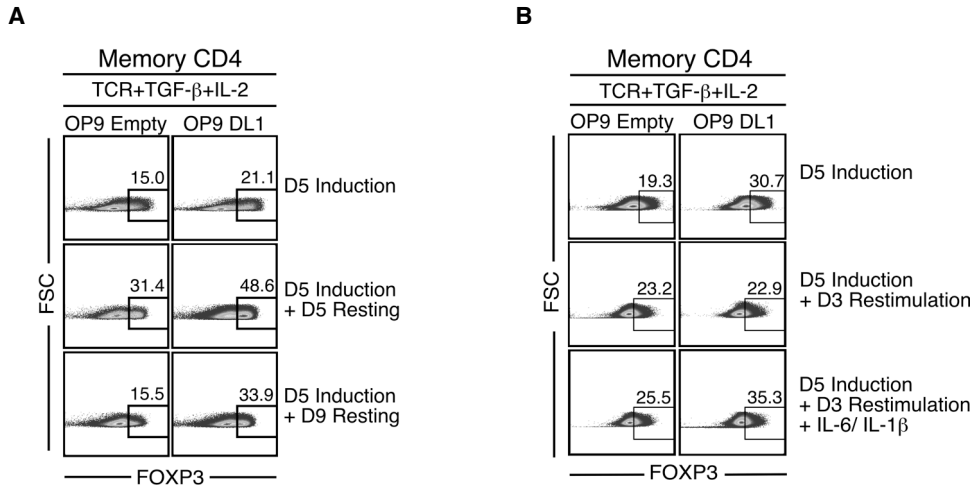


Figure 3. iTreg differentiated from memory CD4 cells exhibited a stable phenotype in long-term cultures and in the presence of pro-inflammatory cytokines. Sort-purified memory CD4 cells ($CD4^{+}CD25^{-}CD127^{hi}CD45RA^{-}$) were TCR-stimulated for 5 days in medium supplemented with TGF-β and IL-2 (TCR+TGF-β+IL-2), in the presence of OP9 cells expressing Delta-like 1 (DL1) or control OP9 stromal cells (Empty). **(A)** Illustrative dot-plots of FOXP3 expression in cells recovered after the induction period as well as after 5 and 9 more days of resting, in medium supplemented with IL-2 and Rapamycin (n=3). **(B)** Illustrative dot-plots of the expression of FOXP3 in cells recovered after the induction period and a further 3 days TCR-restimulation in the presence of IL-2 alone or a combination of IL-2, IL-6 and IL-1β (pro-inflammatory cocktail) (n=3).

Overall, our data indicate that iTreg generated from memory CD4 cells were stable both in long-term cultures and even in the presence of pro-inflammatory cytokines.

Mechanisms mediating DL1 enhancement of iTreg differentiation from human memory CD4 T cells.

Several mechanisms may underlie the DL1-mediated enhancement of FOXP3 induction in memory CD4 cells. Firstly, we hypothesized that DL1 might increase the proliferation and/or promote the preferential survival of $FOXP3^+$ cells during the induction period. However, it is unlikely that either mechanism significantly contributed, as the expression of Ki67 and Bcl2 in the recovered $FOXP3^+$ cells differentiated from sort-purified memory CD4 cells was similar in the presence and absence of DL1 (Figure 4A and 4B, respectively). Similarly, the frequency of $Ki67^+$ cells and the Bcl2 expression levels within $FOXP3^-$ cells were not modulated by DL1 (data not shown). The absence of a DL1 effect on survival was further reinforced by the similar frequency of live cells recovered at day 5 of induction, in the presence or absence of the ligand (data not shown).

The *in vitro* survival of TCR-stimulated circulating Treg was also not altered by the presence of DL1, as assessed by their Bcl2 levels (Supplemental Figure 3E). Notably, the frequency of cycling cells, estimated by the proportion of Ki67 was increased by the ligand (Supplemental Figure 3F), supporting the possibility that Notch signaling is involved in the homeostasis of the human peripheral Treg compartment. Moreover, this result suggests that DL1-mediated Notch signaling outcome is cell type specific.

The PI3K/Akt/mTOR signaling pathway is known to interfere with FOXP3 induction *in vitro* and *in vivo* (11). The phosphorylation levels of S6, a downstream PI3K/Akt/mTOR target, were assessed by flow cytometry in sort-purified memory CD4 cells after TCR-stimulation in the presence of OP9 empty or OP9 DL1 stroma. As a control, cells were either left in medium alone or TCR-stimulated in the presence of Rapamycin, a known mTOR attenuator, and OP9 control cells. We found similar levels of S6 phosphorylation in the presence or absence of DL1, excluding modulation of this pathway as a major contributor to the DL1-mediated enhancement of iTreg conversion (Figure 4C). We also found no impact of DL1 on S6 phosphorylation levels under sub-optimal TCR signal strength as well as on the Akt phosphorylation levels (data not shown). These results excluded the possibility that modulation of PI3K/Akt/mTOR accounted for the positive effects of DL1.

The interaction between Notch and the TGF-β signaling pathway is well described (17-19,

28). We therefore investigated the phosphorylation levels of downstream targets of TGF-β signaling (Smad-2 and Smad-3) in different culture conditions. Sort-purified memory CD4 cells were cultured in complete medium in the absence of serum for 3 hours, and then for a further 2 hours in the presence or absence of TGF-β with either control OP9 or OP9 DL1 cells. We confirmed the expected phosphorylation of Smad-2/-3 in response to TGF-β (Figure 4D). Notably, we found that Smad-2/-3 phosphorylation levels were increased by DL1, even in the absence of exogenous TGF-β, suggesting that Notch cooperatively interacted with TGF-β signaling. Furthermore, DL1 also enhanced the *Smad-3* mRNA expression levels (Figure 4D). Since the addition of a TGF-β blocking antibody led to a reduction of the Smad2/3 phosphorylation induced by DL1, its effect appeared to be at least partly mediated by endogenous TGF-β, possibly produced by the memory CD4 T cells, as supported by our Real-Time PCR data (data not shown).

Finally, DL1 may exert its effects by direct modulation of *Foxp3* gene transcription, since Notch signaling directly targets the Foxp3 promoter via RBP-J and Hes-1 binding sites within it (22). To test this possibility, we quantified *Foxp3* mRNA expression levels in memory CD4 T cells ($CD4^{+}CD25^{-}CD127^{hi}CD45RA^{-}$) after 24 hours culture in conditions favoring FOXP3 acquisition, in the presence of control OP9 or OP9 DL1 cells. The *Foxp3* mRNA expression levels were increased in the presence of DL1 (Figure 4E), supporting our hypothesis.

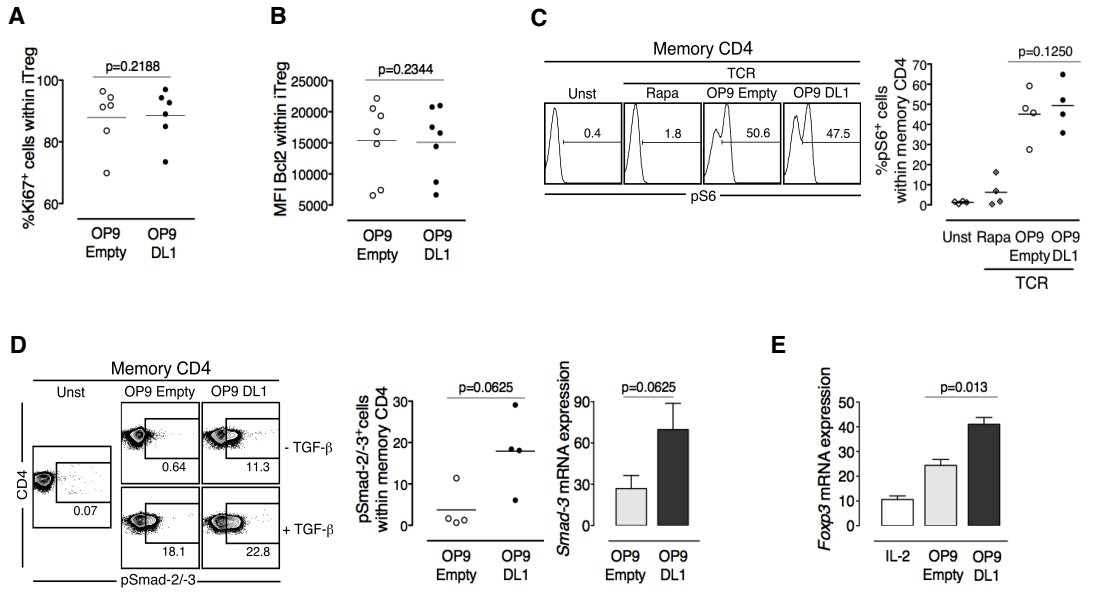


Figure 4. Mechanisms mediating DL1 enhancement of iTreg differentiation from memory CD4 cells. (A-B) Sort-purified memory CD4 cells ($CD4^+CD25^-CD127^{hi}CD45RA^-$) were TCR-stimulated for 5 days in medium supplemented with IL-2 and TGF-β, in the presence of OP9 DL1 (DL1) or control OP9 stroma (Empty). Graphs show the frequency of $Ki67^+$ **(A)** and Bcl2 MFI **(B)** within generated $FOXP3^+$ iTreg (n=6). **(C)** Histograms show S6 phosphorylation levels, as a measure of mTOR activity, assessed after 14 hours TCR-stimulation in the presence of OP9 DL1 or OP9 Empty. Cells left in medium without TCR stimulation (Unstim) or TCR-stimulated in the presence of the mTOR inhibitor Rapamycin (Rapa) and OP9 control stroma were used as controls. Graph shows the frequency of phosphorylated $S6^+$ cells in the described conditions (n=3). **(D)** Illustrative dot-plots of Smad-2/-3 phosphorylation within sort-purified memory CD4 cells stimulated for 2 hours with or without TGF-β, in the presence of OP9 DL1 or OP9 empty, after a 3h resting period in serum-free medium. Graphs show the frequency of Smad-2/-3 phosphorylated cells in the described condition without addition of exogenous TGF-β (n=4) and the *Smad-3* mRNA levels in sort-purified memory CD4 cells TCR-stimulated for 24h in medium supplemented with IL-2, in the presence of OP9 DL1 (DL1) or control OP9 stroma (Empty) (n=4). **(E)** *Foxp3* mRNA levels in sort-purified memory CD4 cells stimulated for 24h as described in (A) (n=3). Cells left in medium with IL-2 alone (IL-2) were used as controls.

Taken together, these data suggest that Notch enhances iTreg differentiation by a dual mechanism: cooperative interaction with the TGF-β pathway and direct modulation of *Foxp3* transcription.

Discussion

This study addressed the efficiency of the *in vitro* conversion of memory CD4 T cells into FOXP3-expressing iTreg and the role of the Notch signaling pathway in modulating this process. Our data demonstrated the feasibility of generating iTreg from human memory CD4 cells, a subset that is likely enriched in cells with auto-reactive specificities in autoimmune patients. Moreover, we showed that DL1-mediated Notch signaling enhances this conversion, offering a new target to facilitate iTreg generation with therapeutic potential. The beneficial DL1 effects were also extended to *in vitro* conversion protocols utilizing conventional naïve CD4 T cells. Additionally, we found that DL1 enhanced the expression levels of molecules associated with Treg function as well as the expansion of circulating human Treg, highlighting a potential role of this signaling pathway in human Treg homeostasis.

A reduced and/or dysfunctional Treg compartment underlies the pathophysiology of many immune-mediated diseases prompting a strong interest in the manipulation of these cells for clinical purposes, particularly in transplantation and autoimmunity. Strategies to obtain sufficient Treg numbers for adoptive transfer rely on extensive *ex vivo* expansion of this subset. However, repeated expansion of Treg may alter their phenotype and function (5). Moreover, peripheral (p)Treg were shown to be ineffective in treating some AID in murine models, due to their unstable phenotype in pro-inflammatory environments (7, 29, 30). Conversely, iTreg have been shown to be stable in inflammatory conditions and resistant to Th17 conversion by IL-6 (7, 31). Thus, there is an increasing interest in iTreg manipulation, with non-regulatory naïve CD4 T cells being used as the starting population in the vast majority of induction studies performed (6-11). Notwithstanding, there is compelling evidence that a significant proportion of circulating Treg in humans may be derived from memory T cells (12-14), as indicated by the marked overlap in their TCR Vβ usage (12). In fact, continuous recruitment from the memory CD4 T cell compartment *in vivo* has been proposed as a major mechanism to maintain a stable pTreg pool over time (12). Accordingly, a few reports show successful *in vitro* induction of FOXP3 in memory CD4 cells (32-34). However there was controversy regarding their phenotype and function, likely due to methodological aspects, mostly related to TCR stimulation strength, suggesting that a suboptimal stimulation provided by lower concentrations of anti-CD3

mAb or Ag may be ideal for efficient iTreg generation (8, 11, 32). Indeed, our results extend the previous data by showing that stable and functional *bona-fide* iTreg can be generated from memory CD4 cells of healthy subjects.

The Notch signaling pathway plays multiple roles in thymic T cell development and peripheral T cell differentiation (35). Accordingly, pathogen-derived signals have been shown to induce or modulate the expression of specific Notch ligands on APC whose interaction with Notch receptors on CD4 T cells have a profound impact on their differentiation towards particular T helper phenotypes (35, 36). The expression of Notch receptors and ligands is nevertheless not restricted to CD4 T cells and APC, respectively. For example, human DC express DL1, Jag1 and Jag2 ligands, in addition to Notch receptors (37). Human Treg were also shown to express Notch1 and Notch4 as well as DL1, Jag1 and Jag2, and thus in principle, being capable of Notch-mediated T-T communication (38). Importantly, the Foxp3 promoter contains Notch-responsive elements (22) and the Treg-associated marker CD25 is also claimed to be a Notch target gene (39). Notably, Systemic Lupus Erythematosus patients with active disease failed to up-regulate Notch1 on T cells upon in *vitro* TCR stimulation, a phenomenon that correlated with decreased CD25 and FOXP3 expression (40).

A role for Notch signaling in human Treg development and expansion has been previously suggested (20, 21, 23). In fact, human cord-blood $CD34^+$ cells differentiate into mature Treg upon co-culture with OP9-DL1, suggesting that Notch is involved in human thymic Treg development (21). Moreover, APC over-expressing human Jag1 promoted the expansion of alloantigen-specific cells with regulatory properties from human naïve CD4 cells (20). In addition, mesenchymal stem cells cultivated with human $CD4^+$ T cells enhanced the recovery of $FOXP3^+$ cells, via a Notch1-mediated mechanism (23). However, these studies did not discriminate whether Notch signaling was promoting Treg expansion and/or Treg *de novo* induction.

We revealed that DL1 impacted upon TCR-stimulated pTreg by increasing their proliferation and expression of Treg function-associated markers, further reinforcing the contribution of the Notch signaling pathway in human Treg homeostasis. Additionally, we showed that *bona-fide* iTreg with a stable and functional phenotype can be efficiently differentiated *in vitro* from human memory CD4 T cells, and this process was enhanced in

the presence of DL1. Based on our results, we suggest that Notch plays a key role in the in *vitro* generation of iTreg, which likely reflects *in vivo* scenarios where their generation from non-regulatory precursors would allow a better control of immune responses. This is the case in the gut, where Notch ligands, including DL1, are highly expressed (41) and where TGF-β-dependent generation of pTreg takes place. Interestingly, we found that DL1 also enhanced FOXP3 and CD39 expression on iTreg generated from conventional memory CD4 cells, even in the absence of exogenous TGF-β. Although we cannot exclude that the TGF-β produced by the T cells themselves contributes to the DL1-mediated effect, these results suggest that even in situations in which TGF-β is limiting, DL1 may help controlling the magnitude of memory CD4 T cell responses.

The observation of a lack of significant DL4 impact on iTreg differentiation from memory cells is possibly unexpected. Nevertheless, DL1 and DL4 have a differential effect on early T cell activation and proliferation upon TCR cross-linking (42), which may affect the efficiency of conversion. Our monitoring of the levels of expression of each ligand on the OP9 stromal cells by FACS revealed a lower expression of DL4 than DL1 (Supplemental Figure 1E), which may have contributed to the much more striking effects of DL1.

Concerning the mechanisms underlying the DL1-mediated enhancement of iTreg differentiation from memory cells, we excluded a significant impact of Notch signaling on iTreg proliferation and/or survival during the induction period. This was possibly unexpected, given the recognized role of Notch in the protection of activated CD4 T cells from apoptosis after an initial phase of clonal expansion, by inducing a broad anti-apoptotic gene expression signature (43). We showed that DL1 cooperatively interacts with the TGF-β signaling pathway, as evidenced by increased phosphorylation of the downstream TGF-β signaling pathway targets Smad-2 and -3. TGF-β is a pleiotropic anti-inflammatory cytokine required for iTreg differentiation. Although direct and indirect mechanisms have been implicated in TGF-β's role in pTreg pool maintenance, recent studies suggest that its downstream targets, specifically Smad-3, directly activate the *Foxp3* gene (44). The interaction between Notch and TGF-β signaling pathways has been repeatedly described and is probably both cell type- and context-dependent. Previous reports showed that Notch intracellular domain increases Smad3 protein at the transcriptional and post-transcriptional levels, as well as Smad nuclear translocation and its

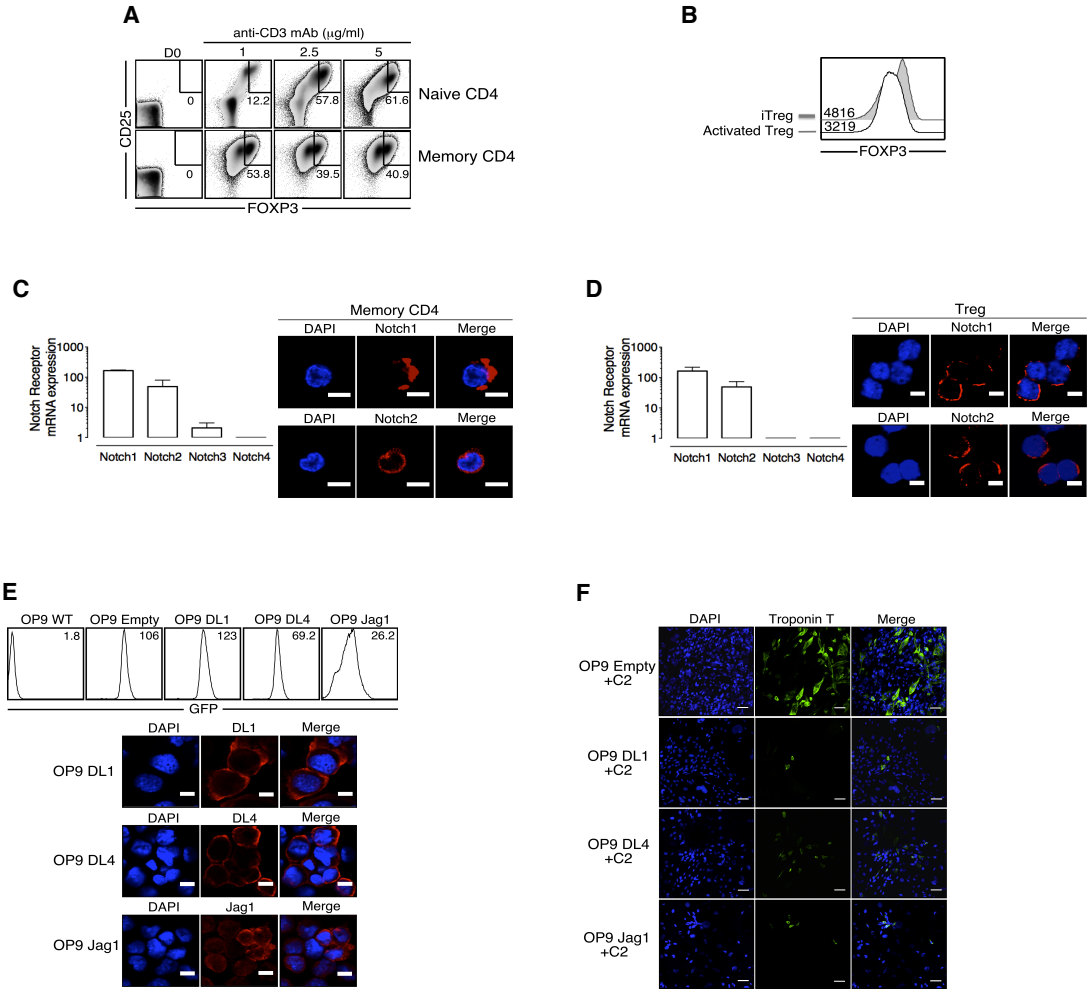
transactivation at promoter sites (18, 28). Moreover, Notch ligands increase Treg suppressive function via the upregulation of TGF-β receptor expression and phosphorylation of Smad3 in effector T cells (19). We therefore hypothesize that the enhanced Smad-2 and -3 phosphorylation we observed in the presence of DL1, an effect particularly evident in the absence of exogenous TGF-β, might occur via up-regulation of TGF-β receptor and transcriptional/pos-transcriptional modulation of Smad3 promoted by the ligand.

Direct targeting of the Foxp3 promoter by Notch is supported by the presence of RBP-J and Hes-1 binding sites within it, in both mice and humans (22). Accordingly, our results indicated increased *Foxp3* mRNA expression in the presence of DL1, supporting an involvement of direct modulation of *Foxp3* gene transcription in the Notch-mediated enhanced conversion of memory CD4 T cells into iTreg.

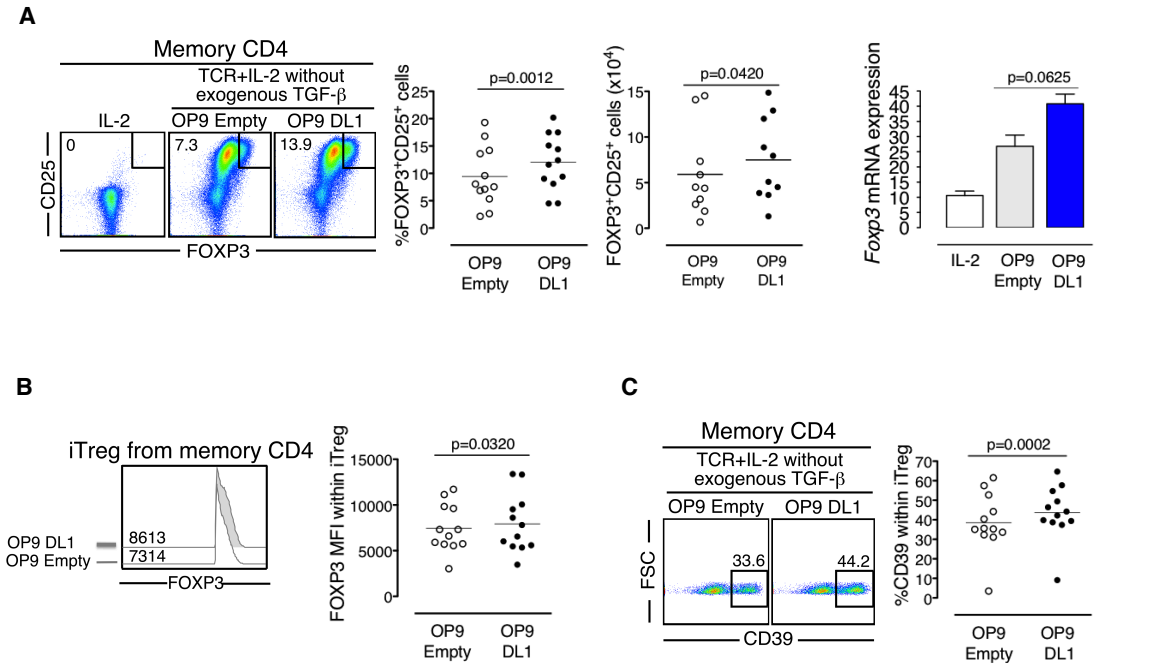
Taken together, our data suggest that DL1 impacts on FOXP3 acquisition by memory CD4 cells through various mechanisms, such as the cooperative interaction with TGF-β signaling pathway and the modulation of *Foxp3* transcription. Other possibilities, such as direct down-regulation of the IL-6 receptor alpha chain on memory CD4 cells by DL1, as recently shown in human $CD34^{+}$ cells (45), cannot be excluded and warrant further investigation.

In conclusion, we provide evidence that manipulation of the Notch signaling pathway, both in Treg expansion and iTreg conversion protocols may help facilitating the use of Treg based-therapies.

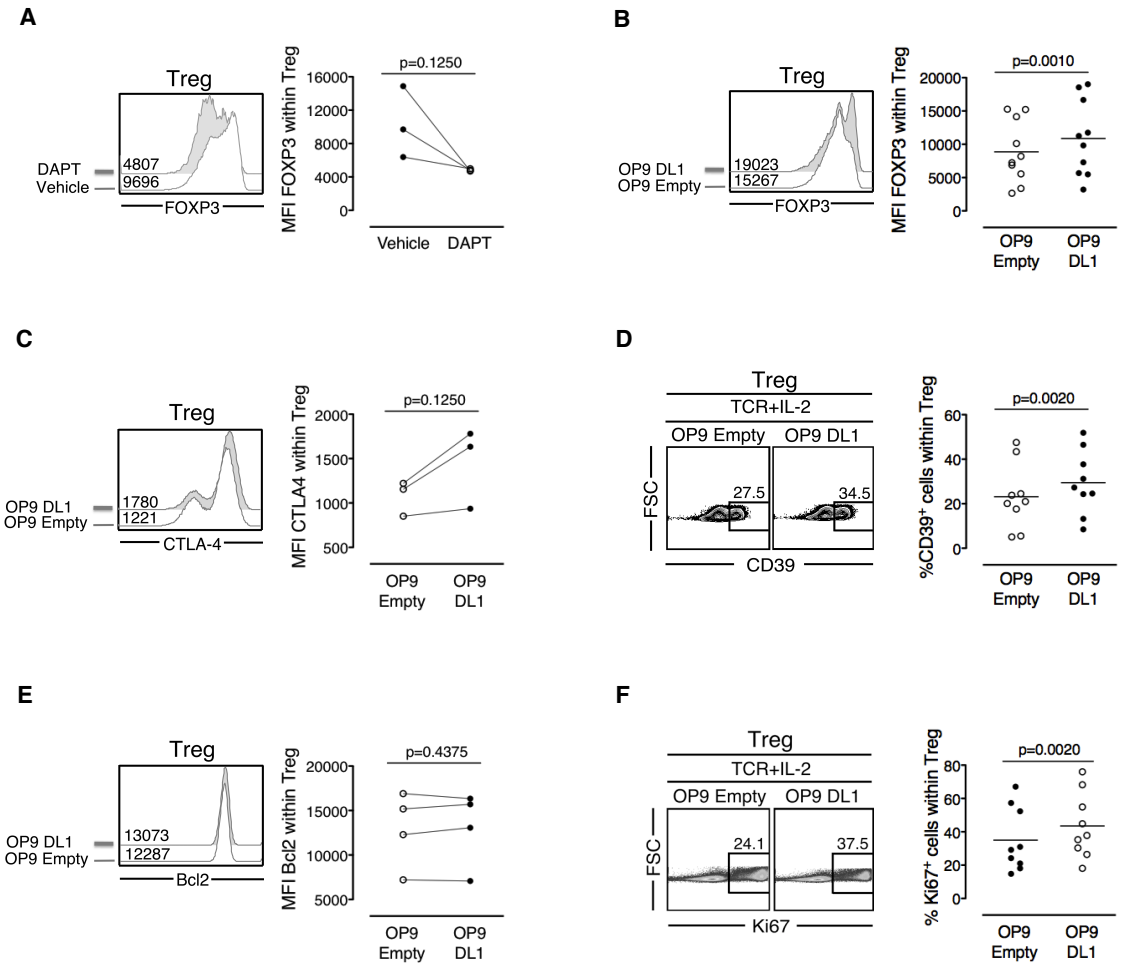
Supplemental Data



Supplemental Figure 1. Methodological validation of the protocol to induce iTreg from naïve and memory CD4 cells, characterization of these cells and the stroma cell lines used. **(A)** Purified naïve ($CD4^+CD25^-CD127^{hi}CD45RA^+$) and memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 T cells were TCR-stimulated for 5 days with the indicated concentrations of plate bound anti-CD3 mAb and soluble anti-CD28 mAb, in the presence of TGF-β and IL-2. Illustrative dot-plots of FOXP3 and CD25 expression post-sorting (D0) and after stimulation. **(B)** Histogram overlay illustrates FOXP3 and CD25 expression within memory CD4 cells after stimulation (iTreg) and in sort-purified Treg ($CD4^+CD25^{hi}CD127^{lo}$) stimulated for 5 days with anti-CD3/anti-CD28 mAbs, plus IL-2 (activated Treg). **(C,D)** Notch receptor mRNA expression levels in freshly-isolated memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 cells (C) and circulating Treg ($CD4^+CD25^{hi}CD127^{lo}$) (D). Notch1 and 2 protein expression on 24h TCR-activated memory $CD4^+$ cells (C) and Treg (D), determined by immunofluorescence, is also shown. Scale bars 5µm; original magnification x630 and x400, in (C) and (D), respectively. **(E)** Histogram shows Notch ligand expression levels on the OP9 stroma cells used, determined by FACS. The expression of DL1, DL4 and Jag1 on the corresponding stroma line, assessed by immunofluorescence, is also shown. Scale bar 10µm; original magnification x200. **(F)** DL1, DL4 and Jag1 expressed by OP9 cells inhibit the differentiation of C2 myoblast cells into myotubes, as indicated by Troponin T immunofluorescence. Scale bar 50µm; original magnification x200.



Supplemental Figure 2. Inducible (i)Treg generated *in vitro* from memory CD4 cells, in the absence of exogenous TGF-β. Sort-purified memory CD4 cells were TCR-stimulated in medium supplemented with IL-2, without addition of exogenous TGF-β, in the presence of OP9 stromal cells expressing the human ligand Delta-like 1 ligand (DL1) or control OP9 stroma (Empty) for 5 days. (A) Graphs depict the frequency (n=12) and number of generated $FOXP3^{+}CD25^{+}$ cells (n=10) as well as *Foxp3* mRNA levels after 24h stimulation (n=3). Representative histogram of FOXP3 MFI (B) and illustrative dot-plots of CD39 expression (C) within generated $FOXP3^{+}$ iTreg. Graphs show FOXP3 MFI and CD39 frequency within converted $FOXP3^{+}$ iTreg (n=12). Cells left in medium with IL-2 alone (IL-2) were used as controls.



Supplemental Figure 3. Notch signaling impact on the phenotype, survival and proliferation of circulating Treg. (A) Sort-purified Treg ($CD4^{+}CD25^{hi}CD127^{lo}$) were TCR-stimulated for 5 days in medium supplemented with IL-2, in the presence or absence (vehicle, DMSO) of the γ-secretase inhibitor DAPT (10μM). Histograms show FOXP3 expression and graph depicts the FOXP3 MFI within recovered cells (n=3). (B-F) Representative histograms, dot-plots and graphs show the analysis of sort-purified Treg, TCR-stimulated for 5 days in medium supplemented with IL-2, in the presence of OP9 cells expressing the human Delta-like 1 ligand (DL1) or control OP9 stromal cells (Empty) for the expression of: (B) FOXP3 (n=10); (C) CTLA-4 (n=3); (D) CD39 (n=9); (E) Bcl2 (n=4); and (F) $Ki67^{+}$ (n=9) within recovered cells.

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3.1.1. Annex 1: DL1-mediated Notch signaling impact on the differentiation of human non-regulatory CD4 thymocytes into FOXP3-expressing cells

The Notch pathaway is an important signaling cascade in several stages of T cell development/ differentiation[1]. In mice, Notch modulates the thymic generation of Treg and the *in vitro* conversion of conventional T cells into the Treg phenotype[2, 3, 4, 5, 6]. In order to better understand the role of Notch signaling on human tTreg development, we evaluated its impact on the differentiation of mature non-regulatory $CD4^+$ thymocytes into FOXP3-expressing cells.

With this purpose, we first performed loss-of-function experiments using the γ-secretase inhibitor DAPT, a pharmacological inhibitor of Notch. Addition of 10μM DAPT to sorted $CD4SPCD25^{neg}CD127^{hi}$ cells cultured with TCR stimulation, TGF-β and IL-2, led to a consistent reduction in the efficiency of conversion (on average 40%, n=4) as monitored by the frequency of $CD25^+FOXP3^+$ cells determined by FACS at day 5 (Figure 1). This result supported the hypothesis that Notch signaling plays a role in the differentiation of human non-regulatory CD4SP thymocytes into Treg.

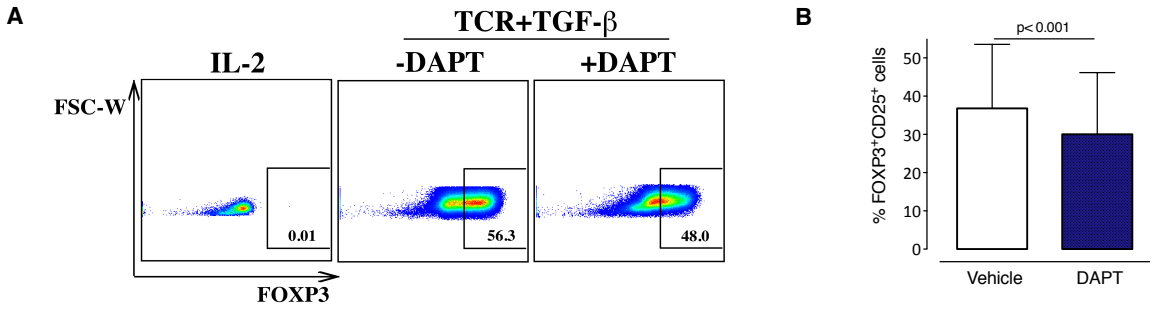

Figure 1. Notch signaling inhibition decreases the *in vitro* differentiation of human thymocytes into Treg. (A) Panel represents a typical FACS profile of the frequency of $FOXP3^+$ cells on live cells arising at Day 5 in cultures of sort-purified Single Positive (SP)$CD4^+CD3^{hi}$ $CD25^-CD127^{hi}$ thymocytes stimulated with plate bound anti-CD3 mAb, anti-CD28 mAb, TGF-β and IL-2, in the absence (-DAPT) or presence (DAPT) of the γ-secretase inhibitor DAPT. The proportion of $FOXP3^+$ cells in $SPCD4^+CD3^{hi}$ $CD25^-CD127^{hi}$ thymocytes left for five days in medium supplemented with IL-2 alone (IL-2) is also shown. **(B)** The frequency of $FOXP3^+CD25^+$ cells in cultures of sort-purified $SPCD4^+CD3^{hi}CD25^-CD127^{hi}$ thymocytes left in IL-2 alone or stimulated with anti-CD3 and anti-CD28 mAbs, TGF-β as well as IL-2, in the presence or absence of DAPT was monitored by flow cytometry at Day 5. Results correspond to a pool of four independent experiments.

Sorted $CD4SPCD25^{neg}CD127^{hi}$ thymocytes were then co-cultured in conditions favoring FOXP3 induction, in the presence of either control OP9 cells (transduced with an empty vector) or stromal cells expressing DL1. We found that DL1 systematically increased the frequency and number of $CD25^+FOXP3^+$ cells in the 9 samples tested (Figure 2). Using a similar co-culture system, we excluded a major contribution of two other Notch ligands, DL4 and JAG1 and focused our attention in DL1. We also found that DL1 enhanced the frequency and number of $FOXP3^+CD25^+$ cells (on average 50%, n=4) even in the absence of exogenous TGF-β, hypothesizing that DL1 may partially substitute for TGF-β in the thymus. Having established that DL1 enhanced the differentiation of non-regulatory CD4SP thymocytes into FOXP3-expressing cells, we next evaluated the phenotype of these cells. Induction in the presence of DL1 led to significantly higher levels of expression of FOXP3 as well as of other Treg lineage-associated markers, namely CD39 (Figure 2).

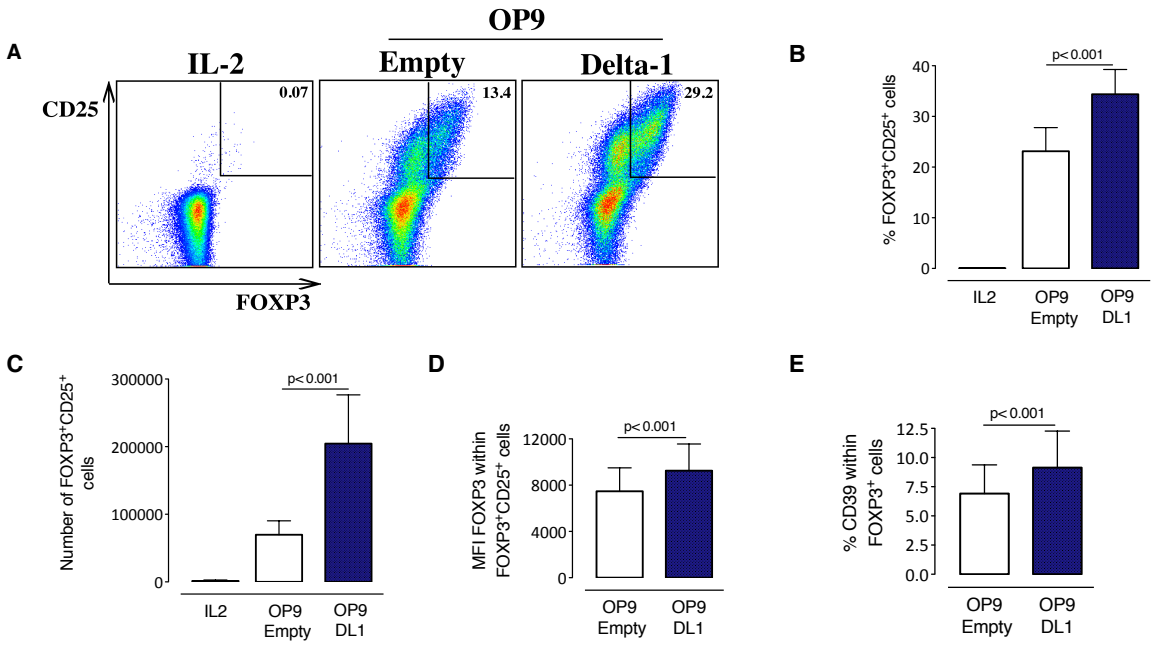

Figure 2. DL1 mediated enhancement of the *in vitro* conversion of human thymocytes into iTreg. (A) SPCD4^{+}CD3^{hi}CD25^{-}CD127^{hi} cells were sort-purified from mononuclear cells isolated from thymic tissue. Cells were cultures for 5 days in the presence of IL-2 alone. Stimulation of SPCD4^{+}CD3^{hi}CD25^{-}CD127^{hi} thymocytes with anti-CD3 mAb, TGF-β and IL-2 in the presence of control OP9 stromal cells transduced with an empty vector (Empty) or in the presence of OP9 stromal cells transduced with Delta-like 1 (Dl1) ligand (Delta-1) was also performed. Representative dot plots show the frequency of CD25^{+}FOXP3^{+} cells in cells recovered from each culture condition, as assessed by flow cytometry. The frequency **(B)** and number of FOXP3^{+}CD25^{+} cells **(C)** arising from SPCD4^{+}CD3^{hi}CD25^{-}CD127^{hi} thymocytes stimulated in the presence of control OP9 stromal cells (Empty) or OP9 cells expressing Dl1 (Delta-1) was monitored at Day 5. The proportion of FOXP3^{+} cells expressing the Treg associated marker CD39 **(D)** and the mean fluorescence intensity (MFI) of FOXP3 on FOXP3^{+} cells **(E)** recovered from each culture condition was also determined. Results correspond to a pool of nine independent experiments.

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3.1.2. Annex 2: DL1-mediated Notch signaling potentiates iTreg conversion from human memory CD4 T cells isolated from autoimmune patients

Autoimmune diseases (AID) are a heterogeneous group of more than 100 chronic diseases that result from an imbalance between self-recognition and protection to non-self. Treg have been show to play a major role in the complex pathogenesis of several AID, hence appearing as an attractive target for use in the most recent therapeutic approaches in autoimmunity.

The capacity of DL1 to enhance iTreg conversion from memory non-regulatory CD4 cells was evaluated in a AID context, using memory conventional CD4 cells isolated from a Systemic Lupus Erythematosus (SLE) patient in stable remission.

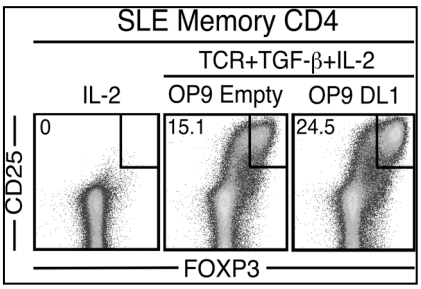

Figure 1. DL1-mediated enhancement of the *in vitro* conversion of memory CD4 cells from an SLE patient into iTreg. Sort-purified memory CD4 cells ($CD4^+CD25^-CD127^{hi}CD45RA^-$) from an SLE patient in stable remission were TCR-stimulated for 5 days in medium supplemented with TGF-β and IL-2 (TCR+TGF-β+IL-2). Cells left in medium with IL-2 alone (IL-2) were used as controls. Representative dot-plots of CD25 and FOXP3 expression on cells stimulated in the presence of control OP9 stroma cells (Empty) or OP9 stroma expressing the human ligand Delta-like 1 (DL1).

We confirmed that memory non-regulatory $CD4^+$ T cells isolated from an SLE patient can efficiently be converted into FOXP3-expressing cells. Moreover, as observed for memory $CD4^+$ T cells from healthy individuals, DL1 enhances the efficiency of iTreg conversion. These additional data support the feasibility of targeting human memory $CD4^+$ T cells in protocols of *in vitro* iTreg conversion in the particular setting of autoimmune pathology, opening new avenues for its treatment.

3.1.3. Annex 3: Targeting self-reactivities in SLE patients

SLE is a systemic AID where the dominant autoimmune response is the production of an array of autoantibodies to self-antigens including nuclear components (DNA, RNA and histones). In SLE, the function of Treg seems defective and their number appears to decrease during the active phases of disease[1, 2, 3]. These observations support the hypothesis that adoptive transfer of Treg is an attractive therapy for SLE. Importantly, in SLE several T cell epitopes have been identified in histones, ribonucleoproteins and anti-DNA antibodies constituting potential specificities to be targeted in immunotherapy[4]. Thus, we aim to use self-reactivities present in conventional CD4 pool of SLE patients and target them to generate antigen-specific Treg for future use in SLE immunotherapy.

In the lead-off experiment below, we sort-purified memory $CD4^{+}CD25^{-}CD127^{hi}$ cells from the peripheral blood of an SLE patient in remission. We thus monitored the proliferative response of this population to a panel of self-peptides relevant in SLE[4]. Irradiated PBMC were used as a source of APC. Our preliminary data showed significant proliferative response of CD25-depleted PBMC from an SLE patient to a cocktail of relevant SLE peptides (Figure 1).

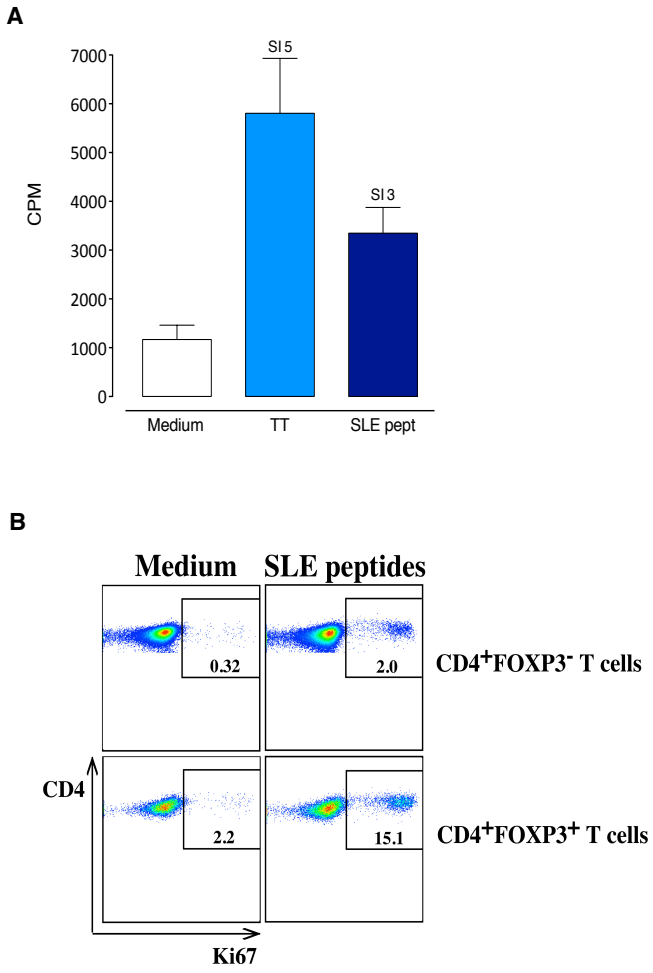

Figure 1: Proliferative response to a cocktail of SLE peptides in a SLE patient. **(A)** Peripheral blood mononuclear cells (PBMC) from an SLE patient in stable remission, with a SELENA-SLEDAI score of 3, were depleted of CD25-expressing cells by magnetic cell sorting and cultured in the presence or absence of the antigen tetanus toxoid (TT) and a cocktail of SLE self-peptides (SmDl 83-119 peptide, H4 71-94 peptide and spliceosomal U1-70K protein 131–151 peptide). Proliferation was measured by tritiated thymidine incorporation at day 6. Stimulation Index (SI) was calculated by dividing the mean counts per minute (cpm) of cells exposed to each antigen by the mean cpm of cells incubated with medium alone. **(B)** Frequency of $Ki67^+$ cells within gated $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells in PBMC of the SLE patient cultured in the absence (Medium) or presence of the SLE peptide cocktail for 6 days, as determined by flow cytometry.

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3.2. Evidence for a two-step developmental program of thymus-derived human Regulatory T cells

Human Regulatory T-cell development is dictated by Interleukin-2 and -15 expressed in a non-overlapping pattern in the thymus

Human Regulatory T-cell development is dictated by Interleukin-2 and -15 expressed in a non-overlapping pattern in the thymus

Iris Caramalho, Vânia Nunes-Silva, Ana R. Pires, Catarina Mota, Ana I. Pinto, Helena Nunes-Cabaço, Russell B. Foxall and Ana E. Sousa

Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Portugal

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Author contribution: Discussion of the experimental design; Data processing for flow cytometry analysis and sample acquisition; Data interpretation; Discussion of the results for the final preparation of the manuscript.

Abstract

Thymus-derived FOXP3-expressing Regulatory T-cells (tTregs) are master orchestrators of physiological and pathological immune responses, thus constituting ideal targets for the treatment of autoimmunity. Despite their clinical importance, the developmental program governing their differentiation in the human thymus remains poorly understood.

Here, we investigated the role of common gamma-chain cytokines in human tTreg differentiation, by performing gain- and loss-of-function experiments in 3D and 2D postnatal thymic cultures. We identified IL-2 and IL-15 as key molecular determinants in this process and excluded a major function for IL-4, IL-7 and IL-21. Mechanistically, IL-2 and IL-15 were equally able to drive tTreg precursor differentiation into $FOXP3^+$ cells, and promote tTreg proliferation and survival. Both cytokines also increased the expression levels of molecules associated with effector function within $FOXP3^+$ subsets, supporting their involvement in tTreg functional maturation. Furthermore, we revealed that IL-2 and IL-15 are expressed in a non-overlapping pattern in the human thymus, with the former produced mainly by mature αβ and γδ thymocytes and the latter by monocyte/macrophages and B lymphocytes.

Our results identify core mechanisms dictating human tTreg development, with IL-2 and IL-15 defining specific niches required for tTreg lineage stabilization and differentiation, with implications for their therapeutic targeting in autoimmune conditions.

Introduction

FOXP3-expressing Regulatory T-cells (Tregs) emerge as a distinct lineage through a process favouring the selection of thymocytes bearing self-reactive T-cell Receptors. Although Tregs can also be generated extra-thymically from conventional T-cells under specific stimulatory conditions, thymus-derived (t)Tregs are considered the major contributors to the peripheral Treg pool [1-3]. The transcription factor FOXP3 is essential for their development and effector function, as demonstrated by the clinical severity and associated mortality of loss-of-function mutations in the gene encoding this transcription factor in humans and mice [1-5]. Importantly, despite the clinical potential of Tregs in the context of autoimmunity, allergy and transplantation tolerance little is known about their ontogeny in the human thymus.

Human T-cell development progresses through a series of sequential stages defined by the surface expression of CD4, CD8 and CD3. T-cell progenitors contained within the early $CD3^{neg}CD4^{neg}CD8^{neg}$ Triple Negative (TN) subset initially acquire CD4 (becoming CD4 Immature Single Positive cells, CD4ISP) and subsequently CD8 expression, giving rise to Double-Positive (DP) thymocytes in the cortex. A progressive increase in surface CD3 expression occurs in parallel with surface TCRαβ in DP cells, followed by final differentiation into CD4 Single-Positive (SP) and CD8SP thymocytes that mature in the medulla [6].

Human Treg development appears to occur concomitantly with the generation of the counterpart $FOXP3^{neg}$ population, with these cells being documented in fetal thymus as early as the 13^{th} week of gestation, prior to their appearance in peripheral sites [7, 8]. FOXP3 induction within developing thymocytes is driven by TCR signaling and associated with positive selection [7-11]. Expression of FOXP3 is indeed clearly detected in post-selection DP thymocytes [7, 8, 11], which we have shown to significantly contribute to the mature $FOXP3^{+}$ pool that is largely composed of CD4SP and some CD8SP thymocytes [11]. Others have shown that human tTregs can be selected by both myeloid and plasmocytoid dendritic cells (DCs) [10, 12, 13]. $FOXP3^{+}$ cells accumulate in the medulla, where the majority of them can be found. Furthermore, FOXP3-expressing mature CD4SP and CD8SP display regulatory properties in both human fetal and postnatal thymus [7, 8, 11, 14, 15].

Treg homeostasis and function in the periphery depends on Interleukin (IL)-2 [16, 17]. Here, we hypothesized that IL-2, and possibly other common-gamma chain (γc) cytokine family members, could play a role in human tTreg development. This family, that shares the use of γc to generate signaling receptor complexes, includes cytokines fundamental for the development and differentiation of immune cells, namely IL-4, IL-7, IL-9, IL-15, and IL-21, in addition to IL-2 [16, 17]. Notably, human tTreg express the α- (CD25) and the β-chain (CD122) of the IL-2 Receptor (R) that are utilised by IL-2 and IL-15 [8, 11]. Moreover, we demonstrated that human FOXP3^{+} thymocytes, although featuring reduced levels of the α-chain of the IL-7R compared to their FOXP3^{neg} counterparts, phosphorylate STAT-5 in response to IL-7 [11]. Moreover, IL-2 increased CD25 and FOXP3 expression levels within FOXP3^{+}DP thymocytes [11]. Thus, existing data support a role for IL-2 and IL-7 in human tTreg development. Importantly, polymorphisms in IL-2, CD25 or IL-2R downstream signaling molecules are associated with impaired Treg number and/or function as well as increased risk of autoimmunity in humans [16], which may be due to defective tTreg generation in addition to reduced peripheral Treg survival. Accordingly, indirect evidence supports a thymic involvement in patients undergoing IL-2 therapy, based on the observed expansion of Tregs expressing CD45RA and the recent thymic emigrant marker CD31 [18-20].

Considerable differences exist between human and murine tTreg development. Human FOXP3^{+} thymocyte development occurs in parallel with the counterpart FOXP3^{neg} population, whereas in mice their appearance is delayed [2, 7, 8]. Moreover, whereas the vast majority of murine tTregs arise at the CD4SP stage, increasing evidence indicates an earlier commitment into the Treg lineage in humans [2, 3, 7-11]. Nonetheless, data generated in murine models support our hypothesis [2, 3, 16, 17].

In this study, we investigated the role of γc cytokines in human tTreg development, by performing gain- and loss-of-function experiments in 3D and 2D postnatal thymic cultures, using recombinant cytokines, specific blocking antibodies and pharmacological inhibitors of their signaling pathway. We identified IL-2 and IL-15, expressed in a non-overlapping pattern, as key γc cytokines, both able to drive human tTreg precursor differentiation into FOXP3^{+} cells as well as to promote tTreg proliferation and survival.

Material and Methods

Samples

Thymic specimens were obtained from thymectomy during pediatric corrective cardiac surgery (newborns to 4-year old) at Santa Cruz Hospital, after parents´ informed consent. Study was approved by the Ethical Boards of the Faculty of Medicine of Lisbon, of Santa Maria and of Santa Cruz Hospitals. Thymocytes and Thymic Epithelial Cells (TECs) were isolated as described [9, 11, 21].

FACS analyses

The following anti-human monoclonal antibodies (BD Biosciences or eBioscience, clones in brackets) were used: CD3 (UCHT1), CD4 (RPA-T4), CD8a (SK1), CD11c (3.9), CD14 (61D3), CD16 (eBioCB16), CD20 (2H7), CD25 (2A3), CD31 (WM59), CD123 (6H6), αβ TCR (IP26), γλ TCR (B1.1), HLA-DR (L243), Bcl-2 (124), CTLA-4 (BNI3), FOXP3 (PCH101), Ki67 (B56), ICOS (ISA-3), Epcam (1B7) and pan-cytokeratin (C-11). After surface staining cells were fixed, permeabilized and stained for FOXP3 and other intracellular molecules using the FOXP3 staining kit (eBioscience). Samples were acquired on a FACSCanto or LSR Fortessa (BD Biosciences) and analyses performed using FlowJo software (TreeStar), after stringent exclusion of cell aggregates, based on area and width parameters of both forward and side scatter, and *dead cells by gating-out* near-IR or violet *LIVE/DEAD® fixable dye* positive cells (Invitrogen). Annexin V and PI staining were performed with the Annexin V apoptosis detection kit (BD Biosciences).

Cell sorting

Cell sorting was performed with a FACSAria III (BD Biosciences). $CD25^{neg}CD127^{hi}$CD4SP and $CD25^{+}CD127^{hi}$CD4SP were purified from total thymocytes as $CD4^{+}CD8^{neg}CD3^{hi}CD127^{hi}$ cells, subdivided according to their CD25 expression level. The lineage markers CD11c, CD14, CD16, CD20, CD56, CD123 and HLA-DR were combined with CD3, CD4 and CD8 staining to sort TN ($Lineage^{neg}$ $CD3^{neg}CD4^{neg}CD8^{neg}$) and CD4ISP thymocytes ($Lineage^{neg}$ $CD3^{neg}CD8^{neg}CD4^{+}$). γδ cells were purified as TCR-

$\gamma\delta^{+}CD3^{+}$. Purification of B cells ($CD20^{+}HLA\text{-}DR^{+}$), pDC ($CD123^{+}HLA\text{-}DR^{lo}$), NK ($CD56^{+}/CD16^{+}CD4^{neg}CD8^{neg}CD3^{neg}$) and monocyte/macrophages ($CD14^{+}$) was performed after CD3-expressing thymocyte depletion using EasySep™ Human CD3 Positive Selection Kit (StemCell Tecnologies). Purity was routinely ≥95%.

Cell Cultures

The following reagents were used: anti-CD3 (clone OKT3), anti-CD28 (clone CD28.2) and mouse IgG1 mAbs, (eBioscience); Dynabeads® Human T-Activator CD3/CD28 (Invitrogen); JAK-3 inhibitor CP690550 (Axon Medchem); STAT-5 inhibitor N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (Calbiochem); anti-CD122 mAb (clone TU27, BioLegend); anti-IL-2 (clone 5334) and anti-IL-15 mAbs (clone 34593), (R&D); IL-2 (AIDS Research and reference Program, Division of AIDS, NIAID, NIH); IL-4 and IL-7 (R&D); IL-15, IL-21 and TGF-β (PeproTech). Complete RPMI and IMDM medium were made by supplementation of respective media with 10% FCS, L-glutamine, sodium pyruvate, Hepes, Non-essential aminoacids, Penicillin/Streptomycin, 2-mercaptoethanol and gentamicin (all from Invitrogen). Total thymocytes ($2x10^{6}$) were co-cultured with primary allogeneic TECs ($4x10^{4}$) in complete RPMI medium alone or supplemented with IL-2, IL-4, IL-7, IL-15 or IL-21. For $CD25^{neg}CD127^{hi}$CD4SP thymocyte differentiation into FOXP3-expressing cells, sorted thymocytes ($2.5x10^{5}$ cells) were stimulated with 1μg/ml plate-bound anti-CD3 mAb, 1μg/ml soluble anti-CD28 mAb and 5ng/ml TGF-β, in complete IMDM medium alone or supplemented with IL-2, IL-4, IL-7, IL-15 or IL-21, for 5 days [22]; parallel cultures consisted of thymocytes TCR-stimulated in the presence of TGF-β and IL-2, to which either IL-4, IL-7, IL-15 or IL-21 was added.

Thymic Organ Cultures (TOCs)

Thymic tissue was cut into ~2 mm^{3} pieces and placed over Isopore membranes (Millipore) supported on Gelfoam [23, 24], under the indicated culture conditions in complete RPMI medium, either alone or supplemented with IL-2, IL-15, anti-IL-2 mAb, anti-IL-15 mAb, JAK-3 inhibitor, STAT-5 inhibitor and anti-CD122 mAb.

Suppression Assays

The $CD25^{high}CD4SP$ populations under test were sort-purified at day 7 from co-cultures of thymocytes with TECs set in the presence of IL-2 (10U/ml) or IL-15 (12.5ng/ml). Control $CD25^{high}CD4$ cells and $CD25^{neg}CD4SP$ (targets) were sorted from an allogeneic thymus. To evaluate their suppressor function, each population was plated at various numbers together with $2.5x10^4$ $CD25^{neg}CD4SP$ thymocytes labeled with CFSE (2.5µM CFSE from Invitrogen, for 5 min at room temperature). Stimulation was provided by CD3/CD28 Dynabeads (bead:cell ratio 1:4, Invitrogen). Proliferation was monitored at day 4 by analyzing target cell CFSE dilution, by FACS.

RT-qPCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the Superscript III Reverse Transcriptase Kit (Invitrogen). The mRNA levels of *Foxp3*, *IL15 R alpha chain; IL-2, IL-15* and *GAPDH* were quantified with TaqMan gene expression kits, according to the manufacturer's instructions, using a 7500 Fast Real-Time PCR System (all from Applied Biosystems). IL-2 and IL-15 expression levels in sorted thymocyte populations were determined after pre-amplification with TaqMan Preamp Master Mix (Applied Biosystems). *Bcl2* (**forward:** 5'-GCACCTGCACACCTGGAT-3' and **reverse:** 5'-CCAAACTGAGCAGAGTCTTCAG-3') and *Bim* (**forward:** 5'-ATGGCAAAGCAACCTTCTGATG-3' and **reverse:** 5'-TCAATGCATTCTCCACACCAGG-3') gene expression were quantified with SYBR® Green PCR Master Mix (Applied Biosystems). Results were analyzed using the ΔΔCt ($2^{-\Delta\Delta Ct}$) method.

Immunohistochemistry

Thymus pieces were preserved in 4% formaldehyde and embedded in paraffin. *Deparaffinised* 3µm samples underwent antigen retrieval (Leica Buffer Ph9) by heat for 15 min. Samples were stained with the following mAb (clone and supplier in brackets): anti-IL-2 (5334, R&D), anti-IL-15 (34593, R&D), anti-CD20 (L26, Dako), anti-CD68 (PG-M1, Dako) and FOXP3 (236/E7, eBiosciences). Single and double immunohistochemistry

stainings were revealed by enzymatic substrate with horseradish peroxidase and alkaline phosphatase, in brown and red respectively. All slides were counterstained with Hematoxylin and mounted with Entellan (single staining) or Glycergel (double staining) from DakoPower. Bright-field images were acquired using a Leica DM 2500 Microscope equipped with a Leica Digital FireWire Camera (DFC) and HC PL FLUOTAR lenses using the Leica Acquire software. FIJI software was used for scaling and Adobe Photoshop software for contrast/brightness correction.

Statistical Analyses

Statistical analyses were performed with the 2-tailed Student's paired test, using GraphPad Prism v5.01 (GraphPad Software Inc.). Data were first transformed with log (base 10) to account for variability between samples before the *t* test was performed. Comparison was always done with the corresponding control thymic culture. Results are expressed as mean±SEM. p-values below 0.05 were considered significant.

Results

Role of γc cytokines in human tTreg development

To mimic human intrathymic T-cell development *in-vitro*, we utilized 3D Thymic Organ Cultures (TOCs) [23, 24] and 2D cultures of total thymocytes with primary Thymic Epithelial Cells (TECs) [21], subsequently referred to as co-cultures. TECs were phenotypically characterized in terms of cytokeratin and EpCAM expression (Supplementary Fig. S1A). Consistent with T-cell differentiation, the frequency of mature CD4SP and CD8SP cells, assessed by FACS, progressively increased during the 7-day culture in both TOCs (Supplementary Fig. S1B) and co-cultures of total or CD4ISP thymocytes with TECs (data not shown and Supplementary Fig. S1C, respectively) set in medium alone.

Firstly, we assessed whether γc cytokine signaling was required for human tTreg development, by evaluating the impact of pharmacological inhibition of JAK-3, a protein tyrosine kinase that specifically associates with γc [17]. JAK-3 blockade significantly reduced the frequency and number of total $CD25^{+}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$CD4SP cells in both culture systems (Figs. 1A and B).

Next, we supplemented TOCs and co-cultures with increasing doses of IL-2, IL-4, IL-7, IL-15 and IL-21. All γc cytokines increased cell recovery and, with the exception of IL-21, augmented thymocyte survival at day 7 (Supplementary Fig. S2). However, whereas IL-4, IL-7 and IL-21 did not substantially impact upon the tTreg compartment (Supplementary Figs. S2C-E), IL-2 and IL-15 increased the frequency and number of recovered $CD25^{+}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$CD4SP cells in both TOCs and co-cultures, as well as the *Foxp3* mRNA expression levels in thymocytes recovered from co-cultures at day 4 (Figs. 1C and D, Supplementary Figs. S3A and B). The impact of IL-15 on Treg frequency and number was apparently independent of IL-2, as it was unaffected by addition of a neutralizing anti-IL-2 mAb (data not shown).

A physiological role for both cytokines was further supported by the reduction in Treg frequency in TOCs upon neutralization of endogenous IL-2 or IL-15 (Fig. 1E and Supplementary Fig. S3C) and CD122 blockade (Fig. 1F and Supplementary Fig. S3D). No modulation of the Treg compartment was observed upon IL-7 neutralization (data not shown).

Signaling via IL-2/IL-15R requires the downstream transcription factor STAT-5 [17]. Addition of a pharmacological STAT-5 inhibitor to TOCs reduced Treg recovery (Fig. 1G and Supplementary Fig. S3E) further supporting the hypothesis that JAK-3/STAT-5 pathway is involved in human tTreg differentiation.

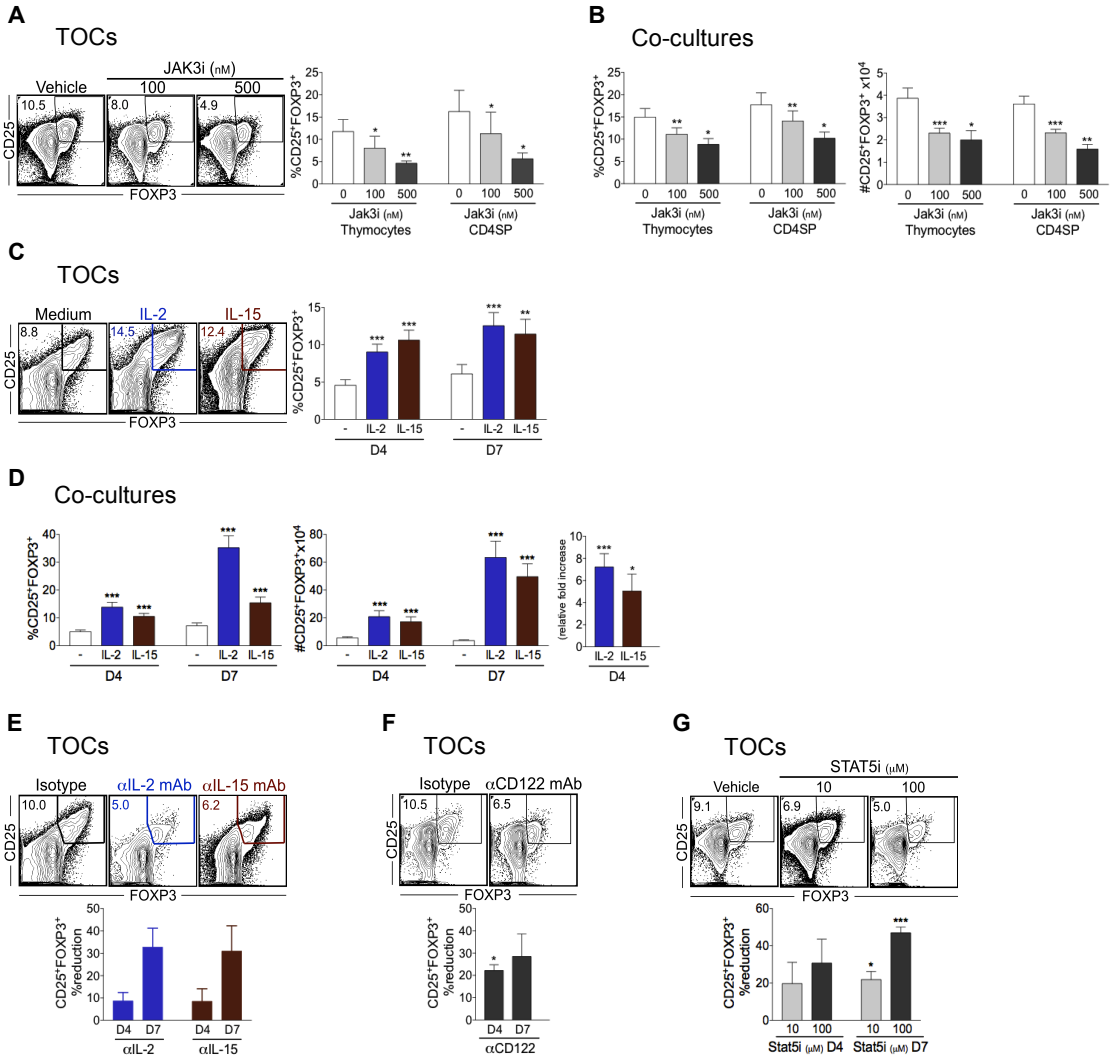


Figure 1: The γc cytokines IL-2 and IL-15 impact on human tTreg development. (A-B) TOC and co-cultures of total thymocytes with TECs were cultured for 7 days in medium alone or supplemented with the indicated concentrations of a JAK-3 inhibitor or DMSO (vehicle): (A) Representative FACS profile of CD25 and FOXP3 expression within TOC recovered cells; graph shows frequency of $FOXP3^+CD25^+$ cells within total and CD4SP thymocytes (n=3-4); (B) Frequency and number of $FOXP3^+CD25^+$ cells within total and CD4SP thymocytes in co-cultures (n=5-6). (C-D) TOCs and co-cultures of total thymocytes with TECs were cultured for 7 days in medium alone or supplemented with IL-2 (10U/ml) or IL-15 (12.5ng/ml): (C) Representative FACS profile of CD25 and FOXP3 expression in cells recovered from TOCs at day 7; graphs depict $FOXP3^+CD25^+$ cell frequency at days 4 and 7 (n=10-11); (D) Proportion and number of $CD25^+FOXP3^+$ cells recovered from co-cultures at days 4 and 7 (n=12); graph on the right shows *Foxp3* mRNA levels in cells harvested at day 4 (n=3). (E-G) Representative contour plots of CD25 and FOXP3 expression in cells recovered at day 7 from TOCs, in medium supplemented with: (E) anti-IL-2 (10μg/ml), anti-IL-15 (10μg/ml) or isotype control mAbs (anti-IgG1, 10μg/ml) (n=3-4)**;** (F) anti-CD122 blocking (10μg/ml) or isotype control mAbs (anti-IgG1, 10μg/ml) (n=4); (G) the indicated concentrations of a STAT-5 inhibitor or DMSO (vehicle) (n=4-5); graphs show the reduction in $FOXP3^+CD25^+$ cell frequency in each test condition at days 4 and 7, in comparison to the corresponding control TOC. Results are expressed as mean±SEM. *p<0.05, ** p<0.01, ***p<0.001.

We and others have shown that in addition to CD4SP, FOXP3 is also expressed by a subset of DP and CD8SP cells in the human thymus [7-9, 11]. We thus investigated the impact of IL-2 and IL-15 on these subsets, and found increased FOXP3 expression in both, upon supplementation with IL-2 and IL-15, irrespective of the culture system used (Fig. 2), as well as a consistent reduction in their frequency when we neutralized endogenous IL-2 or IL-15 (data not shown).

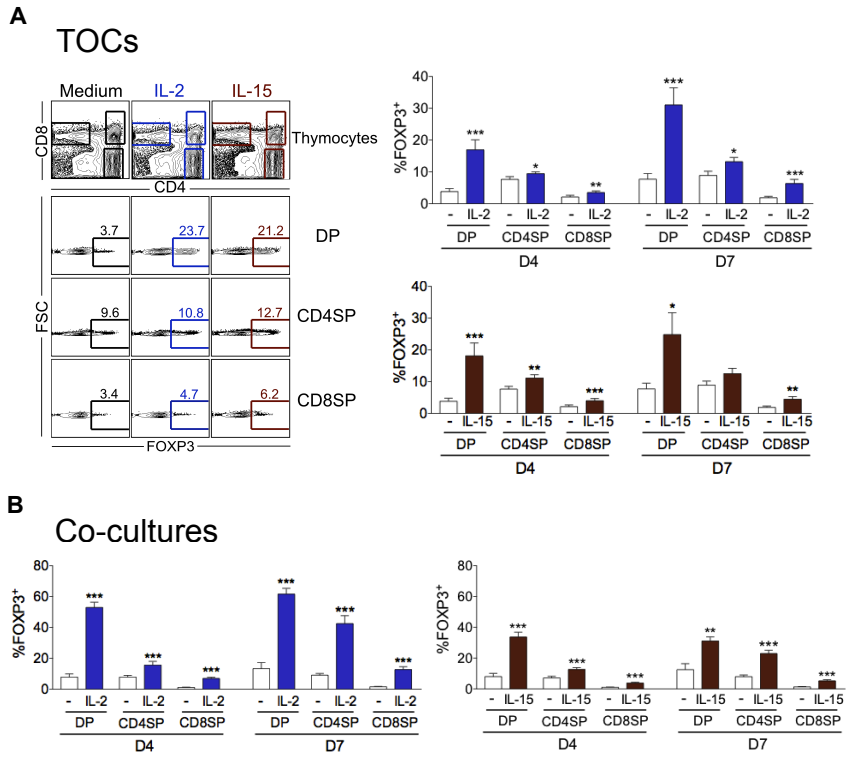


Figure 2: IL-2 and IL-15 enhance FOXP3 expression within DP, CD8SP and CD4SP thymocytes. (A) Representative contour plots of CD4 and CD8 expression within total thymocytes, together with FOXP3$^+$ cells within gated DP, CD4SP and CD8SP thymocytes recovered at day 4 from TOCs in medium alone or supplemented with IL-2 (10U/ml) or IL-15 (12.5ng/ml). Graphs show the frequency of FOXP3$^+$ cells within DP, CD4SP and CD8SP thymocytes recovered from (A) TOCs (n=8-9) or (B) co-cultures (n=12) setup as in (A), at days 4 and 7. Results are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

Overall, our data indicate that IL-2 and IL-15 are the key γc cytokines involved in human tTreg development and that their signaling largely occurs via JAK-3/STAT-5.

IL-2 and IL-15 favour the development of bona-fide Treg

We next investigated how IL-2 and IL-15 supplementation impacted on the phenotype and function of tTreg accumulating in co-cultures, and found that $FOXP3^{+}$DP, $FOXP3^{+}$CD8SP and $FOXP3^{+}$CD4SP thymocytes featured higher expression levels of both FOXP3 and other Treg function-associated molecules such as CD25, CTLA-4 and ICOS, as well as increased frequency of HLA-DR (Fig. 3A and Supplementary Figs. S4A and B). IL-2 and IL-15 also increased FOXP3 and CD25 expression levels within $FOXP3^{+}$ subsets in TOCs (data not shown).

Notably, lower levels of FOXP3 and CD25 expression were found within $FOXP3^{+}$ cells recovered from co-cultures and TOCs in the presence of a neutralizing anti-IL-2 or anti-IL-15 mAb (Supplementary Fig. S4C and data not shown), further supporting a physiological role for these cytokines in tTreg functional maturation.

We next evaluated the functional capacity of mature CD4SP Treg accumulating in co-cultures, using a standard *in-vitro* suppression assay [22]. $CD25^{bright}$CD4SP cells recovered at day 7 from IL-2- or IL-15-supplemented co-cultures inhibited the *in-vitro* proliferation of $CD25^{neg}$CD4SP targets, in a dose dependent manner, at least as efficiently as $CD25^{bright}$CD4SP control cells (Fig. 3B), demonstrating they possess functional properties of *bona-fide* Treg.

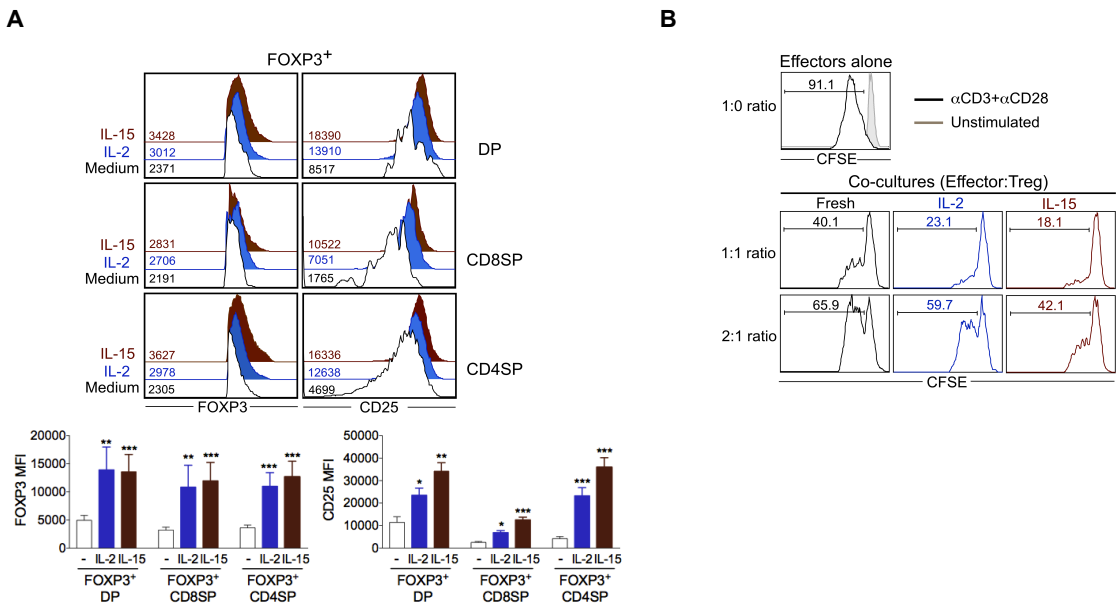


Figure 3: FOXP3$^+$ cells accumulating in IL-2- and IL-15-supplemented co-cultures display phenotypic and functional properties of Treg. (A) Representative histogram overlays of FOXP3 and CD25 (numbers inside histograms indicate MFI) within FOXP3$^+$DP, FOXP3$^+$CD8SP and FOXP3$^+$CD4SP cells recovered at day 7 from co-cultures of thymocytes with TECs in medium alone or supplemented with 10U/ml IL-2 or 12.5ng/ml IL-15; graphs show the data from different thymuses (n=12). (B) Proliferation of CFSE-labeled CD25^{neg}CD4SP thymocytes unstimulated (filled lines) or stimulated (open lines) with CD3/CD28 Dynabeads (1:0 ratio; Target:Treg ratio) for 4 days either alone or in the presence of decreasing numbers of CD25^{bright}CD4SP cells (1:1 and 2:1 ratio). The CD25^{bright}CD4SP cells tested were either sort-purified from freshly-isolated thymocytes (control of suppression) or sort-purified from day 7 co-cultures of total thymocytes with TECs, cultured in the presence of exogenous 10U/ml IL-2 or 12.5ng/ml IL-15 (n=2). Numbers correspond to the frequency of target cells that have divided at least once during the culture period. Results are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

IL-2 and IL-15 induce tTreg proliferation and survival

To determine mechanism(s) underlying the IL-2 and IL-15 effect on human tTreg development, we first tested whether these cytokines impacted upon the proliferation of FOXP3$^+$ cells, by assessing the frequency of Ki67 in cells recovered from co-cultures. Both cytokines considerably enhanced the proliferation of FOXP3$^+$ cells at day 4 (Fig. 4A and Supplementary Fig. S5), irrespectively of whether they were DP, CD4SP or CD8SP. Additionally, IL-2 and IL-15 increased Bcl2 expression levels within all FOXP3$^+$ subsets more efficiently than in their FOXP3^{neg} counterparts (Fig. 4B and Supplementary Fig. S5). Furthermore, whereas *Bcl2* mRNA levels increased with IL-2 and IL-15, *Bim* mRNA expression was unaffected by cytokine supplementation (Fig. 4C). Accordingly, IL-2 and

IL-15 consistently decreased the frequency of Annexin-V^{+} and PI^{+} cells within CD25^{+} thymocytes, used here as correlate of FOXP3^{+} cells as this readout precluded their direct labeling, recovered from co-cultures at 24h (Figs. 4D and E and Supplementary Fig. S5) but had no significant effect on their CD25^{neg} counterparts.

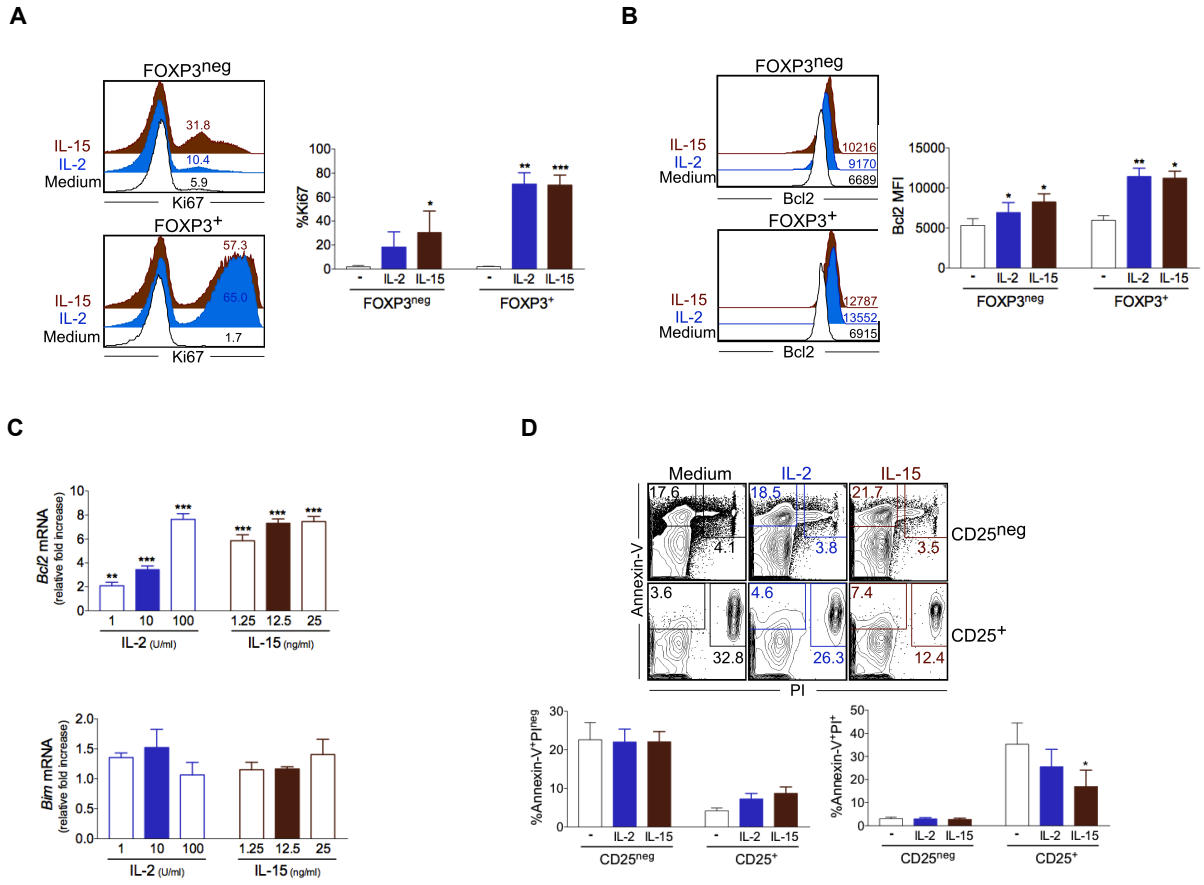


Figure 4: IL-2 and IL-15 favour the preferential proliferation and survival of human FOXP3^{+} thymocytes. (A, B, D) Total thymocytes were co-cultured with TECs, in medium alone or supplemented with either IL-2 (10U/ml) or IL-15 (12.5ng/ml). Frequency of Ki67^{+} cells (A) and Bcl-2 MFI (B) within FOXP3^{neg} and FOXP3^{+} cells, at day 4 (n=3-4). (C) Quantification of *Bim* and *Bcl2* mRNA levels by qPCR in total thymocytes recovered at 24 hours from co-cultures in medium supplemented with the indicated concentrations of IL-2 and IL-15, in comparison to control co-cultures in medium alone (n=3). (D) Frequency of Annexin-V^{+}PI^{neg} and Annexin-V^{+}PI^{+} cells within CD25^{neg} and CD25^{+} cells recovered at 24 hours (n=3). Numbers in histograms correspond to either percentage (Ki67, Annexin-V/PI) or MFI (Bcl2). Results are expressed as mean±SEM. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Collectively, our data indicate that IL-2 and IL-15 promote the preferential proliferation and survival of FOXP3^{+} cells.

IL-2 and IL-15 drive human tTreg precursor differentiation into FOXP3$^+$ cells

Notably, a significant increase in the frequency of CD25$^+$FOXP3$^+$ cells as well as in their levels of FOXP3 protein and mRNA expression was already observed at 24h in co-cultures supplemented with IL-2 or IL-15 (Fig. 5A), a time-point at which the impact of cytokines upon proliferation was negligible (Fig. 5B). These results raised the possibility that IL-2 and IL-15 could directly drive thymocyte differentiation into tTreg.

To investigate this hypothesis, we first used a classical *in-vitro* assay for Treg differentiation in which sort-purified non-regulatory CD4SP thymocytes (sorted as CD25^{neg}CD127^{hi}CD4SP) were TCR-stimulated in the presence of TGF-β [22]. Notably, cells cultured under these conditions did not acquire FOXP3, in media without γc cytokines. However, addition of either IL-2, IL-15 and to a lesser extent IL-7, but not IL-4 or IL-21, readily promoted FOXP3 expression. Moreover, IL-4 and IL-21 seemed to antagonize FOXP3 acquisition in the presence of TGF-β and IL-2 (Fig. 5C). Our data suggest that IL-2 and IL-15 are particularly efficient at inducing FOXP3 expression in TCR-stimulated non-regulatory human thymocytes and demonstrate that TCR/CD28 activation alone, even in the presence of TGF-β, was insufficient to promote acquisition of FOXP3 expression by these cells.

Next, we sort-purified CD127^{hi}CD4SP thymocytes according to their level of CD25 expression, and showed that CD25$^+$CD127^{hi}CD4SP cells did not require provision of TCR triggering to progress to the FOXP3$^+$ stage, a step induced equally well by IL-2 or IL-15, with approximately 40% of cells expressing FOXP3 at day 3 (Fig. 5D). Thus, CD25$^+$CD127^{hi}CD4SP cells (surrogates of CD25$^+$FOXP3^{neg}CD4SP cells) are highly enriched in tTreg precursors prone to differentiate into FOXP3$^+$ cells upon stimulation with IL-2 or IL-15 alone. We estimated that CD25$^+$FOXP3^{neg} cells represent 2.6±0.2% (n=19) of CD3^{hi}CD4SP thymocytes and observed a trend towards a direct relationship between their frequency and the proportion of CD25$^+$FOXP3$^+$CD4SP cells (r=0.38; p=0.0565). Notably, the counterpart CD25^{neg}CD127^{hi}CD4SP population lacked the capacity to differentiate into tTreg, failing to up-regulate FOXP3 upon exposure to IL-2 or IL-15 (Fig. 5D). We also found that following overnight stimulation with anti-CD3 mAb, alone or together with anti-CD28 mAb, roughly 2 and 19% of the CD25^{neg}CD127^{hi}CD4SP thymocytes, respectively, acquired CD25 (with no concomitant FOXP3 expression) and

that upon further exposure to IL-2 and IL-15, only cells pre-exposed to both stimuli acquired expression of FOXP3 (Fig. 5E).

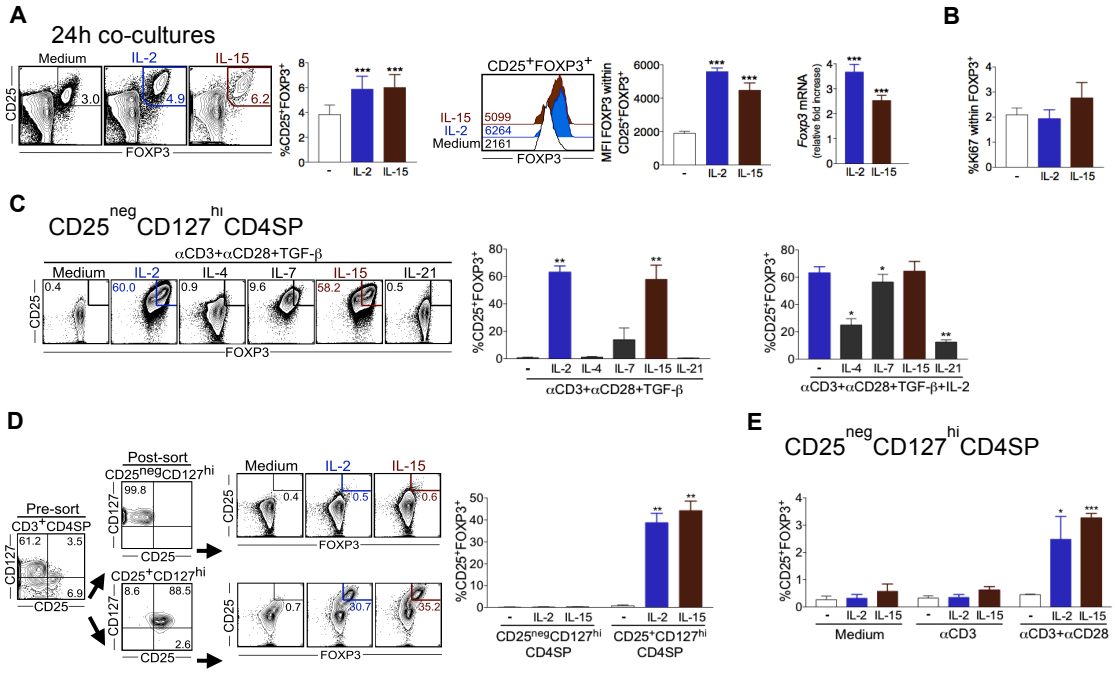


Figure 5: IL-2 and IL-15 promote human tTreg precursor differentiation into tTreg. (A-B) 24h co-culture of total thymocytes with TECs in medium alone or supplemented with IL-2 (10U/ml) or IL-15 (12.5ng/ml): (A) Representative FACS profile of FOXP3 and CD25 expression and histogram overlay of FOXP3 expression levels within $CD25^{+}FOXP3^{+}$ cells in recovered thymocytes; graphs show $CD25^{+}FOXP3^{+}$cell frequency recovered (n=6), their FOXP3 expression levels (n=6), and *Foxp3* mRNA levels in recovered thymocytes (n=5); (B) Frequency of Ki67 within $FOXP3^{+}$ cells recovered from the 24h co-cultures (n=6). (C) Sort-purified $CD25^{neg}CD127^{hi}CD4SP$ thymocytes were stimulated for 5 days with plate-bound anti-CD3 mAb, soluble anti-CD28 mAb and TGF-β, in the presence or absence of the γc cytokines IL-2 (10U/ml), IL-4 (10ng/ml), IL-7 (10ng/ml), IL-15 (12.5ng/ml) or IL-21 (25ng/ml). Representative contour plots of CD25 and FOXP3 expression are shown, with graphs depicting the frequency of recovered $CD25^{+}FOXP3^{+}$ cells (left, n=3) and after adding 10U/ml IL-2 (right, n=3). (D) Representative contour plots illustrating the sorting strategy and purity of $CD25^{neg}CD127^{hi}CD4SP$ and $CD25^{pos}CD127^{hi}CD4SP$ cells, and their levels of CD25 and FOXP3 expression upon culture for 3 days in medium alone or supplemented with IL-2 (10U/ml) or IL-15 (12.5ng/ml); graph shows differentiated $CD25^{+}FOXP3^{+}$ cell frequency (n=3-5). (E) Sort-purified $CD25^{neg}CD127^{hi}CD4SP$ were left in medium or stimulated overnight with plate-bound anti-CD3 mAb (1μg/ml) alone or in combination with soluble anti-CD28 mAb (1μg/ml), washed, and then cultured for 3 days in medium alone or supplemented with IL-2 (10U/ml) or IL-15 (12.5ng/ml); graphs show $CD25^{+}Foxp3^{+}$ cell frequency in each culture condition (n=4). Results are expressed as mean±SEM. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Overall, our data support a 2-step model in which initial TCR and co-stimulatory signals give rise to $CD25^{+}FOXP3^{neg}$CD4SP tTreg precursors that on subsequent exposure to IL-2 or IL-15 differentiate into $CD25^{+}FOXP3^{+}$ cells.

Non-overlapping pattern of IL-2 and IL-15 expression in the human thymus

There are, to our knowledge, no data regarding the pattern of expression of either IL-2 or IL-15 in the human thymus. Using immunohistochemistry, we showed that IL-2 was expressed both in the cortex and medulla, with a predominantly "patchy" distribution (Figs. 6A-D and Supplementary Fig. S6A). IL-15 presented a more diffuse pattern, mostly restricted to the medulla, although a few cytokine niches were identifiable in both cortex and medulla (Figs. 6A, C and D and Supplementary Fig. S6A). The specificity of the cytokine staining was confirmed by lack of positive labeling with the isotype control Ab (Fig. 6B), and by its loss following pre-incubation of each anti-cytokine Ab with its cytokine target (Fig. 6C). Importantly, there was a mostly non-overlapping pattern of IL-2 and IL-15 expression that was reproducibly detected in all thymuses tested (Figs. 6A and D). We also detected $FOXP3^{+}$ cells in the vicinity of IL-2- and IL-15- enriched areas, as assessed by single stainings performed in sequential cuts (Supplementary Fig. S6A), since technical limitations precluded co-immunostaining and double immunofluorescence for FOXP3 and cytokines. Immunohistochemistry data also suggested that IL-2 was expressed at considerably higher levels than IL-15, an observation corroborated by quantitative RT-PCR (qPCR) measurement of *IL-2* and *IL-15* mRNA expression in total thymocytes (Fig. 6E), although the putative contribution of stromal cells was not evaluated here.

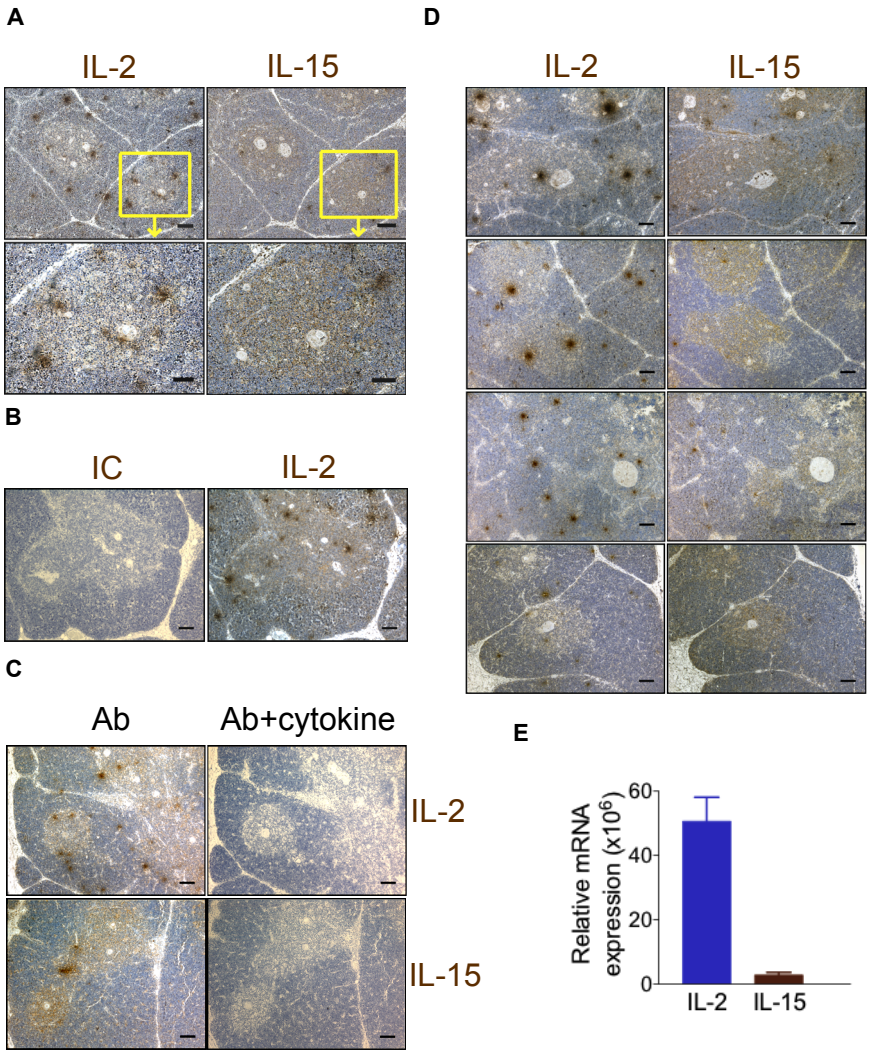


Figure 6: IL-2 and IL-15 are expressed thymus in non-overlapping niches in the human. Illustrative immunohistochemistry staining of paraffin-embedded serial sections of human thymus with: (A) human anti-IL-2 and anti-IL-15 mAbs with lower set of images corresponding to a zoom of the region indicated by the yellow box in the upper ones; (B) human anti-IL-2 mAb or the corresponding isotype control antibody (IC: mouse IgG1); (C) anti-IL-2 mAb or anti-IL-15 mAb, with or without pre-incubation of the antibody with its corresponding cytokine ligand, as indicated; (D) human anti-IL-2 and anti-IL-15 mAbs in 4 additional thymuses to illustrate the reproducibility of the staining pattern. Scale bars in A, 100 and 50 μm (upper and lower images, respectively); original magnification x400. Scale bars in B, C and D correspond to 100 μm; original magnification x100. (E) Quantification of *IL-2* and *IL-15* mRNA levels in total thymocytes, by qPCR (n=2). Results are expressed as mean±SEM.

We next investigated which populations within the human thymus were responsible for IL-2 and/or IL-15 secretion. qPCR analyses of sort-purified thymocyte subsets suggested that mature thymocytes were the major IL-2 source, particularly CD4SP and CD8SP, but also indicated a contribution of γδ and NK cells (Fig. 7A). Intracellular staining for IL-2 showed that 1.5±0.3% of thymocytes (n=13) produced IL-2 after PMA/ionomycin stimulation. IL-2 production was mostly confined to $CD3^+$ thymocytes (Fig. 7B), particularly CD4SP (1.8±0.4%, n=13) and CD8SP (4.1±0.6%, n=13). Additionally, a reasonable proportion of γδ (15.7±4.7%, n=3) and NK cells (17.9±4.2%, n=6) also produced IL-2 (Fig. 7B), although they only constituted 0.5±0.05 and 0.2±0.09% of total thymocytes, respectively. Therefore, CD4SP, CD8SP and $CD3^{bright}$DP thymocytes are the main contributors to thymic IL-2, followed by γδ and NK cells (Fig. 7C). On the other hand, most thymic IL-15 appeared to be produced by monocyte/macrophage $CD14^+$ cells, and to a lesser extent, B lymphocytes (Fig. 7D). In accordance with a non-overlapping pattern of IL-2 and IL-15 expression, CD4SP and CD8SP thymocytes that constituted major sources of IL-2, did not express IL-15. Moreover, whereas TECs were negative for IL-2, they readily expressed IL-15 (Fig. 7D). Taking into consideration our immunohistochemistry data, we expect that IL-15 expression in TECs to be mostly restricted to mTECs (Figs. 6A, C, D and Supplementary Fig. S6A). Since IL-15 needs to be presented in *trans* by IL-15Rα$^+$ cells to neighbouring IL-2Rβ-expressing cells [25], we confirmed by qPCR that thymic populations with the potential to secrete IL-15 expressed the IL-15Rα chain (Fig. 7E).

Additionally, we performed co-immunostaining for IL-15 and the macrophage marker CD68, or the B cell marker CD20, due to the lack of a reliable anti-IL-15 mAb for flow cytometry and our inability to detect IL-15 by immunofluorescence. Immunohistochemistry data supported a contribution of both populations to thymic IL-15 (Figs. 7F and G). Indeed, macrophages and B cells were found within ~90% and 33% of the IL-15 niches, respectively. Interestingly, whereas macrophages localized in both cortex and medulla, B cells were mostly found in the medulla (Supplementary Fig. S6B). No co-localization of macrophages or B cells with IL-2 was observed (Fig. S6C), as predicted by our qPCR data (Fig. 7A).

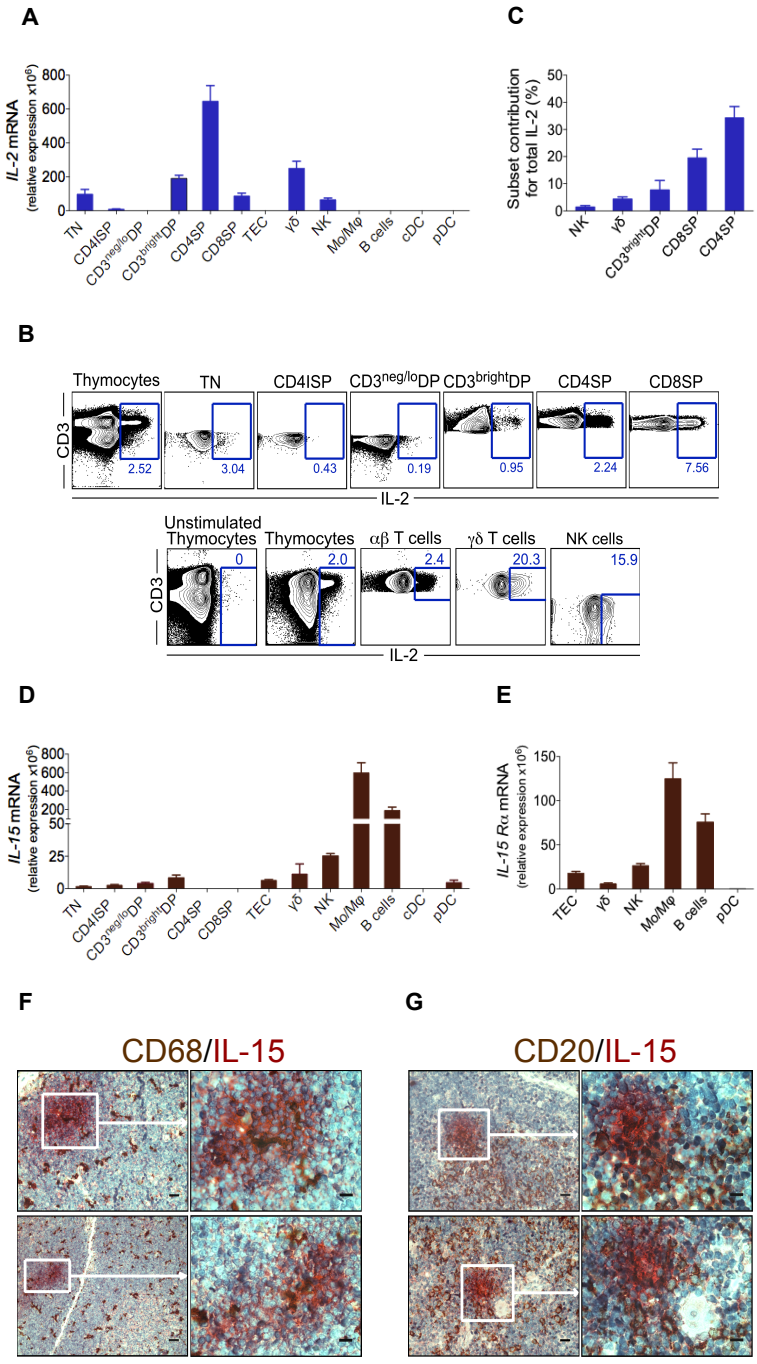


Figure 7: Cellular sources of IL-2 and IL-15 in the human thymus. Quantification of *IL-2* mRNA levels by qPCR (A) in the indicated sort-purified thymocyte populations (n=2-4), and representative contour plots of IL-2 expression by flow cytometry (B) following short-time PMA/Ionomycin stimulation of freshly-collected thymocytes, with unstimulated total thymocytes shown as controls (n=3-13). (C) Contribution of the indicated thymocyte subsets to the thymic IL-2 pool (n=3-13). Quantification of *IL-15* (D) and *IL-15-Ralpha* (E) mRNA levels by qPCR in the indicated sort-purified thymocyte populations (n=2-4). Double immunohistochemistry for IL-15 and CD68 (F) or IL-15 and CD20 (G) performed in thymic sections (n=3); the right-hand panel corresponds to a zoom of the indicated region, labelled in white, in the left-hand panel; scale bars, 20 μm and 10μm in right and left panels, respectively; original magnification x400. Results are expressed as mean±SEM.

In summary, our data indicate that IL-2 and IL-15 are expressed in the human thymus in a mostly non-overlapping pattern, and produced by distinct populations.

Discussion

In the present study we revealed the key contribution of IL-2 and IL-15 to human tTreg development and characterized their mechanism of action, their pattern of expression and the populations responsible for their production in the human thymus.

This study is, to the best of our knowledge, the first that directly assesses the role of γc cytokines in human tTreg development, a still poorly defined process. Pharmacological JAK-3 blockade led, on average, to a 50% reduction in Treg frequency and number, supporting a significant contribution of γc signaling to tTreg differentiation. Of the γc cytokines tested, IL-2 and IL-15 had the strongest effects, both being capable of driving tTreg precursor differentiation into $FOXP3^+$ cells and promoting tTreg preferential expansion and survival. IL-2 and IL-15 also increased the expression level of molecules associated with effector function within all $FOXP3^+$ subsets, suggesting their involvement in tTreg functional maturation. We also established that IL-2 and IL-15 are expressed in the human thymus in a mostly non-overlapping fashion and identified mature αβ and γδ cells or monocyte/macrophages and B lymphocytes as the main producers of IL-2 or IL-15, respectively. These results suggest that in addition to TCR ligands, IL-2 and IL-15 define extra niches required for human tTreg lineage stabilization and differentiation.

Despite the differences between human and murine tTreg development, evidence also supports a requirement for γc cytokines in mice [2, 3, 16, 17]. In fact, γC-deficient animals display a dramatic decrease in T-cells, harboring almost no $Foxp3^+$ cells [26]. Moreover, whereas IL-2-deficient animals feature a 50% reduction in tTregs [26, 27], mice deficient for both IL-2 and IL-15 or for the IL-2Rβ chain bear almost none [28]. Mechanistically, while some authors suggest that IL-2 and IL-15 directly drive Foxp3 expression in mice [29], others propose these cytokines are mainly required for tTreg survival [30, 31]. Here, we provide evidence that IL-2 and IL-15 are required for lineage commitment, functional programming, survival and proliferation of Tregs during their thymic development in humans. These cytokines were previously shown to enhance the proliferation and survival of human circulating Tregs as well as to increase their FOXP3 expression levels [32-34]. Our results thus suggest that the capacity of IL-2 and IL-15 to modulate the survival, phenotype and proliferation of human Tregs is acquired during their thymic differentiation, which is in agreement with their constitutive CD25 and CD122 expression. They also indicate that augmented thymic development may account for the increased Treg

frequency and number observed in humans upon IL-2 therapy in the context of autoimmunity, cancer therapy, hematopoietic stem cell transplantation and HIV infection [18-20, 35]. Our study also raises a note of caution regarding the proposed administration of IL-15 to boost anti-tumor immunity in humans [36], and highlights IL-21 as a safer therapeutic option.

We demonstrated that the tTreg compartment was reduced by ~50% upon STAT-5 inhibition. This dependency may explain the incapacity of IL-4 and IL-21 to positively impact on tTreg development, since neither is very efficient at activating STAT-5, utilizing instead STAT-6 or STAT-3, respectively [17]. IL-4 and IL-21 may actually antagonize the differentiation of $CD25^{+}FOXP3^{+}$ cells, as we observed upon TCR stimulation of $CD25^{neg}CD127^{hi}$CD4SP cells in presence of TGF-β and IL-2. These results are in agreement with, and extend previous reports using human thymocytes [13] and peripheral cells [37, 38]. The lack of a clear positive impact of IL-7, known to efficiently activate STAT-5, on tTreg development is *surprising*. We observed that short-term exposure to IL-7 increased Treg frequency as well as their levels of FOXP3 and Bcl2 expression (data not shown), although less efficiently than IL-2 or IL-15. However, although IL-7 sustained the proliferation of $FOXP3^{+}$ cells, its positive effect on Tregs was diluted by its preferential expansion of $FOXP3^{neg}$ cells (data not shown).

Our findings support a TCR and co-stimulation signaling requirement for $CD25^{+}FOXP3^{neg}$CD4SP tTreg precursor generation. We propose that, subsequently, tTreg differentiation enters a TCR/co-stimulation independent stage in which exposure to either IL-2 or IL-15 drives tTreg precursor acquisition of FOXP3 expression, with concomitant lineage stabilization and differentiation into mature tTreg. These results are compatible with a 2-step model previously proposed in mice [29], highlighting a considerable evolutionary conservation in key mechanisms governing human and mouse tTreg differentiation.

Recent data challenge the role of $CD25^{+}FOXP3^{neg}$CD4SP cells as a mandatory intermediate population in murine tTreg development, with an alternative pathway via $CD25^{neg}FOXP3^{+}$CD4SP being proposed [31, 39]. We confirmed that in humans $CD25^{neg}FOXP3^{+}$CD4SP are present (1.6±0.2% of CD4SP thymocytes, n=19). In agreement with the possibility they constitute an additional population of human tTreg

precursors, their FOXP3 MFI is lower than within $CD25^{+}FOXP3^{+}CD4SP$ cells (1714±152 versus 2465±247, respectively, n=19). Moreover, we found a direct correlation between their frequency and the proportion of $CD25^{+}FOXP3^{+}CD4SP$ cells (r=0.680; p=0.0007). Unfortunately, tools currently available for humans do not allow direct testing of the potential of $CD25^{neg}FOXP3^{+}CD4SP$ to differentiate into mature $CD25^{+}FOXP3^{+}$ cells upon IL-2 or IL-15 stimulation. In fact, although their CD127 MFI is higher than that of $CD25^{+}FOXP3^{+}CD4SP$ cells, it is not distinctive enough to permit their isolation. New tools are also required to assess the TCR repertoire of $CD25^{+}FOXP3^{neg}$, $CD25^{neg}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$ cells within $CD3^{hi}CD4SP$ thymocytes in order to provide a characterization of tTreg precursors and establish a temporal framework for human tTreg development.

Given the dependency of tTreg differentiation on IL-2 or IL-15, it was critical to identify their thymic source. We found that IL-2 and IL-15 are expressed in the human thymus in distinct locations, as reported in mice [40]. Notably, enriched areas of IL-2 and IL-15 production designated here as cytokine niches, were present in the human thymus. A similar pattern of IL-2 secretion was previously reported in human tonsils [41] and in murine thymus [42]. We expect these niches, likely resulting from post-secretion binding of cytokine to extracellular matrix components [43], to locally increase cytokine availability and prolong their *in-vivo* half-life. Of physiological relevance, we show that tTregs are found in close proximity to IL-2- and IL-15-producing cells.

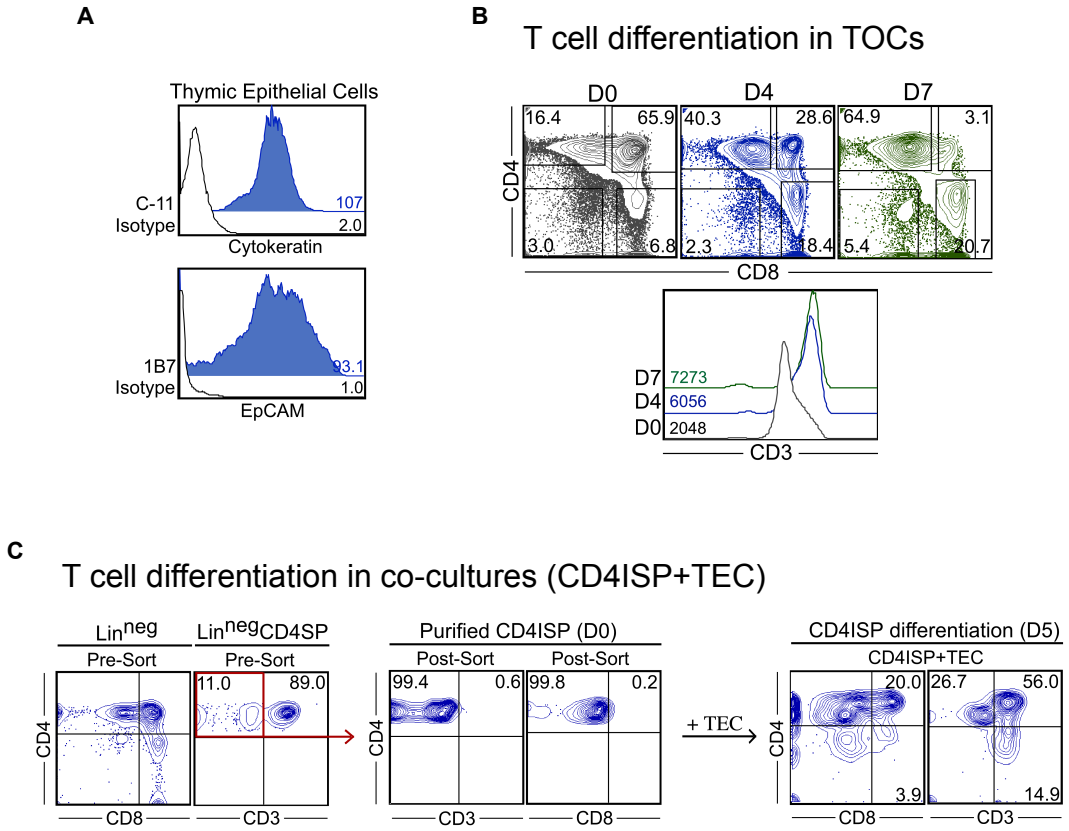
We estimated that ~1.5% of human thymocytes had the potential to secrete IL-2 and identified mature αβ and γδ thymocytes as its main producers, a phenomenon conserved in the murine thymus [44]. We propose that the main IL-15 sources are monocyte/macrophages and B lymphocytes, populations shown in humans to produce IL-15 in the periphery [45-47], as well as TECs, which in mice secrete IL-15 [48]. Interestingly, the majority of human $CD25^{bright}CD4SP$ cells express CCR8, migrating in response to CCL1/I-309 secreted by macrophages [49]. Moreover, B lymphocytes were recently shown to select tTreg in mice [50]. Another important issue for future studies will be the comparison of the TCR repertoire of tTreg that differentiate in presence of IL-2 or IL-15 and the possible role of cells that produce these cytokines in mediating tTreg selection.

Our results support the possibility of the therapeutic manipulation of tTreg by targeting IL-2 and/or IL-15 pathways, and, in this way, potentially help control autoimmune manifestations. The impact of these cytokines on the human thymus has not, to our knowledge, been taken into account in clinical trials involving them, an aspect that deserves a reappraisal.

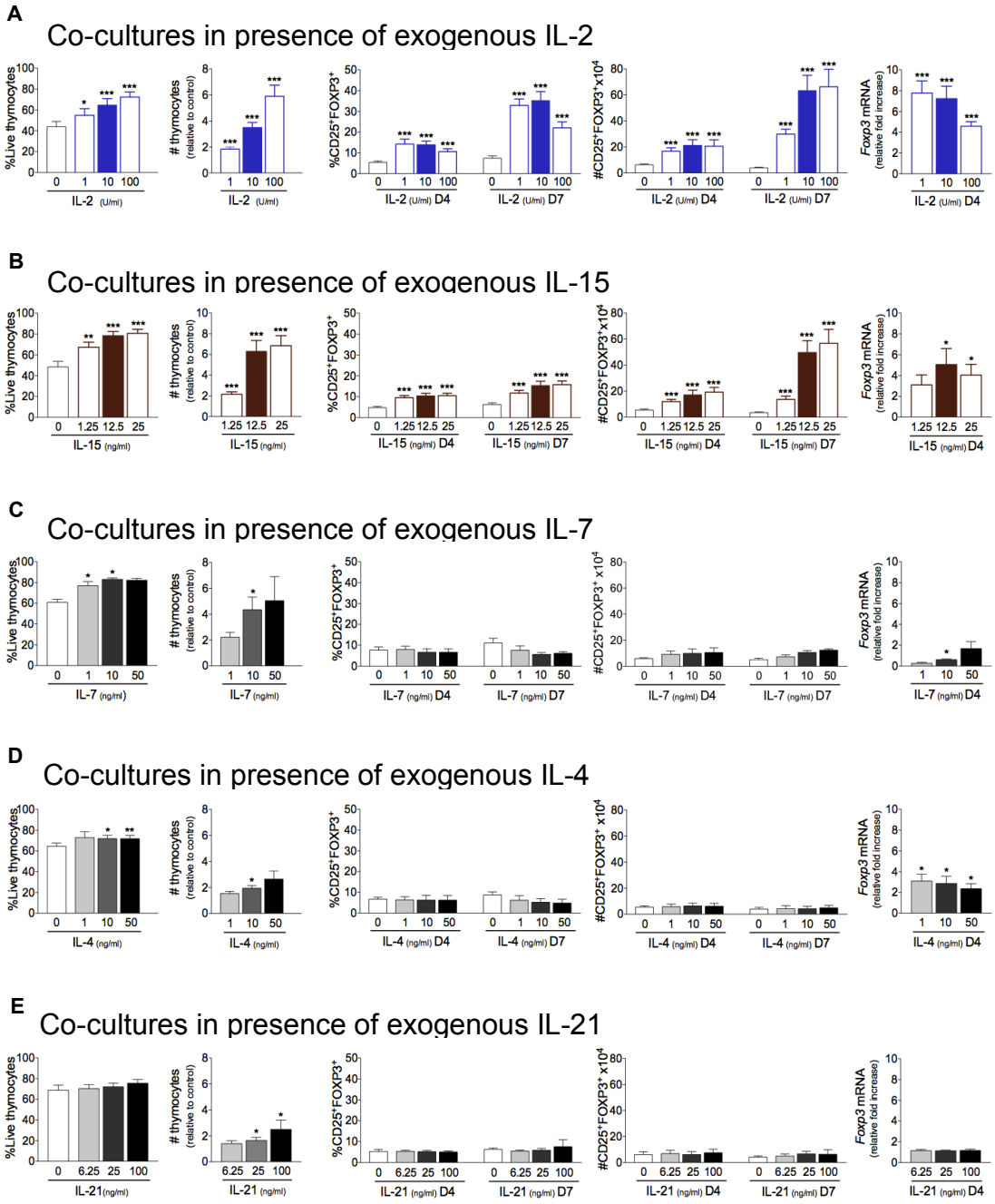
Conclusions

Collectively our data suggest that following human tTreg precursor generation, subsequent exposure to either IL-2 or IL-15 drives their final differentiation into $FOXP3^+$ cells. These cytokines then act by enhancing tTreg survival and proliferation as well as by fostering their functional maturation. The physiological role of these cytokines is further supported by our demonstration of their production in the human thymus in a non-overlapping pattern by distinct cell populations. Ultimately, we expect that the ability to increase tTreg output via administration of IL-2 or IL-15 constitutes a novel approach for the management of autoimmunity.

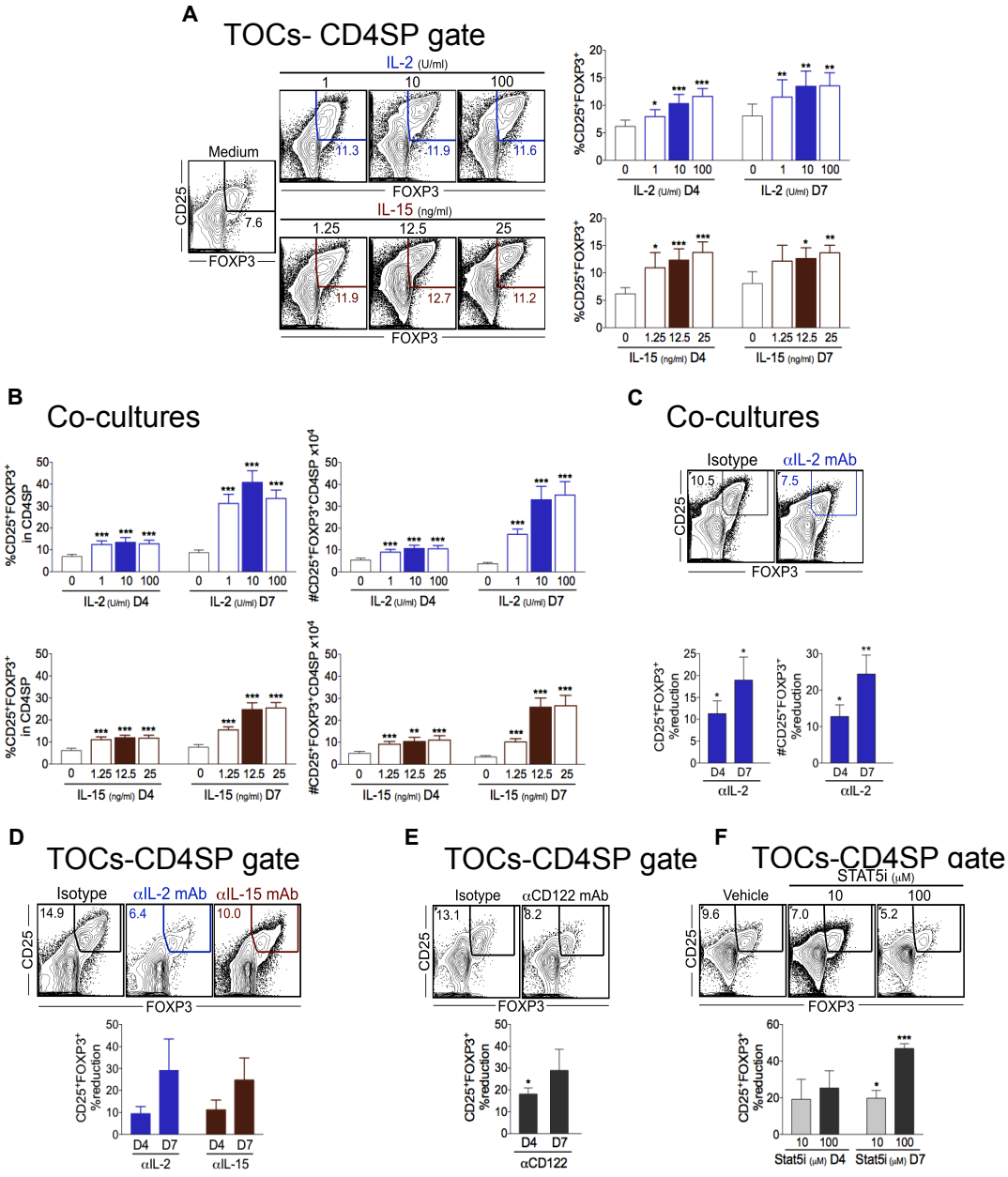
Supplemental Data



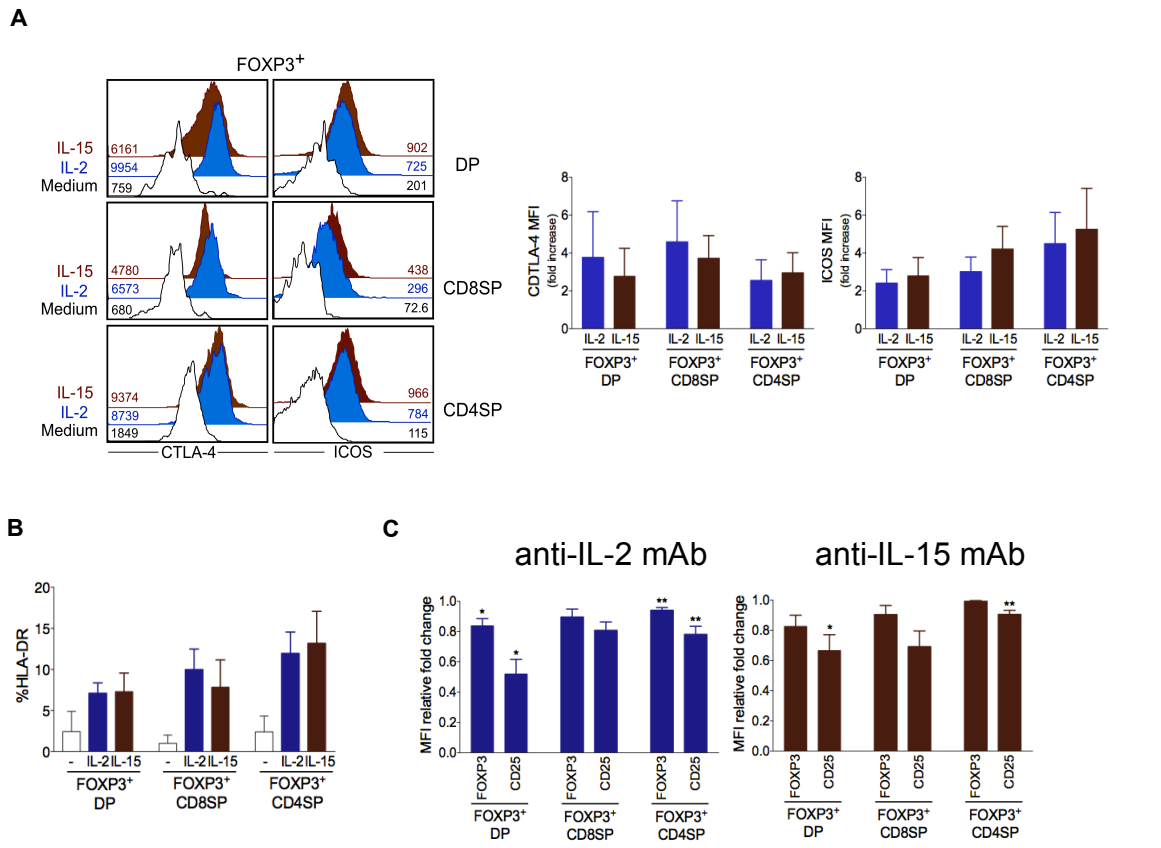
Supplementary Fig. S1: Human T cell development in 2D and 3D cultures. (A) Phenotype of primary allogeneic TECs assessed by flow cytometry at day 7 of differentiation, using anti-EpCAM (clone 1B7) and pan-cytokeratin (clone C-11) mAbs (filled lines). Staining with the isotype control antibody is also shown (mouse IgG1, open lines). (B) Representative flow cytometric profile of CD4 and CD8 expression on thymocytes recovered from TOCs at days 0, 4 and 7. Histogram overlay shows CD3 expression levels on thymocytes recovered at these same time points. (C) Sort-purified CD4ISP thymocytes were co-cultured for 5 days with primary allogeneic TECs. Representative contour plots show the sorting strategy and the purity after sorting (D0) as well as the expression of CD4, CD8 and CD3 determined by flow cytometry in cells recovered from co-cultures at day 5 (D5). Lin=Lineage (CD14, CD16, CD20, HLA-DR, CD11c and CD123).



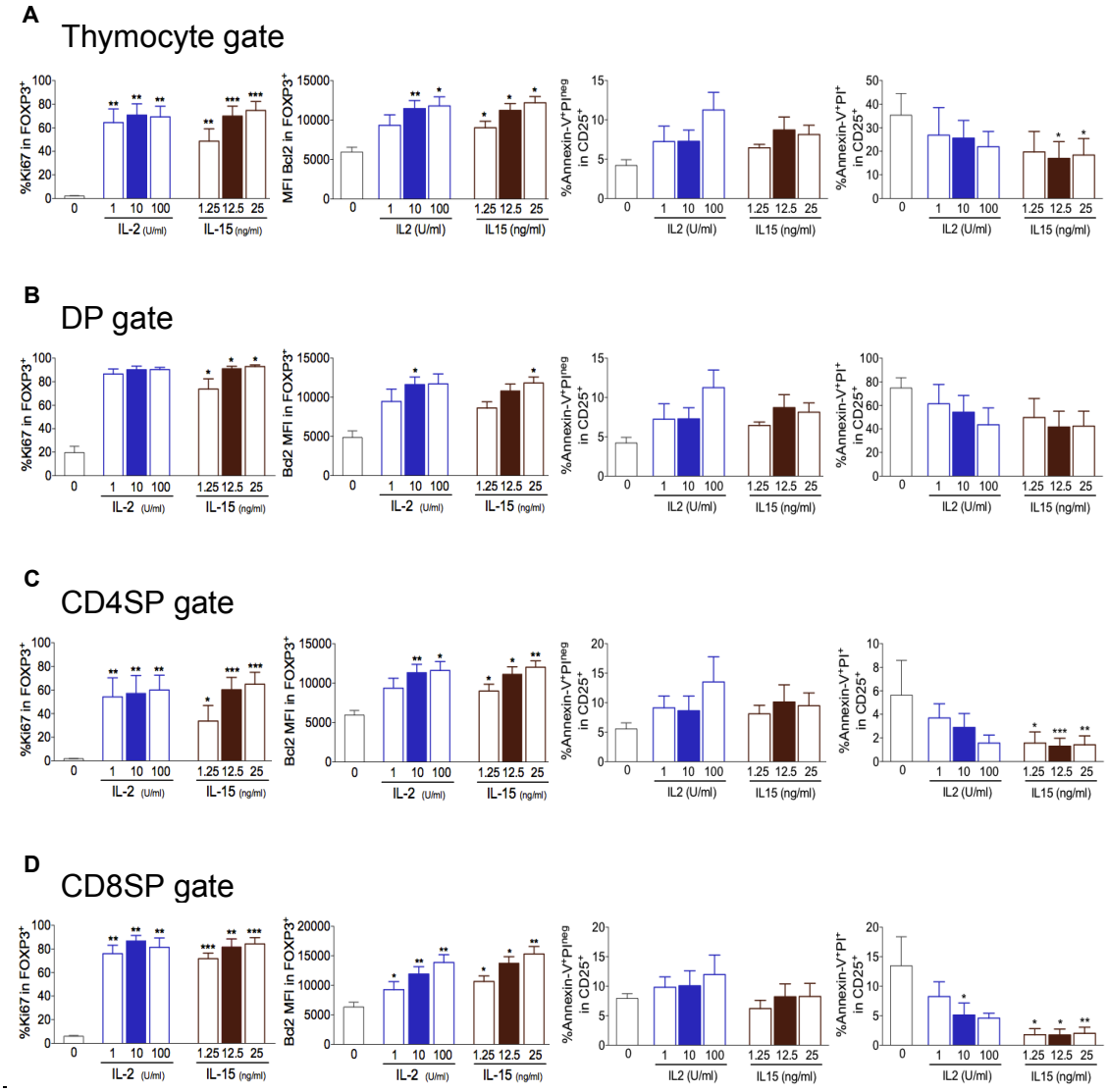
Supplementary Fig. S2: IL-4, IL-7 and IL-21 do not have a major impact on Treg recovery in 2D cultures. Total thymocytes were cultured with primary TECs in medium alone or supplemented with the indicated concentrations of IL-2 (A), IL-15 (B), IL-7 (C), IL-4 (D) or IL-21 (E). Frequency and number of live cells recovered determined by flow cytometry at day 7 (n=3-12) as well as frequency and number of $CD25^{+}FOXP3^{+}$ cells recovered at days 4 and 7 in co-cultures are depicted (n=3-12); graphs on the right show the *Foxp3* mRNA levels in thymocytes recovered at day 4 (n=2-3). Results are expressed as mean±SEM. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.



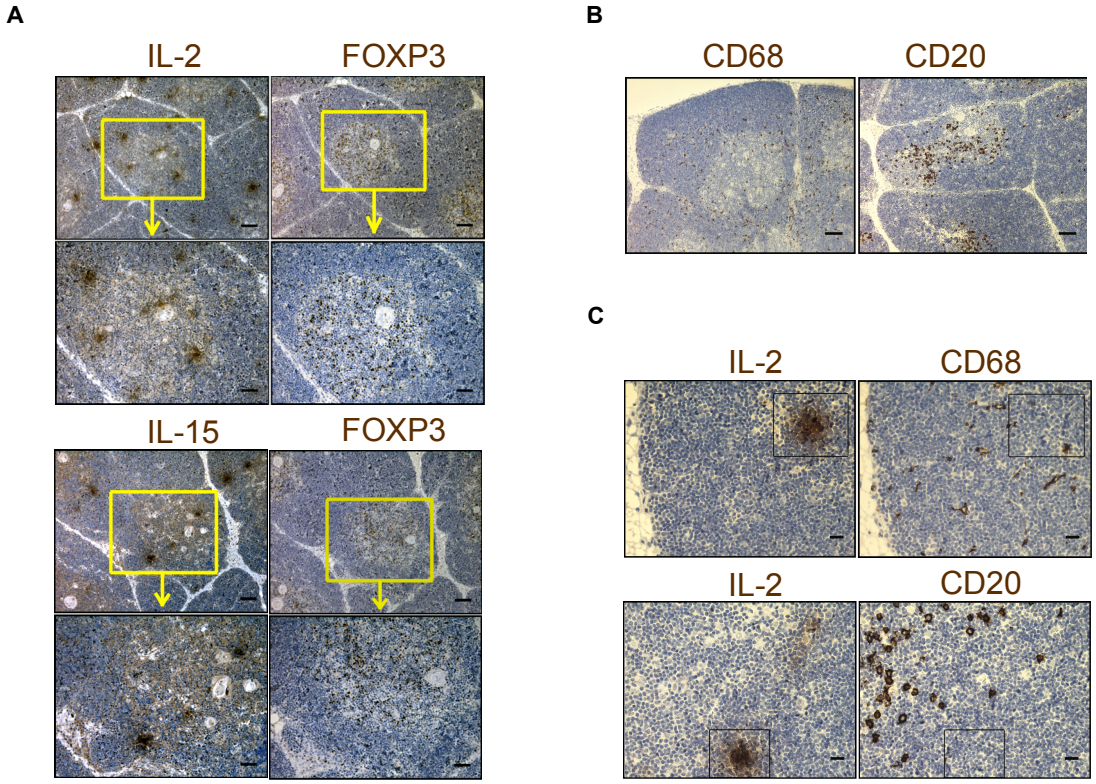
Supplementary Fig. S3: IL-2 and IL-15 enhance human Treg differentiation/expansion in 2D and 3D cultures. (A) Representative FACS profile of CD25 and FOXP3 expression in CD4SP thymocytes recovered at day 7 from TOCs cultured in medium alone or supplemented with the indicated concentrations of IL-2 or IL-15; graphs show the frequency of $CD25^+FOXP3^+$ cells within CD4SP recovered from TOCs at days 4 and 7 (n=7-11). (B) Proportion of $CD25^+FOXP3^+$ cells within CD4SP thymocytes and number of $CD25^+FOXP3^+CD4SP$ cells recovered at days 4 and 7 in co-cultures of total thymocytes with TECs in medium alone or supplemented with the indicated concentrations of IL-2 or IL-15 (n=12). (C-F) Representative contour plot of CD25 and FOXP3 expression within total (C) or CD4SP thymocytes (D-F) recovered at day 7 from co-cultures (C) or TOCs (D-F) in medium supplemented with: (C) anti-IL-2 mAb (1μg/ml) or isotype control anti-IgG1 mAb (1μg/ml) (n=11-12); (D) anti-IL-2 mAb (10μg/ml), anti-IL-15 mAb (10μg/ml) or isotype control anti-IgG1 mAb (10μg/ml) (n=3-4); (E) anti-CD122 blocking mAb (10μg/ml) or control anti-IgG1 mAb (10μg/ml) (n=4); (F) the indicated concentrations of the STAT-5 inhibitor or DMSO (vehicle control) (n=4-5). Graphs show the reduction in the frequency of $FOXP3^+CD25^+$ cells within total (C) or CD4SP thymocytes (D-F) in each test condition at days 4 and 7, in comparison to the corresponding control culture. In (C) reduction in number of $FOXP3^+CD25^+$ cells is also depicted. Results are expressed as mean±SEM. *p < 0.05, **p < 0.01, ***p<0.001.



Supplementary Fig. S4: IL-2 and IL-15 enhance the expression of molecules associated with Treg function. (A) Representative histogram overlays of CTLA-4 and ICOS expression (numbers inside histograms correspond to MFI) within FOXP3$^+$DP, FOXP3$^+$CD8SP and FOXP3$^+$CD4SP cells recovered at day 7 from co-cultures of thymocytes with TECs in medium alone or supplemented with either 10U/ml IL-2 or 12.5ng/ml IL-15; graphs show the data from different thymuses in comparison with control co-cultures set in medium alone (n=3). (B) Frequency of HLA-DR$^+$ cells within FOXP3$^+$DP, FOXP3$^+$CD8SP and FOXP3$^+$CD4SP cells recovered at day 7 in co-culture of total thymocytes with TECs in medium alone or supplemented with 10U/ml IL-2 or 12.5ng/ml IL-15 (n=3). (C) Graphs represent the fold change in FOXP3 and CD25 expression levels within the indicated FOXP3$^+$ thymocyte subsets at day 7 in co-cultures set in the presence of an anti-IL-2 (1µg/ml) or an anti-IL-15 blocking mAb (1µg/ml) in comparison to control thymic cultures (anti-IgG1 mAb, 1µg/ml) (n=10-12). Results are expressed as mean±SEM. *$p < 0.05$, **$p < 0.01$.



Supplementary Fig. S5: IL-2 and IL-15 impact on the proliferation and survival of human FOXP3$^+$DP, FOXP3$^+$CD4SP and FOXP3$^+$CD8SP thymocytes. Analysis performed within total thymocytes (A), DP (B), CD4SP (C) and CD8SP (D) thymocytes after co-culture of total thymocytes with TECs, in medium alone or supplemented with the indicated concentrations IL-2 and IL-15 for 4 days; graphs show the frequency of Ki67$^+$ and Bcl2 MFI in gated FOXP3$^+$ cells as well as the frequency of Annexin-V$^+$PI^{neg} and Annexin-V^{neg}PI$^+$ cells in gated CD25$^+$ cells, recovered at day 4 (Ki67 and Bcl2) or 24h (Annexin-V/PI) (n=3-4). Results are expressed as mean±SEM. *p < 0.05, **p < 0.01, ***p<0.001.



Supplementary Fig. S6: IL-2 and IL-15 expression in the human thymus. Illustrative immunohistochemistry staining in fixed serial thymic sections with: (A) anti-IL-2 or anti-IL-15, and anti-FOXP3 mAbs; (B) anti-CD68 mAb or anti-CD20 mAb; and (C) anti-IL-2 mAb or anti-CD68 mAb, as well as anti-IL-2 mAb or anti-CD20 mAb. In (A) the lower set of images correspond to a zoom of the region indicated by the yellow box in the upper ones. Scale bars, 100 and 50 μm (upper and lower images, respectively); original magnification x100. In (B) scale bar corresponds to 100 μm; original magnification x100. In (C) scale bars correspond to 20μm, respectively; original magnification x400.

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CHAPTER 4. CONCLUSIONS AND FUTURE PERSPECTIVES

The understanding of the mechanisms that underly immunological self-tolerance establishment and maintenance is still one of the major challenges of immunology and medicine. The precise control of the quality and magnitude of adaptive immune responses to self and non-self antigens holds an unquestionable therapeutic potential in several clinical scenarios such as allergy, transplantation, cancer, chronic infection and autoimmunity.

Regulatory T Cells (Treg) have emerged as key effectors of peripheral tolerance, with particular relevance in the prevention of autoimmunity, both in mice and in humans[1, 2, 3, 4]. There is cumulative evidence that a reduced and/or dysfunctional Treg compartment underlies the pathophysiology of several immune-mediated diseases. Therefore, there is a strong interest in manipulating this subset to achieve targeted immunosuppression, particularly in the fields of transplantation tolerance and autoimmunity[5, 6, 7, 8, 4]. Accordingly, several clinical trials using Treg-based immunotherapy are currently ongoing[5, 6, 7, 8, 4]. Since large numbers of Treg are required for clinical efficacy and they constitute a minor subset of peripheral $CD4^+$ T cells, a large effort has gone into developing clinical protocols for efficient *ex vivo* expansion of human Treg.

Protocols aiming the efficient expansion of Treg for clinical application face great challenges. First, it has been difficult to unequivocally identify and isolate human Treg from patients because of the lack of unique marker(s), as isolation based on FOXP3 expression precludes the obtention of viable cells[9]. Second, Treg exibit a weak proliferative potential, and repeated expansion has been associated with the risk of altering their phenotype and function[10]. Third, thymus-derived (t)Treg, particularly from autoimmune patients, have been demonstrated to feature considerable plasticity, and hence may acquire a pathogenic effector phenotype upon *ex vivo* expansion[11, 12, 13]. Finally, this strategy may not be ideal when the Treg pool features intrinsic defects, as is often the case in autoimmune contexts[4]. Therefore, novel and optimized strategies to generate *bona-fide in vitro* induced (i)Treg are a promising approach towards the establishment of Treg adoptive immunotherapy. At the same time, a deeper and more detailed knowledge of human Treg development, physiology and homeostasis will assuredly provide major progress in Treg manipulation for clinical purposes.

The overall aim of this work was to adress the efficiency of the *in vitro* conversion of memory CD4 into FOXP3-expressing cells and the role of the Notch signaling pathway in regulating this process. Additionally, this project intends to contribute to a better understanding of the rules dictating human Treg thymic development and peripheral homeostasis. Ultimately, we aimed not only to extend our knowledge on human Treg physiology but also, through this goal, to facilitate and improve Treg-based immunotherapy, particularly in autoimmune contexts.

In section 1 of the chapter of Results, we showed that stable and functional bona-fide iTreg can be generated from memory $CD4^+$ T cells of healthy subjects and that Delta-like (DL)1-mediated Notch signaling activation enhances this conversion. The benefical DL1 effects were also extended to conventional naïve $CD4^+$ T cells, supporting the feasibility of differentiating iTreg from human non-regulatory $CD4^+$ T cells in order to facilitate the establishment of adoptive Treg-based cellular therapies.

Treg reduction and/or dysfunction underlies the pathophysiology of several imune-mediated diseases in different clinical settings. Therefore, a great interest has arised in manipulating this subset for clinical purposes. As with any clinical intervention, safety is a major concern when considering immunotherapy with Treg. The strategies currently employed for Treg adoptive transfer rely on extensive *ex vivo* expansion of this subset, a strategy that faces a wide range of constraints. Some groups reported that repeated expansion of Treg alters their phenotype and function[10]. Moreover, tTreg were shown to be innefective in treating some autoimmune diseases (AID) in murine models, likely as a consequence of their unstable phenotype in pro-inflammatory environments[14, 15]. Importantly, iTreg have been shown to be stable in inflammatory conditions and resistant to Th17 conversion by interleukin (IL)-6[11, 16]. There is therefore a raising interest on iTreg induction from non-regulatory subsets, for use in the appropriated clinical scenarios. For instance, there is currently ongoing a United States (US) National Institute of Health registered phase I single center dose escalation study to determine the efficacy and tolerability of *in vitro* generated iTreg in adult patients undergoing non-myeloablative human leucocyte antigen (HLA)-identical sibling donor peripheral blood stem cell transplantation for the treatment of a high risk malignancy (NCT01634217, Masonic Cancer Center, University of Minnesota).

The vast majority of the induction studies used naïve non-regulatory T cells as the starting population. Nonetheless, a significant proportion of the circulating Treg compartment is thought to result from the conversion of memory $CD4^+$ T cells into Treg in response to antigenic stimulation *in vivo*[17, 18, 19]. Accordingly, human Treg and $CD4^+$ T cells have been shown to display, on average, 80% homology in their T cell receptors (TCR) Vβ usage[17]. Also, few groups reported successful *in vitro* induction of FOXP3 on memory $CD4^+$ T cells[20, 21, 22], with controversial results regarding their phenotype and function. Possible technical reasons may explain the previous generation of unstable and ineffective transforming growth factor beta (TGF-β)-induced iTreg in previous studies, namely the strength of TCR signaling, with data suggesting that a suboptimal stimulation provided by lower concentrations of anti-CD3 monoclonal antibody (mAb) or antigen (Ag) is probably ideal for efficient iTreg generation[23, 24, 20]. Importantly, in the particular setting of autoimmunity, conventional memory T cells are thought to be enriched in self-reactive T cells, rendering this subset as the ideal substrate for the induction of iTreg with relevant specificities. Our results show for the first time that bona-fide iTreg can be consistently generated from memory $CD4^+$ T cells of healthy subjects, displaying a stable and functional phenotype.

The Notch signaling pathway has undoubtedly been shown to play crucial roles in thymic and peripheral T cell differentiation, as well as in the modulation of T cell-mediated immune responses[25]. In this regard, the induction of specific Notch ligands by pathogen-derived signals in antigen presentig cells (APC) and their interaction with Notch receptors in $CD4^+$ T cells are clearly involved in the differentiation towards particular T helper phenotypes[25].

Interestingly, it has been previously shown that human Treg express Notch1 and Notch4 as well as DL1, Jagged (JAG)1 and JAG2[26]. Our data have shown that human Treg express Notch1 and 2. Moreover, loss-of-function experiments using DAPT, a well-known pharmacological inhibitor of the Notch signaling pathway, in cultures of sorted memory CD4 T cells with TCR stimulation, TGF-β and IL-2 and no extrinsic Notch ligands, led to a consistent decrease in the frequency and number of recovered iTreg. Therefore, Notch-mediated T-T communication seems to take place in the differentiation and homeostasis of Treg. Supporting a role for Notch signaling in Treg physiology, the Foxp3 promoter has

Notch-responsive elements[27], and the Treg-associated marker CD25 is claimed to be a Notch target[28].

We showed that the human Notch ligand DL1 increases the efficiency of conversion of conventional memory and naïve $CD4^+$ T cells into iTreg, in agreement with previous reports suggesting an important role for Notch signaling in murine iTreg differentiation[29, 30].

Additionally, we showed that DL1 enhanced the *in vitro* differentiation of human memory CD4 T cells into *bona-fide* iTreg with a stable and functional phenotype. These striking results suggest that Notch plays a key role in the in *vitro* generation of iTreg, with likely relevance *in vivo*. Notch ligands including DL1 are in fact highly expressed in the gut[31], where TGF-β-dependent generation of pTreg has been fully established. In human inflammatory bowel disease, Notch1 mucosal expression was shown to differ in inflamed and non-inflamed mucosa and to increase in response to beneficial anti-tumor necrosis factor α (TNFα) treatment[32]. Moreover, hepatitis B virus infection was shown to increase Notch1 and Foxp3 expression in intrahepatic T cells[33]. It has also been shown that Notch signaling regulation of T cell proliferation in T-cell acute lymphoblastic leukemia is related to Foxp3 expression[34]. Additionally, Systemic Lupus Erytemathosus (SLE) patients with active disease failed to upregulate Notch1 on T cells upon in *vitro* TCR stimulation, a phenomenon that correlates with decreased CD25 and FOXP3 expression[35]. Interestingly, we found that DL1 also enhanced FOXP3 and CD39 expression on iTreg generated from memory CD4 T cells, even in the absence of exogenous TGF-β. Assuming the possibility that the TGF-β produced by the T cells themselves contributes to the DL1-mediated effect, these results raise the appealing hypothesis that even in situations in which TGF-β is limiting, DL1 may help controlling the magnitude of local immune responses.

We thus postulate that Notch plays a proeminent role in the in *vitro* conversion of iTreg, likely extended to in *vivo* scenarios where the generation of Treg from non-regulatory precursors is required for a better control of immune/ inflammatory responses.

One major hurdle facing the clinical usage of Treg, particularly in autoimmune settings, is the problem of their stability. The ultimate goal in Treg immunotherapy is the generation of stable iTreg that lack the plasticity to convert to pathogenic effector cells. This is

particularly important in autoimmune settings because of the constant threat of reemerging autoimmunity. We showed that iTreg converted from memory $CD4^+$ T cells in the presence of DL1 displayed a considerable stable phenotype in long-term cultures and in the presence of pro-inflammatory cytokines, which is particularly relevant for clinical purposes in the setting of highly inflammatory scenarios as autoimmunity. It has been proposed that the Treg-specific demethylated region (TSDR) on TGF-β-induced iTreg but not on tTreg is methylated, contributing for their instability[36]. However, it was recently shown that the methylation *status* within the Foxp3 *loci* does not exclusively account for Treg stability and/or function[37], since efficient human TGF-β induced iTreg displaying a methylated Foxp3 *locus* were shown to be successfuly supressive[38]. It would be, nevertheless, relevant to assess the methylation status on our iTreg in further studies. Notably, it was recently shown that Notch1 activation converted high-grade adenoma into low-grade adenoma via suppression of Wnt target gene expression in cancer cells through epigenetic modification recruiting histone methyltransferase SET domain bifurcated 1, demonstrating an uncovered function of Notch signaling in mediating epigenetic modulation[39].

Despite the initial enthusiasm generated by clinical trials employing the adoptive transfer of expanded Treg, the aimed widespread application of Treg-based therapy encountered limitations related to the technical difficulty of expanding an homogenous and functionally stable population for *in vivo* use[11]. In fact, the recent evidence that human circulating Treg can lose FOXP3 expression and acquire a T cell effector phenotype during *in vitro* expansion has been hampering the potential of this approach[10]. We therefore assessed the impact of DL1 on the homeostatic proliferation and phenotype of TCR-stimulated circulating Treg. Our results show that DL1 increases Treg proliferation, reinforcing a possible role of Notch in the homeostasis of the human peripheral Treg compartment. Importantly, we also demonstrated that DL1 enhances the Treg expression of function-related molecules, contributing to the maintenance of their phenotype, a property worth to be explored in protocols aiming the efficient Treg expansion for immunotherapy purposes.

We have also investigated the mechanisms accounting for the DL1-mediated enhancement of iTreg differentiation from memory cells. Our results suggest that DL1 cooperatively interacts with the TGF-β signaling pathway, as evidenced by increased phosphorylation of

the TGF-β pathway downstream targets Smad-2 and -3. In fact, Notch intracellular domain increases Smad3 protein at transcriptional and post-transcriptional level[40, 41, 42]. Notch1 activation also enhances TGF-β signaling by increasing Smad nuclear translocation and its transactivation at promoter sites[43]. Moreover, Notch ligands significantly increase Treg suppressive effect on effector T cells via the upregulation of transforming growth factor beta receptor (TGF-βR)II expression and phosphorylation of Smad3[43]. We therefore hypothesize that the enhanced Smad-2 and -3 phosphorylation observed in the presence of DL1 (and absence of exogenous TGF-β) might occur via upregulation of TGF-βR and transcriptional/ pos-transcriptional modulation of *Smad3* promoted by the ligand.

The direct targeting of the Foxp3 promoter by Notch has been supported by presence of recombination signal binding protein for immunoglobulin kJ region (RBPJ) and Hes-1 binding sites within the murine and the human promoters[27]. Accordingly, our results demonstrate increased *Foxp3* mRNA expression in the presence of DL1 supporting that direct modulation of *Foxp3* gene transcription is involved in the Notch-mediated enhanced conversion of memory $CD4^+$ T cells into iTreg. Taken together, our data suggest that the DL1 impact on FOXP3 acquisition by memory $CD4^+$ T cells involves multiple mechanisms, such as the cooperative interaction with TGF-β signaling pathway and the modulation of Foxp3 transcription. Other possibilities, such as the downregulation of proinflammatory cytokine receptors (namely IL-6 receptor α (IL-6R α), on memory $CD4^+$ cells by DL-1 were not excluded and may be worth to be investigated[44].

Taken together, these data indicate that bona-fide iTreg can be differentiated *in vitro* from human memory CD4 cells and that DL1 increases the efficiency of this conversion, associated with a stable and functional phenotype.

The work of Maillard's group and others has firmly established an impact of Notch signaling on hematopoietic progenitors and alloimmune T cell regulation in the settings of allogeneic bone marrow and hematopoietic cell transplantation. *Ex vivo* culture of multipotent blood progenitors with immobilized DL ligands induces supraphysiological Notch signals that enhance progenitor expansion[45], attenuate myelosuppression and enhance T cell reconstitution in preclinical models. At the same time, Notch inhibition in donor-derived T cells or transient blockade of DL ligands after transplantation have been shown to markedly decrease graft-versus-host-disease (GVHD) incidence and severity in

mouse allo-hematopoietic stem cell transplantation (HSCT) models[46]. These distinct, not mutually exclusive interventions, with Notch manipulation in the immune system are a mirror of the different expected effects of Notch modulation according to the cell-type and context. In GVHD scenario much effort has gone into manipulating Notch in the perspective of its effects on pathogenic alloimmune T cells, which does not contradict the promising results obtained with the modulation of Notch for Treg manipulation[47].

SLE is a chronic multisystemic AID with a wide spectrum of clinical manifestations and substantial negative impact on the quality of life, resulting in a significant healthcare cost. Recent studies in Europe and the US indicate an increase in the incidence and improvement of survival rates in SLE, with concomitant increase in the prevalence of the disease[48]. Despite the improved survival of SLE patients in recent years, life-expectancy remains low compared to that of age and sex-matched populations[48]. In addition to mortality associated with disease activity, cardiovascular and infectious diseases associated largely with therapeutic regimens, in particular with broad immunosuppression, are a major complication[49]. Therefore, the development of targeted therapies, with potential intervention in disease pathogenesis, clinical efficacy and reduction of adverse effects associated with indiscriminate imunossupression, is crucial. There is increased evidence that a disturbed T-cell homeostasis plays a critical role in the development of SLE and, although studies on Treg in the peripheral blood of SLE patients have generated some conflicting data inherent to the the large heterogeneity in study methodologies, Treg function in SLE has been reported to be defective and their numbers appear to be decreased during the active phases of disease[1, 50]. These observations support the hypothesis that adoptive transfer of Treg would be an appropriate approach regarding SLE treatment. In fact, it is currently ongoing a clinical trial to evaluate the safety and effectiveness of autologous ex vivo selected and expanded polyclonal Treg in adults with cutaneous lupus (NCT02428309, Maria Dall'Era, National Institute of Allergy and Infectious Diseases, University of California). In this regard, to validate the *in vitro* efficacy of the protocols we previously established in a relevant AID setting, we purified peripheral memory non-regulatory $CD4^+$ T cells from an SLE patient in clinical remission and applied the previously optimized protocol for FOXP3 induction. Our results showed that the induction of Treg from memory non-regulatory cells from an SLE patient was effective and it was enhanced in the presence of the Notch ligand DL1. The iTreg

generated in the presence of DL1 expressed higher levels of CD39, a feature particular interesting in this setting since CD39-expressing Treg have been described as nearly absent in a subset of lupus patients with untreated minimally active disease presenting reduced adenosine-dependent Treg-mediated supression[51]. Furthermore, FOXP3 expression within induced iTreg appeared to be stable, validanting our approach in a relevant autoimmune scenario (data not shown).

As previously discussed, there are several distinct pathologies in which immunotherapy with polyclonal Treg may play a protective role. In the particular setting of AID, there is however clear evidence that Ag-specific Treg can be more effective[52]. The decision to use polyclonal or Ag-specific Treg will depend not only on the autoimmune disease in question but also on the autoimmune patient immunological profile. Organ-specific autoimmune diseases usually target well-known autoantigens, which would clearly favour the use of Ag-specific Treg[2]. In systemic autoimmune diseases such as SLE, a myriad of diffuse antigens and different cell types are targeted, likely making polyclonal adoptive Treg an adequate and effective approach. Notwithstanding, several T cell epitopes have been identified in SLE patients, such as histones, ribonucleoproteins and anti-DNA antibodies[53], making these attractive specificities to be targeted in optimized immunotherapy strategies using Ag-specific Treg.

The major challenge in Ag-specific Treg therapy is dictated by the difficulty in isolating sufficient numbers of Ag-specific Treg, given their reduced frequency in the periphery, further impaired by the limited availability of appropriate peptide–MHC multimers[2]. Despite these challenges, several groups are developing protocols aiming the utilization of Ag-specific Treg immunotherapy in autoimmunity. Arbour et al.[54] are currently investigating conditions for the expansion of myelin-specific T cells isolated from patients with Multiple Sclerosis (MS) as a possible source of Ag-specific Treg for clinical trials. Research is also underway to examine the use of Ag-specific Treg in the treatment of Type 1 Diabetes (T1D)[55]. In fact, Tarbell et al. successfully used antigen-loaded dendritic cells (DC) to expand islet-specific Treg, which upon transfer were capable of reversing hyperglycemia in 50% of treated diabetic mice[56].

The current technology still doesn't provide a robust and effective method for isolating and expanding a sufficient number of Ag-specific Treg for clinical use. Therefore, in the future

we propose to use self-reactivities present in the conventional CD4 pool of autoimmune patients and target them in *in vitro* conversion protocols to generate Ag-specific Treg.

Finally, it will be fundamental to evaluate the stability and function of the generated iTreg populations in preclinical humanized animal models, before any attempt of translation in humans.

Altogether, we expect our work to foster the development of cellular therapy employing Treg particularly in the setting of autoimmunity, providing a better therapeutic approach to the current immunosuppression.

In section 2 of the chapter of Results, we revealed the major contribution of IL-2 and IL-15 to human tTreg development, with clear insights into their mechanism of action, their pattern of expression and the populations responsible for their production in the human thymus.

Our results showed that pharmacological inhibition of JAK3, required for γc cytokine signaling, markedly reduced Treg development in thymic organ cultures (TOC) and co-cultures of thymocytes with primary thymic epitelial cells (TEC). In gain of function experiments, addition of exogenous IL-2 and IL-15 (but not IL-4, IL-7 and IL-21) to thymic cultures increased the frequency and number of Treg as well as their FOXP3 expression level, both at protein and transcriptional level. Moreover, the effects of IL-2 and IL-15 on tTreg development required signal transducer and activator of transcription (STAT)5 as well as the IL-2 receptor β chain.

Importantly, Treg arising in IL-2 and IL-15 supplemented cultures expressed higher levels of function-associated molecules and displayed efficient suppressor function. IL-2 and IL-15 additionally impacted on the survival and proliferation of differentiated tTreg. Notably, IL-2 and IL-15 also were also capable of commiting tTreg precursors into the Treg lineage. Hence, our results clearly establish IL-2 and IL-15 as two crucial common gamma chain (γc) cytokines in human tTreg development, with direct impact on lineage commitment, functional maturation, survival and proliferation.

Remarkably, we showed that IL-2 and IL-15 are expressed in the human thymus in non-

overlapping niches, with mature thymocytes and γδ cells appearing as the main IL-2 source whereas IL-15 production is mostly confined to macrophages and B cells.

We hence propose that human thymic Treg development follows a two step-model, highlighting a considerable evolutionary conservation in the key mechanisms governing human and mouse tTreg differentiation[57]. In this hypotetic model, TCR signals on developing thymocytes may be sufficient to induce CD25 expression, allowing these cells to respond to either IL-2 and/or IL-15, with consequent STAT5 activation and *FOXP3* transcription. FOXP3 expression would then act in conjunction with STAT5, ensuring the stabilization of the Treg lineage program.

In the future, it will interesting to compare the TCR repertoire of tTreg that differentiate in presence of IL-2 or IL-15 and to further investigate the possible role of the thymic celular populations that produce these cytokines in mediating tTreg selection.

The role for IL-2 in human tTreg development antecipated by our results can explain the observed increase in $CD45^{+}CD31^{+}$ Treg upon administration of IL-2 in several contexts[58, 59, 60, 61]. Indeed, IL-2 has recently become a promising immunotherapeutic agent in different scenarios[62]. IL-2 is known to promote both effector T cells (Teff) and Treg responses. Notwithstanding, several proof-of-concept clinical trials have demonstrated that low-dose IL-2 specifically expands and activates Treg without boosting Teff responses, presumedly because Treg are more sensitive to IL-2[62]. The first clinical trial with IL-2 administration in Hepatitis C Virus (HCV)-induced vasculitis[63] has shown low doses of IL-2 to be safe, and to be associated with a marked increase in the proportion and number of Treg that correlated with clinical improvement, with no obvious impact upon conventional $CD4^{+}$ T cells. Importantly, in chronic GVHD low-dose interleukin-2 has been safely administered with preferential Treg expansion and clinical amelioration[60,61]. Many other clinical trials with IL-2 administration for selective expansion of Treg in diferent pathologies (e.g. T1D and alopecia areata) are currently ongoing with encouraging preliminary results. Recently, TRANSREG proposes to assess the safety and biological efficacy of low-dose IL2 as a Treg inducer in a set of eleven autoimmune and auto-inflammatory diseases (Rheumatoid Arthritis, Ankylosing Spondylitis, SLE, Psoriasis, Behcet's Disease, Wegener's Granulomatosis, Takayasu's Disease, Crohn's Disease, Ulcerative, Colitis, Autoimmune Hepatitis, Sclerosing Cholangitis), with the objective of selecting diseases in which further therapeutic will be beneficial (NCT01988506,

Assistance Publique - Hôpitaux de Paris). Thus, in a phase of increasing clinical applicability, it is essential to scrutinize the role of IL-2 in thymic Treg development and peripheral homeostasis.

Importantly, our results also question the adequacy and safety of IL-15 administration to boost anti-tumor imune responses in humans[64], and suggest that IL-21 is probably a safer therapeutic choice. In fact, several clinical trials are currently ongoing with recombinant IL-15 administration in haematologic and solid tumors[65]. Notwithstanding, IL-15 has been shown paradoxically to participate in the development of some leukemias and solid tumors, namely by inhibiting apoptosis of tumor cells and supporting their survival, proliferation and migration[66, 65]. The IL-15's impact on tTreg development and differentiation may contribute to this effect.

Overall, our work has provided important insights into human tTreg development, proposing IL-2 or IL-15 administration as a novel approach for the treatment of autoimmunity.

In conclusion, as with any proposed therapy, numerous arguments can be made for and against cellular therapy with Treg, and safety is always a major concern. Notwithstanding, and particularly in the context of autoimmunity, it is extremely important to maintain the pursuit for better targeted therapies, associated with greater efficacy and fewer adverse effects. Importantly, we have shown that memory conventional $CD4^+$ T cells are a reliable source of stable and efficient bona-fide iTreg and that Notch signaling manipulation through DL1 enhances the efficiency of this conversion. We additionally clarified some of the molecular and cellular requirements for human thymic Treg development, which should facilitate the manipulation of this subset for clinical applications. Altogether, this work has contributed to a better understading of the intricate *in vivo* and *in vitro* Treg physiology, contributing to the progress of Treg based-therapies.

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CHAPTER 5. PUBLISHED ARTICLES INCLUDED IN THE THESIS

The Journal of Immunology

Delta-like 1–Mediated Notch Signaling Enhances the In Vitro Conversion of Human Memory CD4 T Cells into FOXP3-Expressing Regulatory T Cells

Catarina Mota,*,† Vânia Nunes-Silva,* Ana R. Pires,* Paula Matoso,* Rui M. M. Victorino,*,† Ana E. Sousa,* and Iris Caramalho*

FOXP3-expressing regulatory T cells (Treg) are essential for the prevention of autoimmunity and were shown to be reduced and/or dysfunctional in several autoimmune diseases. Although Treg-based adoptive transfer represents a promising therapy, the large cell number required to achieve clinical efficacy constitutes an important limitation. Therefore, novel strategies to generate bona fide in vitro–induced Treg (iTreg) are critical. In this study, we report that human memory CD4 T cells can be efficiently converted into iTreg, and that Delta-like 1 (DL1)–mediated Notch signaling significantly enhances this process. The iTreg generated in the presence of DL1 featured higher levels of Treg function–associated molecules and were efficient suppressors. Importantly, these iTreg displayed a stable phenotype in long-term cultures, even in the presence of proinflammatory cytokines. Additionally, DL1 potentiated FOXP3 acquisition by memory CD4 cells through the modulation of the TGF-β signaling pathway and of *Foxp3* transcription. Our data demonstrate that iTreg can be efficiently induced from memory CD4 cells, a subset enriched in relevant specificities for targeting in autoimmune diseases, and that DL1 enhances this process. DL1 also enhanced the proliferation and Treg function–associated marker expression of ex vivo–stimulated human circulating FOXP3$^+$ cells. Manipulation of the Notch signaling pathway constitutes a promising approach to boost the in vitro generation of iTreg and ex vivo Treg expansion, thus facilitating the establishment of effective Treg-based adoptive therapy in autoimmune diseases. ***The Journal of Immunology*****, 2014, 193: 5854–5862.**

Regulatory T cells (Treg) are crucial in the induction of self-tolerance and prevention of autoimmunity. These cells were shown to be decreased in number and/or to display compromised function in several human autoimmune diseases (1), in agreement with experimental animal models of autoimmunity, demonstrating that defects in the Treg compartment contribute to disease development (2). The potential of harnessing Treg for immunotherapeutic purposes in distinct clinical settings is compelling, and adoptive transfer of Treg is considered a method for so-called "intelligent immunosuppression" (3). Adoptive transfer of autologous Treg appears, in fact, to provide most of the benefits of efficient immunosuppression without the adverse effects of standard immunosuppressive drugs (3). The adoptive transfer of Treg in experimental mouse models of autoimmunity led to disease prevention and remission (2, 4), and the first clinical trials, involving the adoptive transfer of human Treg in type I diabetes, are currently ongoing, with promising preliminary results (3).

However, because large numbers of Treg are required for clinical efficacy, and as they constitute a minor subset of peripheral CD4 T cells, a large amount of effort has gone into developing clinical protocols for the ex vivo expansion of human Treg. The current approaches have suffered some setbacks, namely the weak proliferative potential of Treg, the risk of altering Treg phenotype and decreasing their immunosuppressive activity associated with repeated expansion, and the inadequacy of this strategy when the Treg pool features intrinsic defects (3, 5).

The establishment of efficient protocols enabling the generation of bona fide in vitro–induced Treg (iTreg) could overcome these problems. In protocols of in vitro conversion, naive CD4 T cells are the usual target population, and in fact many reports, both in mice and in humans, show that these cells can acquire high levels of FOXP3 expression associated with regulatory properties (6–11). FOXP3 expression is currently the best available marker to identify Treg and is required for their development and function (1, 2). The potential of human conventional memory CD4 cells to acquire in vitro a stable Treg phenotype remains poorly explored, although a significant proportion of the circulating Treg compartment is thought to result from the conversion of memory CD4 cells into Treg in response to antigenic stimulation in vivo (12–14). Moreover, human Treg and memory CD4 T cells have been shown to display, on average, 80% homology in their TCR Vβ usage (12). Importantly, conventional memory T cells in autoimmune patients are likely enriched in self-reactive T cells, making them particularly relevant substrates for the induction of iTreg with relevant specificities in the setting of autoimmunity.

The Notch signaling pathway is an evolutionarily conserved signaling cascade involved in cell differentiation processes at

*Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisbon, Portugal; and †Hospital Universitário de Santa Maria, Centro Hospitalar Lisboa Norte, 1649-035 Lisbon, Portugal

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Address correspondence and reprint request to Iris Caramalho, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisbon, Portugal. E-mail address: iriscaramalho@fm.ul.pt

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Abbreviations used in this article: DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DL1, Delta-like 1; DL4, Delta-like 4; iTreg, in vitro–induced Treg; Jag1, Jagged 1; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; pTreg, peripheral Treg; Treg, regulatory T cell.

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distinct developmental stages. There is solid experimental data in mice demonstrating a crucial role for Notch signaling in the thymic generation of Treg, their peripheral expansion and function, as well as in the in vitro conversion of conventional T cells into iTreg (15–19). In humans, there is evidence suggesting a role of Notch signaling in thymic Treg development as well as in peripheral Treg expansion (20–23), although the putative role of Notch signaling in iTreg conversion has never been formally assessed.

In the present study, we investigated the capacity of human nonregulatory memory CD4 T cells to differentiate in vitro into bona fide FOXP3-expressing cells and the role of the Notch signaling pathway in modulating this conversion. Our results showed that Notch enhanced the conversion of memory CD4 T cells into stable and efficient iTreg, supporting a role, particularly for Delta-like 1 (DL1), in human Treg conversion. DL1 also enhanced the proliferation of ex vivo–stimulated human circulating FOXP3$^+$ cells as well as their expression levels of molecules associated with effector function. Manipulation of the Notch signaling pathway may therefore constitute a promising approach to facilitate the establishment of Treg-based therapies.

Materials and Methods

Flow cytometry

The following anti-human Abs (with clones in parentheses) were used: CD4–Alexa Fluor 450 (RPA-T4), CD39-allophycocyanin (eBioA1), FOXP3–PerCP-Cy5.5 (PCH101), CD8-FITC (RPA-T8), CD14-FITC (61D3), CD19-FITC (HIB19), CD45RO-PE (UCHL1), CD127-allophycocyanin–eFluor 780 (eBioRDR5), CD45RA–PercCP-Cy5.5 (HI100), CD4-allophycocyanin (RPA-T4), S6 (pS235/pS236)–Alexa Fluor 488 (N7-548), and Akt (pS477)–Alexa Fluor 488 (H89-61) (all from eBioscience, San Diego, CA); Ki67-PE (B56), CTLA-4–PE (BNI3), and CD25-PE-Cy7 (clone 2A3) (BD Biosciences, Franklin Lakes, NJ); purified Smad2 (pSer$^{465/467}$)/Smad3 (pSer$^{423/425}$) (D6G10) (Cell Signaling Technology, Danvers, MA); Bcl2-FITC (124) (Dako, Glostrup, Denmark); and anti-rabbit IgG–Alexa Fluor 546 (Invitrogen, Carlsbad, CA). After surface staining for 30 min at 4°C with optimal dilutions of each mAb, cells were fixed, permeabilized, and stained for intracellular molecules using the Foxp3 staining kit from eBioscience, according to the manufacturer's instructions. Eight- to 10-parameter acquisition was performed on an LSR Fortessa flow cytometer (BD Biosciences). For phosphorylation level assessment, cells were fixed and permeabilized using the BD Phosflow staining kit (BD Biosciences), stained for 30 min at 4°C with optimal dilutions of each mAb, and acquired with a FACSCalibur (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Ashland, OR) after exclusion of dead cells using Live/Dead fixable viability dye (Molecular Probes, Eugene, OR) and doublets using a plot of forward scatter height versus amplitude. The FOXP3^{bright} population gate was always defined in each experiment, using the TCR stimulation and TGF-β culture condition and this gate subsequently used to identify this population in all additional culture conditions performed.

Cell purification

A negative selection kit was used to obtain CD4 T cells (RosetteSep human CD4$^+$ T cell enrichment cocktail, StemCell Technologies, Grenoble, France) from buffy coats of healthy subjects, according to the manufacturer's instructions. After surface staining, the following populations were sort-purified on a FACSAria cell sorter (BD Biosciences) with >95% purity: memory CD4$^+$CD25$^-$CD45RA$^-$CD127^{hi}, naive CD4$^+$CD25$^-$CD45RA$^+$CD127^{hi},and Treg CD4$^+$CD25^{hi}CD127^{lo}. The study was approved by the Ethical Board of the Faculdade de Medicina da Universidade de Lisboa.

OP9 stromal cell line

OP9 stromal cells expressing the human Notch ligand DL1, Delta-like 4 (DL4), or Jagged 1 (Jag1) and control OP9 cells transduced with the corresponding empty GFP vector (OP9 Empty) were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 50 μg/ml gentamicin (all from Invitrogen). OP9 monolayers were prepared 24 h before their use in coculture experiments by plating 10G irradiated stromal cells in 48-well culture flat-bottom plates in complete DMEM medium.

Cell culture

Human T cells were cultured in IMDM medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 50 μg/ml gentamicin, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, and 1% nonessential amino acids (IMDM complete medium). Sort-purified memory, naive, and Treg CD4 cells were TCR stimulated with either 1 or 2 μg/ml plate-bound anti-CD3 mAb (clone OKT3, eBioscience), 1 μg/ml soluble anti-CD28 mAb (clone CD28.2, eBioscience), and 50 IU/ml IL-2 (AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) at a density of 2.5 × 10^5 cells/well in complete IMDM, for 5 d, in the presence or absence of 5 ng/ml TGF-β (PeproTech, Rocky Hill, NJ). Similar culture conditions were used in the presence of OP9 stromal cells, except that memory cells and Treg were TCR stimulated with 2 and 1 μg/ml soluble anti-CD3 mAb, respectively, plus soluble anti-CD28 mAb, whereas naive CD4 cells were stimulated with CD3/CD28 Dynabeads (Invitrogen) at a 1:16 bead/T cell ratio.

For assessment of iTreg phenotypic stability, memory cells stimulated for 5 d under FOXP3-inducing conditions, were washed, replated, and cultured for 9 more days in complete IMDM supplemented with 50 IU/ml IL-2 and 100 nM rapamycin (Sigma-Aldrich), or restimulated with CD3/CD28 Dynabeads (1:2 ratio of beads/T cells) for 3 more days in medium supplemented with IL-2 (12.5 IU/ml) only or with IL-2 (12.5 IU/ml), 20 ng/ml IL-6 (PeproTech), and 20 ng/ml IL-1β (PeproTech). FOXP3 expression levels on recovered iTreg were evaluated by FACS.

A C2 cell differentiation assay in the presence of OP9 cells was performed as described (24).

In vitro suppression assay

Memory CD4 cells stimulated for 5 d under FOXP3-favoring conditions, in the presence of control OP9 or OP9 DL1 cells, were washed and replated in medium supplemented with 50 IU/ml IL-2 and 100 nM rapamycin and cultured for 5 more days before use in the in vitro suppression assay. Sort-purified CD25$^-$ CD4 cells (targets) were resuspended at 2.5 × 10^6/ml in serum-free RPMI 1640, stained with 2.5 μM CFSE (Invitrogen) for 5 min at room temperature (reaction stopped by the addition of 20% FCS), washed once in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, and stimulated either alone (1:0 ratio) or in the presence of the differentiated iTreg population at the ratios of 1:1, 2:1, and 4:1 (target/iTreg). Their CFSE labeling intensity was assessed at day 4 of culture by flow cytometry. As controls, sort-purified Treg from the same healthy donor as the target cells were used. The inhibition index was calculated as follows: [(% proliferating target cells when plated alone − % proliferating target cells when cocultured with Treg)/% proliferating targets cells when plated alone] × 100.

Signaling experiments

For assessment of PI3K/Akt/mammalian target of rapamycin (mTOR) signaling pathway modulation, sort-purified memory cells were stimulated at a density of 1 × 10^6 cells/ml in complete RPMI medium for 14 h with CD3/CD28 Dynabeads, at a 1:2 ratio (beads/T cells), in the presence of OP9 empty or OP9 DL1 cells. As a control, cells were left in medium alone or stimulated with beads and 100 nM rapamycin in the presence of OP9 control stroma. For assessment of TGF-β signaling pathway modulation, memory cells were cultured at 2.5 × 10^5 cells/well in complete RPMI medium without FBS for 3 h. Cells were then stimulated with or without TGF-β (5 ng/ml, PeproTech) in the presence of control OP9 or OP9 DL1 for 2 more hours.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen). The mRNA levels of FOXP3 and GAPDH were quantified in duplicates with TaqMan gene expression kits, according to the manufacturer's instructions, using a 7500 Fast real-time PCR system (all from Applied Biosystems, Foster City, CA). *Smad3* (forward, 5′-TGCCTTTCACTCCCCCGAT-3′, reverse, 5′-TGCCCCGTCTTCTTGAGTTTC-3′), *Notch1* (forward, 5′- CGGGTCCACCAGTTTGAATG-3′, reverse, 5′-GTTGTATTGGTTCGGCACCAT-3′), *Notch2* (forward, 5′-TTGAGAGTTATACTTGCTTGTGTGC-3′, reverse, 5′-GATACACTCGTCAATGTCAATGG-3′), *Notch3* (forward, 5′-ATGCAGGATAGCAAGGAGGA-3′, reverse, 5′-AAGTGGTCCAACAGCAGCTT-3′), and *Notch4* (forward, 5′-ACCTGCTCAACGGCTTCCA-3′, reverse: 5′-AGCTTCTGCACTCATCGATATCCTC-3′) gene expression were quantified with SYBR Green PCR Master Mix (Applied Biosystems). The relative expression of *FOXP3*, *Notch1* to *Notch4*, and *Smad3* were normalized to GAPDH

expression, and the fold change in transcription was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence

Cells were adhered to poly-L-lysine–coated slides, fixed with 4% paraformaldehyde, and permeabilized with either 0.1% saponin (Notch ligand and receptor stainings) or 0.5% Triton X-100 (troponin T staining). The following primary Abs were used (clones in parentheses): anti-Notch1 mAb (MHN1-519; BioLegend, San Diego, CA); anti-Notch2 (C651.6DbHN) and anti–troponin T (CT3) mAbs (Developmental Studies Hybridoma Bank, Iowa City, IA); anti-DL1 (MHD1-314) and anti-DL4 (MHD4-46) mAbs (provided by Dr. Hideo Yagita, Juntendo University, Tokyo, Japan); and anti-Jag1 Ab (H-114; Santa Cruz Biotechnology, Santa Cruz, CA).

Appropriate fluorochrome-conjugated secondary Abs were obtained from Invitrogen. DAPI was used as for nuclear counterstaining and slides were mounted in Mowiol. Confocal images were acquired with a Zeiss 710 confocal point-scanning microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.01 (GraphPad Software, San Diego, CA) using a Wilcoxon matched-pairs signed rank test. Results are expressed as means ± SEM.

Results

DL1-mediated Notch signaling potentiates iTreg conversion from human memory CD4 T cells

Our first aim was to assess whether human memory CD4 T cells are a reliable source of Treg precursors. With this in mind, naive ($CD4^+CD25^-CD127^{hi}CD45RA^+$) and memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 T cells were sort-purified from healthy subjects and stimulated in vitro with increasing concentrations of plate-bound anti-CD3 mAb in the presence of anti-CD28 mAb, TGF-β, and IL-2. The inclusion of CD127 in the sorting strategy guaranteed an effective exclusion of contaminating FOXP3-expressing cells from the starting populations (25, 26) (Supplemental Fig. 1A). A considerable proportion of memory as well as naive CD4 T cells acquired FOXP3 expression (Supplemental Fig. 1A). Only $FOXP3^{bright}$ cells were considered for the analysis to ensure their regulatory phenotype. Of note, the anti-TCR Ab requirements for conversion of naive and memory CD4 T cells were different, with the lowest concentration of anti-CD3 mAb tested (1 μg/ml) being the best suited for in vitro conversion protocols using memory CD4 T cells (Supplemental Fig. 1A). Importantly, the levels of FOXP3 expression in memory CD4 T cells after induction were equal to or higher than those observed in sort-purified Treg activated with anti-CD3 and anti-CD28 mAbs for the same length of time (Supplemental Fig. 1B). Our induction protocol consistently resulted in the acquisition of FOXP3 expression by a significant proportion of memory CD4 cells in all individuals tested (30 ± 3.3% of cells acquiring FOXP3 expression [n = 10] as compared with 40 ± 9.2% [n = 10] when starting with naive CD4 T cells).

To investigate the potential role of Notch in the in vitro conversion of human conventional CD4 into iTreg, we first performed loss-of-function experiments using the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), a well-known pharmacological inhibitor of the Notch signaling pathway. Addition of 10 μM DAPT to sorted memory CD4 T cells cultured with TCR stimulation, TGF-β and IL-2, led to a consistent decrease in the frequency and number of $FOXP3^+CD25^+$ cells recovered at day 5 (Fig. 1A), as well as in their *Foxp3* mRNA expression levels (22.3 ± 4.4%, n = 2). These results support the hypothesis that Notch signaling plays a role in the in vitro conversion of memory CD4 into iTreg, leading us to evaluate the impact of distinct human Notch ligands, namely DL1, DL4, and Jag1, on the induction of FOXP3 expression, after confirming Notch receptor expression on T cells (Supplemental Fig. 1C). Purified memory CD4 cells were cocultured with either control OP9 cells (transduced with an empty vector) or stromal cells separately expressing functional DL1, DL4, and Jag1 (see Supplemental Fig. 1E, 1F), in conditions favoring FOXP3 induction. From the ligands we tested, only DL1 systematically increased the efficiency of FOXP3 acquisition by conventional memory CD4 T cells, excluding a major role for DL4 and Jag1 (Fig. 1B). Coculture of sort-purified memory CD4 cells with OP9 DL1 in FOXP3-inducing conditions significantly increased both the frequency and number of $FOXP3^+CD25^+$ cells in the 23 individuals tested (Fig. 1B). Of note, the comparison of OP9 empty versus OP9 DL1 revealed that DL1 neither modulated the survival of $FOXP3^-$ cells (Bcl2 mean fluorescence intensity [MFI], 14,403 ± 1,474 versus 13,509 ± 1,385, n = 7; cell recovery, $21.1 \times 10^4 \pm 5 \times 10^4$ versus $19.6 \times 10^4 \pm 3.6 \times 10^4$, n = 23) nor their differentiation profile as assessed by CCR6/CXCR3 expression (data not shown). Moreover, addition of DAPT significantly diminished the frequency of converted FOXP3-expressing cells (27.2 ± 2.5 in empty versus 34.2 ± 3.5 in DL1 versus 24.0 ± 5.3 in DL1 + DAPT, n = 6), suggesting that Notch receptor cleavage is required for the DL1 effect and reinforcing its role in human iTreg conversion using the OP9 system.

DL1 also potentiated the iTreg generation from human naive CD4 T cells. In fact, DAPT-mediated inhibition of Notch signaling resulted in decreased conversion efficiency (Fig. 1C), and the presence of DL1 led to an increased frequency of iTreg generated from naive CD4 T cells (Fig. 1D).

Overall, these results support the feasibility of targeting human memory and naive CD4 T cells in protocols of in vitro iTreg conversion and show that DL1 increases the efficiency of this process.

DL1 enhances the expression levels of Treg function–associated molecules

Having established that DL1 enhances the in vitro conversion of memory CD4 cells into FOXP3-expressing cells, we next evaluated the phenotype and function of these iTreg. Induction in the presence of DL1 led to significantly higher levels of expression of FOXP3 (Fig. 2A), as well as of other Treg lineage–associated markers, namely CTLA-4 (Fig. 2B) and CD39 (Fig. 2C).

Standard in vitro suppression assays were used to evaluate the suppressive function of iTreg (27), using as a control population freshly isolated circulating Treg ($CD4^+CD25^{hi}CD127^{lo}$, 80–90% of them expressing FOXP3). The iTreg generated from memory CD4 cells, both in the absence and presence of DL1, efficiently inhibited the proliferation of target cells in a dose-dependent manner (Fig. 2D).

Similar results were obtained when targeting human naive CD4 cells, with iTreg generated in the presence of DL1 displaying higher levels of FOXP3 and CD39 expression (Fig. 2E and 2F, respectively) and efficient suppressive activity in vitro (data not shown).

We also found that DL1 affected iTreg induction even in the absence of exogenous TGF-β, significantly increasing both the frequency and number of $FOXP3^+CD25^+$ cells and enhancing the expression levels of FOXP3 and CD39 as well as of *Foxp3* mRNA (Supplemental Fig. 2). Importantly, the effects of DL1 on iTreg conversion and phenotype were nevertheless more marked in the presence of exogenous TGF-β.

We also evaluated the impact of the Notch signaling pathway on sort-purified circulating Treg, whose expression of Notch receptors we confirmed by qRT-PCR and immunofluorescence (Supplemental Fig. 1D). First, we assessed the effect of Notch signaling

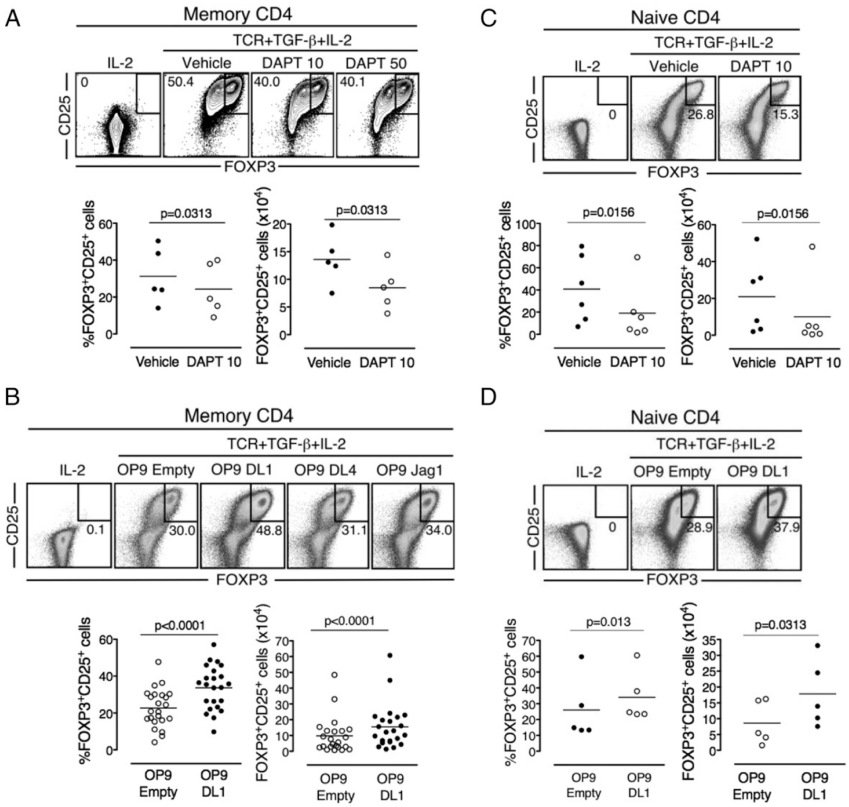

FIGURE 1. DL1 mediated enhancement of the in vitro conversion of memory and naive CD4 cells into iTreg. Sort-purified memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) and naive ($CD4^+CD25^-CD127^{hi}CD45RA^+$) CD4 cells were TCR stimulated for 5 d in medium supplemented with TGF-β and IL-2 (TCR + TGF-β + IL-2). Cells left in medium with IL-2 alone (IL-2) were used as controls. (**A** and **C**) Dot plots illustrate FOXP3 and CD25 expression in the presence or absence (vehicle, DMSO) of the γ-secretase inhibitor DAPT (at the indicated concentration in μM) within recovered live cells in memory (A) and naive (C) CD4 cultures, and graphs show the frequency and number of generated $FOXP3^+CD25^+$ cells ($n = 5$ and $n = 6$ for memory and naive CD4 cultures, respectively). (**B** and **D**) Representative dot plots of CD25 and FOXP3 expression on memory (B) and naive (D) CD4 cells stimulated in the presence of control OP9 stroma cells (Empty) or OP9 stroma expressing the human ligand DL1. Cocultures of memory CD4 cells with DL4 or Jag1 stroma cells are also depicted in (B). Graphs show the frequency and number of $FOXP3^+CD25^+$ cells generated in the presence of OP9 DL1 or OP9 empty ($n = 23$ and $n = 5$, memory and naive CD4 cultures, respectively).

pathway inhibition using DAPT and found that it systematically decreased FOXP3 expression levels in TCR-stimulated sort-purified Treg ($CD4^+CD25^{hi}CD127^{lo}$) from healthy subjects (Supplemental Fig. 3A). Notably, DL1 also consistently enhanced the expression of FOXP3, CTLA-4, and CD39 on these cells (Supplemental Fig. 3B–D).

Overall, our data demonstrated that DL1, in addition to increasing the efficiency of conversion, also enhanced the expression of FOXP3 and other Treg-associated molecules related to effector function, both in the absence and presence of exogenous TGF-β. Moreover, they further support the hypothesis that bona fide iTreg can be differentiated in vitro from human memory CD4 cells.

Treg induced in the presence of DL1 display a stable phenotype

Next we evaluated the stability of the FOXP3-expressing phenotype of iTreg generated from memory CD4 cells, specifically in a proinflammatory context, which is of utmost importance for their therapeutic use. After 5 d stimulation in conditions favoring FOXP3 induction (induction period), differentiated cells were left for a further 9 d in medium supplemented with IL-2 and rapamycin only (resting period), and the expression of FOXP3 was assessed at two separate time points (days 5 and 9). FOXP3 expression was maintained in iTreg at both time points (day 5 induction, 14.9 ± 3.8 versus 32.7 ± 8.4; day 5 resting, 31.6 ± 10.1 versus 38.4 ± 6.3; day 9 resting, 35.6 ± 12.0 versus 40.2 ± 8.7; empty versus DL1, $n = 3$), suggesting a stable phenotype in long-term cultures, even in the absence of TCR stimulation and exogenous TGF-β (Fig. 3A). Of note, the increased FOXP3 frequency we observed in long-term cultures was not associated with the preferential survival of $FOXP3^+$ cells (data not shown). Additionally, we evaluated the behavior of iTreg in the presence of proinflammatory cytokines. Cells recovered after the 5 d induction period were TCR restimulated in the presence or absence of IL1-β and IL-6 for 3 more days. We observed that $FOXP3^+$ expression was maintained in both culture conditions (day 5 induction, 43.4 ± 15.4 versus

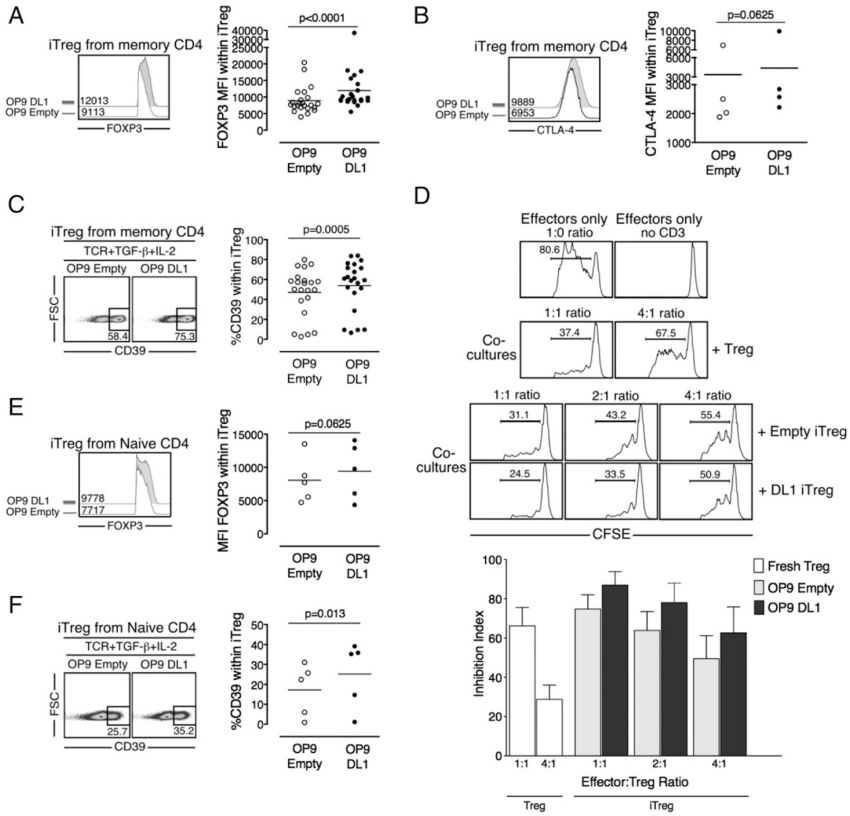

FIGURE 2. Levels of Treg function–associated molecules and suppressive function of iTreg generated from memory and naive CD4 cells in the presence of DL1. Sort-purified memory ($CD4^+CD25^-CD127^{hi}CD45RA^-$) and naive ($CD4^+CD25^-CD127^{hi}CD45RA^-$) CD4 cells were TCR stimulated for 5 d in medium supplemented with TGF-β and IL-2 (TCR + TGF-β + IL-2) in the presence of OP9 cells expressing DL1 or control OP9 stromal cells (Empty). (**A** and **E**) Representative histogram of FOXP3 expression and graph of FOXP3 MFI within generated $FOXP3^+$ cells from memory (A) and naive (E) CD4 cultures ($n = 23$ and $n = 5$ for memory and naive CD4 cultures, respectively). (**B**) Representative histogram of CTLA-4 expression and graph of CTLA-4 MFI within converted $FOXP3^+$ cells from memory CD4 cells ($n = 4$). (**C** and **F**) Illustrative dot plots of the expression of CD39 and graph of the frequency of $CD39^+$ cells within differentiated $FOXP3^+$ iTreg from memory (C) and naive (F) CD4 cells ($n = 22$ and $n = 5$ for memory and naive CD4 cultures, respectively). (**D**) Suppressive function of iTreg differentiated from memory CD4 cells assessed after an additional 5-d resting period in medium supplemented with IL-2 and rapamycin using allogeneic target cells ($CD4^+CD25^-$ cells isolated from the peripheral blood of a healthy subject). Representative histograms show the CFSE intensity of target cells when stimulated alone (1:0 ratio) or at the indicated ratios of iTreg or freshly isolated Treg from the same donor of target cells. Graph shows the inhibition index of fresh Treg and iTreg ($n = 3$), calculated as described in *Materials and Methods*.

55.8 ± 13.0; day 3 restimulation, no IL-1β and IL-6, 53.9 ± 18.6 versus 52.2 ± 16.1; day 3 restimulation, in presence of IL-1β and IL-6, 52.7 ± 16.2 versus 57.3 ± 13.5; empty versus DL1, $n = 3$) (Fig. 3B).

Overall, our data indicate that iTreg generated from memory CD4 cells were stable both in long-term cultures and even in the presence of proinflammatory cytokines.

Mechanisms mediating DL1 enhancement of iTreg differentiation from human memory CD4 T cells

Several mechanisms may underlie the DL1-mediated enhancement of FOXP3 induction in memory CD4 cells. First, we hypothesized that DL1 might increase the proliferation and/or promote the preferential survival of $FOXP3^+$ cells during the induction period. However, it is unlikely that either mechanism significantly contributed, as the expression of Ki67 and Bcl2 in the recovered $FOXP3^+$ cells differentiated from sort-purified memory CD4 cells was similar in the presence and absence of DL1 (Fig. 4A and 4B, respectively). Similarly, the frequency of $Ki67^+$ cells and the Bcl2 expression levels within $FOXP3^-$ cells were not modulated by DL1 (data not shown). The absence of a DL1 effect on survival was further reinforced by the similar frequency of live cells recovered at day 5 of induction, in the presence or absence of the ligand (data not shown).

The in vitro survival of TCR-stimulated circulating Treg was also not altered by the presence of DL1, as assessed by their Bcl2 levels (Supplemental Fig. 3E). Notably, the frequency of cycling cells, estimated by the proportion of Ki67, was increased by the ligand (Supplemental Fig. 3F), supporting the possibility that Notch signaling is involved in the homeostasis of the human peripheral

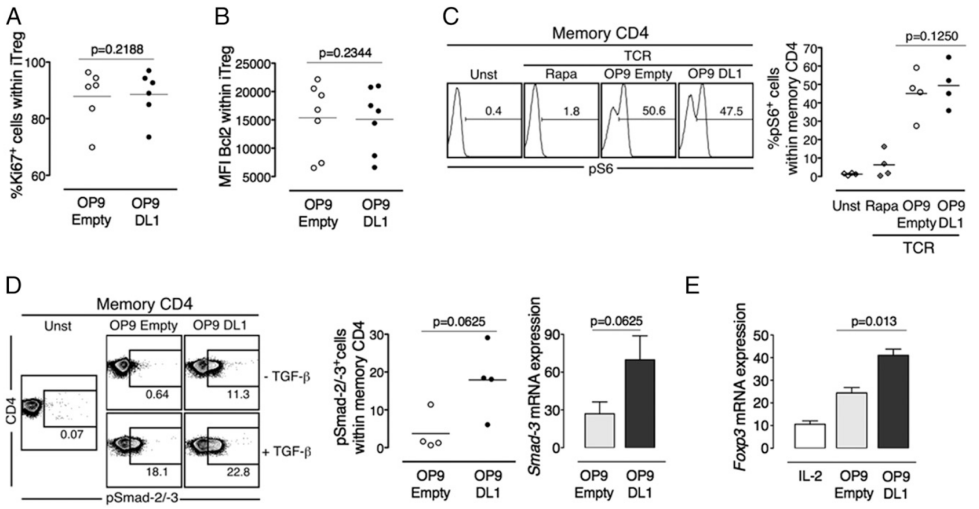

FIGURE 4. Mechanisms mediating DL1 enhancement of iTreg differentiation from memory CD4 cells. (**A** and **B**) Sort-purified memory CD4 cells ($CD4^+CD25^-CD127^{hi}CD45RA^-$) were TCR stimulated for 5 d in medium supplemented with IL-2 and TGF-β in the presence of OP9 DL1 (DL1) or control OP9 stroma (Empty). Graphs show the frequency of $Ki67^+$ (A) and Bcl2 MFI (B) within generated $FOXP3^+$ iTreg ($n = 6–7$). (**C**) Histograms show S6 phosphorylation levels, as a measure of mTOR activity, assessed after 14 h TCR stimulation in the presence of OP9 DL1 or OP9 empty. Cells left in medium without TCR stimulation (Unstim) or TCR stimulated in the presence of the mTOR inhibitor rapamycin (Rapa) and OP9 control stroma were used as controls. Graph shows the frequency of phosphorylated $S6^+$ cells in the described conditions ($n = 4$). (**D**) Illustrative dot plots of Smad2/3 phosphorylation within sort-purified memory CD4 cells stimulated for 2 h with or without TGF-β, in the presence of OP9 DL1 or OP9 empty, after a 3-h resting period in serum-free medium. Graphs show the frequency of Smad2/3 phosphorylated cells in the described condition without addition of exogenous TGF-β ($n = 4$) and the *Smad3* mRNA levels in sort-purified memory CD4 cells TCR-stimulated for 24 h in medium supplemented with IL-2 in the presence of OP9 DL1 (DL1) or control OP9 stroma (Empty) ($n = 4$). (**E**) *Foxp3* mRNA levels in sort-purified memory CD4 cells stimulated for 24 h as described in (A) ($n = 3$). Cells left in medium with IL-2 alone (IL-2) were used as controls.

a major mechanism to maintain a stable pTreg pool over time (12). Accordingly, a few reports show successful in vitro induction of FOXP3 in memory CD4 cells (32–34). However, there was controversy regarding their phenotype and function, likely due to methodological aspects, mostly related to TCR stimulation strength, suggesting that a suboptimal stimulation provided by lower concentrations of anti-CD3 mAb or Ag may be ideal for efficient iTreg generation (8, 11, 32). Indeed, our results extend the previous data by showing that stable and functional bona fide iTreg can be generated from memory CD4 cells of healthy subjects.

The Notch signaling pathway plays multiple roles in thymic T cell development and peripheral T cell differentiation (35). Accordingly, pathogen-derived signals have been shown to induce or modulate the expression of specific Notch ligands on APC whose interaction with Notch receptors on CD4 T cells have a profound impact on their differentiation toward particular T helper phenotypes (35, 36). The expression of Notch receptors and ligands is nevertheless not restricted to CD4 T cells and APCs, respectively. For example, human dendritic cells express DL1, Jag1 and Jagged 2 ligands, in addition to Notch receptors (37). Human Treg were also shown to express Notch1 and Notch4 as well as DL1, Jag1, and Jagged 2, and thus, in principle, being capable of Notch-mediated T–T communication (38). Importantly, the Foxp3 promoter contains Notch-responsive elements (22), and the Treg-associated marker CD25 is also claimed to be a Notch target gene (39). Notably, systemic lupus erythematosus patients with active disease failed to upregulate Notch1 on T cells upon in vitro TCR stimulation, a phenomenon that correlated with decreased CD25 and FOXP3 expression (40).

A role for Notch signaling in human Treg development and expansion has been previously suggested (20, 21, 23). In fact, human cord blood $CD34^+$ cells differentiate into mature Treg upon coculture with OP9-DL1, suggesting that Notch is involved in human thymic Treg development (21). Moreover, APCs overexpressing human Jag1 promoted the expansion of alloantigen-specific cells with regulatory properties from human naive CD4 cells (20). Additionally, mesenchymal stem cells cultivated with human $CD4^+$ T cells enhanced the recovery of $FOXP3^+$ cells via a Notch1-mediated mechanism (23). However, these studies did not discriminate whether Notch signaling was promoting Treg expansion and/or Treg de novo induction.

We revealed that DL1 affected TCR-stimulated pTreg by increasing their proliferation and expression of Treg function–associated markers, further reinforcing the contribution of the Notch signaling pathway in human Treg homeostasis. Additionally, we showed that bona fide iTreg with a stable and functional phenotype can be efficiently differentiated in vitro from human memory CD4 T cells, and this process was enhanced in the presence of DL1. Based on our results, we suggest that Notch plays a key role in the in vitro generation of iTreg, which likely reflects in vivo scenarios where their generation from nonregulatory precursors would allow a better control of immune responses. This is the case in the gut, where Notch ligands, including DL1, are highly expressed (41) and where TGF-β–dependent generation of pTreg takes place. Interestingly, we found that DL1 also enhanced FOXP3 and CD39 expression on iTreg generated from conventional memory CD4 cells, even in the absence of exogenous TGF-β. Although we cannot exclude that the TGF-β produced by the T cells themselves

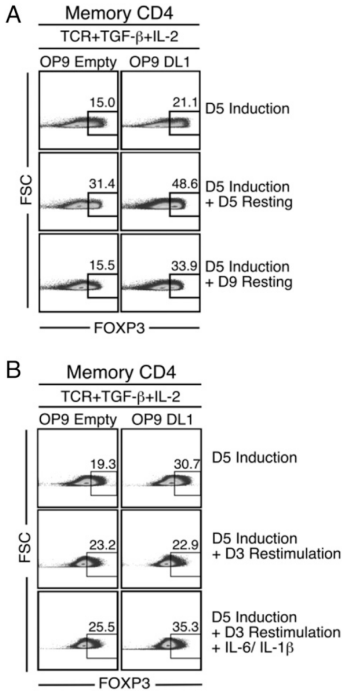

FIGURE 3. iTreg differentiated from memory CD4 cells exhibited a stable phenotype in long-term cultures and in the presence of proinflammatory cytokines. Sort-purified memory CD4 cells ($CD4^+CD25^-CD127^{hi}CD45RA^-$) were TCR stimulated for 5 d in medium supplemented with TGF-β and IL-2 (TCR + TGF-β + IL-2), in the presence of OP9 cells expressing DL1 or control OP9 stromal cells (Empty). (**A**) Illustrative dot plots of FOXP3 expression in cells recovered after the induction period as well as after 5 and 9 more days of resting, in medium supplemented with IL-2 and rapamycin ($n = 3$). (**B**) Illustrative dot plots of the expression of FOXP3 in cells recovered after the induction period and a further 3 d TCR restimulation in the presence of IL-2 alone or a combination of IL-2, IL-6, and IL-1β (proinflammatory mixture) ($n = 3$).

Treg compartment. Moreover, this result suggests that DL1-mediated Notch signaling outcome is cell type specific.

The PI3K/Akt/mTOR signaling pathway is known to interfere with FOXP3 induction in vitro and in vivo (11). The phosphorylation levels of S6, a downstream PI3K/Akt/mTOR target, were assessed by flow cytometry in sort-purified memory CD4 cells after TCR stimulation in the presence of OP9 empty or OP9 DL1 stroma. As a control, cells were either left in medium alone or TCR stimulated in the presence of rapamycin, a known mTOR attenuator, and OP9 control cells. We found similar levels of S6 phosphorylation in the presence or absence of DL1, excluding modulation of this pathway as a major contributor to the DL1-mediated enhancement of iTreg conversion (Fig. 4C). We also found no impact of DL1 on S6 phosphorylation levels under suboptimal TCR signal strength as well as on the Akt phosphorylation levels (data not shown). These results excluded the possibility that modulation of PI3K/Akt/mTOR accounted for the positive effects of DL1.

The interaction between Notch and the TGF-β signaling pathway is well described (17–19, 28). We therefore investigated the phosphorylation levels of downstream targets of TGF-β signaling (Smad2 and Smad3) in different culture conditions. Sort-purified memory CD4 cells were cultured in complete medium in the absence of serum for 3 h, and then for a further 2 h in the presence or absence of TGF-β with either control OP9 or OP9 DL1 cells. We confirmed the expected phosphorylation of Smad2/3 in response to TGF-β (Fig. 4D). Notably, we found that Smad2/3 phosphorylation levels were increased by DL1, even in the absence of exogenous TGF-β, suggesting that Notch cooperatively interacted with TGF-β signaling. Furthermore, DL1 also enhanced the *Smad3* mRNA expression levels (Fig. 4D). Because the addition of a TGF-β blocking Ab led to a reduction of the Smad2/3 phosphorylation induced by DL1, its effect appeared to be at least partly mediated by endogenous TGF-β, possibly produced by the memory CD4 T cells, as supported by our real-time PCR data (data not shown).

Finally, DL1 may exert its effects by direct modulation of *Foxp3* gene transcription, as Notch signaling directly targets the Foxp3 promoter via RBP-J and Hes-1 binding sites within it (22). To test this possibility, we quantified *Foxp3* mRNA expression levels in memory CD4 T cells ($CD4^+CD25^-CD127^{hi}CD45RA^-$) after 24 h culture in conditions favoring FOXP3 acquisition, in the presence of control OP9 or OP9 DL1 cells. The *Foxp3* mRNA expression levels were increased in the presence of DL1 (Fig. 4E), supporting our hypothesis.

Taken together, these data suggest that Notch enhances iTreg differentiation by a dual mechanism: cooperative interaction with the TGF-β pathway and direct modulation of *Foxp3* transcription.

Discussion

This study addressed the efficiency of the in vitro conversion of memory CD4 T cells into FOXP3-expressing iTreg and the role of the Notch signaling pathway in modulating this process. Our data demonstrated the feasibility of generating iTreg from human memory CD4 cells, a subset that is likely enriched in cells with autoreactive specificities in autoimmune patients. Moreover, we showed that DL1-mediated Notch signaling enhances this conversion, offering a new target to facilitate iTreg generation with therapeutic potential. The beneficial DL1 effects were also extended to in vitro conversion protocols utilizing conventional naive CD4 T cells. Additionally, we found that DL1 enhanced the expression levels of molecules associated with Treg function as well as the expansion of circulating human Treg, highlighting a potential role of this signaling pathway in human Treg homeostasis.

A reduced and/or dysfunctional Treg compartment underlies the pathophysiology of many immune-mediated diseases, prompting a strong interest in the manipulation of these cells for clinical purposes, particularly in transplantation and autoimmunity. Strategies to obtain sufficient Treg numbers for adoptive transfer rely on extensive ex vivo expansion of this subset. However, repeated expansion of Treg may alter their phenotype and function (5). Moreover, peripheral Treg (pTreg) were shown to be ineffective in treating some autoimmune diseases in murine models owing to their unstable phenotype in proinflammatory environments (7, 29, 30). Conversely, iTreg have been shown to be stable in inflammatory conditions and resistant to Th17 conversion by IL-6 (7, 31). Thus, there is an increasing interest in iTreg manipulation, with nonregulatory naive CD4 T cells being used as the starting population in the vast majority of induction studies performed (6–11). Notwithstanding, there is compelling evidence that a significant proportion of circulating Treg in humans may be derived from memory T cells (12–14), as indicated by the marked overlap in their TCR Vβ usage (12). In fact, continuous recruitment from the memory CD4 T cell compartment in vivo has been proposed as

contributes to the DL1-mediated effect, these results suggest that even in situations in which TGF-β is limiting, DL1 may help controlling the magnitude of memory CD4 T cell responses.

The observation of a lack of significant DL4 impact on iTreg differentiation from memory cells is possibly unexpected. Nevertheless, DL1 and DL4 have a differential effect on early T cell activation and proliferation upon TCR cross-linking (42), which may affect the efficiency of conversion. Our monitoring of the levels of expression of each ligand on the OP9 stromal cells by FACS revealed a lower expression of DL4 than DL1 (Supplemental Fig. 1E), which may have contributed to the much more striking effects of DL1.

Concerning the mechanisms underlying the DL1-mediated enhancement of iTreg differentiation from memory cells, we excluded a significant impact of Notch signaling on iTreg proliferation and/or survival during the induction period. This was possibly unexpected, given the recognized role of Notch in the protection of activated CD4 T cells from apoptosis after an initial phase of clonal expansion, by inducing a broad antiapoptotic gene expression signature (43). We showed that DL1 cooperatively interacts with the TGF-β signaling pathway, as evidenced by increased phosphorylation of the downstream TGF-β signaling pathway targets Smad2 and Smad3. TGF-β is a pleiotropic anti-inflammatory cytokine required for iTreg differentiation. Although direct and indirect mechanisms have been implicated in the role of TGF-β in pTreg pool maintenance, recent studies suggest that its downstream targets, specifically Smad3, directly activate the *Foxp3* gene (44). The interaction between Notch and TGF-β signaling pathways has been repeatedly described and is probably both cell type– and context-dependent. Previous reports showed that Notch intracellular domain increases Smad3 protein at the transcriptional and posttranscriptional levels, as well as Smad nuclear translocation and its transactivation at promoter sites (18, 28). Moreover, Notch ligands increase Treg suppressive function via the upregulation of TGF-β receptor expression and phosphorylation of Smad3 in effector T cells (19). We therefore hypothesize that the enhanced Smad2 and Smad3 phosphorylation we observed in the presence of DL1, an effect particularly evident in the absence of exogenous TGF-β, might occur via upregulation of TGF-β receptor and transcriptional/posttranscriptional modulation of Smad3 promoted by the ligand.

Direct targeting of the Foxp3 promoter by Notch is supported by the presence of RBP-J and Hes-1 binding sites within it, in both mice and humans (22). Accordingly, our results indicated increased *Foxp3* mRNA expression in the presence of DL1, supporting an involvement of direct modulation of *Foxp3* gene transcription in the Notch-mediated enhanced conversion of memory CD4 T cells into iTreg.

Taken together, our data suggest that DL1 affects FOXP3 acquisition by memory CD4 cells through various mechanisms, such as the cooperative interaction with TGF-β signaling pathway and the modulation of *Foxp3* transcription. Other possibilities, such as direct downregulation of the IL-6 receptor α-chain on memory CD4 cells by DL1, as recently shown in human $CD34^+$ cells (45), cannot be excluded and warrant further investigation.

In conclusion, we provide evidence that manipulation of the Notch signaling pathway, both in Treg expansion and iTreg conversion protocols, may help facilitating the use of Treg-based therapies.

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Disclosures

The authors have no financial conflicts of interest.

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Human regulatory T-cell development is dictated by Interleukin-2 and -15 expressed in a non-overlapping pattern in the thymus

Iris Caramalho*, Vânia Nunes-Silva, Ana R. Pires, Catarina Mota, Ana I. Pinto, Helena Nunes-Cabaço, Russell B. Foxall, Ana E. Sousa

Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Portugal

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ABSTRACT

Thymus-derived FOXP3-expressing regulatory T-cells (tTregs) are master orchestrators of physiological and pathological immune responses, thus constituting ideal targets for the treatment of autoimmunity. Despite their clinical importance, the developmental program governing their differentiation in the human thymus remains poorly understood.

Here, we investigated the role of common gamma-chain cytokines in human tTreg differentiation, by performing gain- and loss-of-function experiments in 3D and 2D postnatal thymic cultures. We identified IL-2 and IL-15 as key molecular determinants in this process and excluded a major function for IL-4, IL-7 and IL-21. Mechanistically, IL-2 and IL-15 were equally able to drive tTreg precursor differentiation into FOXP3$^+$ cells, and promote tTreg proliferation and survival. Both cytokines also increased the expression levels of molecules associated with effector function within FOXP3$^+$ subsets, supporting their involvement in tTreg functional maturation. Furthermore, we revealed that IL-2 and IL-15 are expressed in a non-overlapping pattern in the human thymus, with the former produced mainly by mature αβ and γδ thymocytes and the latter by monocyte/macrophages and B lymphocytes.

Our results identify core mechanisms dictating human tTreg development, with IL-2 and IL-15 defining specific niches required for tTreg lineage stabilization and differentiation, with implications for their therapeutic targeting in autoimmune conditions.

1. Introduction

FOXP3-expressing regulatory T-cells (Tregs) emerge as a distinct lineage through a process favoring the selection of thymocytes bearing self-reactive T-cell receptors. Although Tregs can also be generated extra-thymically from conventional T-cells under specific stimulatory conditions, thymus-derived (t)Tregs are considered the major contributors to the peripheral Treg pool [1–3]. The transcription factor FOXP3 is essential for their development and effector function, as demonstrated by the clinical severity and associated mortality of loss-of-function mutations in the gene encoding this transcription factor in humans and mice [1–5]. Importantly, despite the clinical potential of Tregs in the context of autoimmunity, allergy and transplantation tolerance, little is known about their ontogeny in the human thymus.

Human T-cell development progresses through a series of sequential stages defined by the surface expression of CD4, CD8 and CD3. T-cell progenitors contained within the early CD3^{neg}CD4^{neg}CD8^{neg} triple negative (TN) subset initially acquire CD4 (becoming CD4 Immature Single Positive cells, CD4ISP) and subsequently CD8 expression, giving rise to double-positive (DP) thymocytes in the cortex. A progressive increase in surface CD3 expression occurs in parallel with surface TCRαβ in DP cells, followed by final differentiation into CD4 single-positive (SP) and CD8SP thymocytes that mature in the medulla [6].

Human Treg development appears to occur concomitantly with the generation of the counterpart FOXP3^{neg} population, with these cells being documented in fetal thymus as early as the 13th week of gestation, prior to their appearance in peripheral sites [7,8]. FOXP3 induction within developing thymocytes is driven by TCR signaling and associated with positive selection [7–11]. Expression of FOXP3 is indeed clearly detected in post-selection DP thymocytes [7,8,11],

* Corresponding author. Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal. Tel.: +351 27999525; fax: +351 7999527.
E-mail address: iriscaramalho@medicina.ulisboa.pt (I. Caramalho).

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which we have shown to significantly contribute to the mature FOXP3$^+$ pool that is largely composed of CD4SP and some CD8SP thymocytes [11]. Others have shown that human tTregs can be selected by both myeloid and plasmacytoid dendritic cells (DCs) [10,12,13]. FOXP3$^+$ cells accumulate in the medulla, where the majority of them can be found. Furthermore, FOXP3-expressing mature CD4SP and CD8SP display regulatory properties in both human fetal and postnatal thymus [7,8,11,14,15].

Treg homeostasis and function in the periphery depend on Interleukin (IL)-2 [16,17]. Here, we hypothesized that IL-2, and possibly other common-gamma chain (γc) cytokine family members, could play a role in human tTreg development. This family, that shares the use of γc to generate signaling receptor complexes, includes cytokines fundamental for the development and differentiation of immune cells, namely IL-4, IL-7, IL-9, IL-15, and IL-21, in addition to IL-2 [16,17]. Notably, human tTreg express the α- (CD25) and the β-chain (CD122) of the IL-2 receptor (R) that are utilized by IL-2 and IL-15 [8,11]. Moreover, we demonstrated that human FOXP3$^+$ thymocytes, although featuring reduced levels of the α-chain of the IL-7R compared to their FOXP3^{neg} counterparts, phosphorylate STAT-5 in response to IL-7 [11]. Moreover, IL-2 increased CD25 and FOXP3 expression levels within FOXP3$^+$DP thymocytes [11]. Thus, existing data support a role for IL-2 and IL-7 in human tTreg development. Importantly, polymorphisms in IL-2, CD25 or IL-2R downstream signaling molecules are associated with impaired Treg number and/or function as well as increased risk of autoimmunity in humans [16], which may be due to defective tTreg generation in addition to reduced peripheral Treg survival. Accordingly, indirect evidence supports a thymic involvement in patients undergoing IL-2 therapy, based on the observed expansion of Tregs expressing CD45RA and the recent thymic emigrant marker CD31 [18–20].

Considerable differences exist between human and murine tTreg development. Human FOXP3$^+$ thymocyte development occurs in parallel with the counterpart FOXP3^{neg} population, whereas in mice their appearance is delayed [2,7,8]. Moreover, whereas the vast majority of murine tTregs arise at the CD4SP stage, increasing evidence indicates an earlier commitment into the Treg lineage in humans [2,3,7–11]. Nonetheless, data generated in murine models support our hypothesis [2,3,16,17].

In this study, we investigated the role of γc cytokines in human tTreg development, by performing gain- and loss-of-function experiments in 3D and 2D postnatal thymic cultures, using recombinant cytokines, specific blocking antibodies and pharmacological inhibitors of their signaling pathway. We identified IL-2 and IL-15, expressed in a non-overlapping pattern, as key γc cytokines, both able to drive human tTreg precursor differentiation into FOXP3$^+$ cells as well as to promote tTreg proliferation and survival.

2. Materials and methods

2.1. Samples

Thymic specimens were obtained from thymectomy during pediatric corrective cardiac surgery (newborns to 4-year old) at Santa Cruz Hospital, after parents' informed consent. Study was approved by the Ethical Boards of the Faculty of Medicine of Lisbon, of Santa Maria and of Santa Cruz Hospitals. Thymocytes and thymic epithelial cells (TECs) were isolated as described [9,11,21].

2.2. FACS analyses

The following anti-human monoclonal antibodies (BD Biosciences or eBioscience, clones in brackets) were used: CD3 (UCHT1), CD4 (RPA-T4), CD8a (SK1), CD11c (3.9), CD14 (61D3), CD16 (eBioCB16), CD20 (2H7), CD25 (2A3), CD31 (WM59), CD123 (6H6), αβ TCR (IP26), γλ TCR (B1.1), HLA-DR (L243), Bcl-2 (124), CTLA-4 (BNI3), FOXP3 (PCH101), Ki67 (B56), ICOS (ISA-3), Epcam (1B7) and pan-cytokeratin (C-11). After surface staining cells were fixed, permeabilized and stained for FOXP3 and other intracellular molecules using the FOXP3 staining kit (eBioscience). Samples were acquired on a FACSCanto or LSR Fortessa (BD Biosciences) and analyses performed using FlowJo software (TreeStar), after stringent exclusion of cell aggregates, based on area and width parameters of both forward and side scatter, and dead cells by gating-out near-IR or violet LIVE/DEAD® fixable dye positive cells (Invitrogen). Annexin V and PI staining were performed with the Annexin V apoptosis detection kit (BD Biosciences). The gate of the FOXP3$^+$ population was always defined in each experiment using the control culture condition and subsequently applied to all the additional culture conditions performed.

2.3. Cell sorting

Cell sorting was performed with a FACSAria III (BD Biosciences). CD25^{neg}CD127^{hi}CD4SP and CD25$^+$CD127^{hi}CD4SP were purified from total thymocytes as CD4$^+$CD8^{neg}CD3^{hi}CD127^{hi} cells, subdivided according to their CD25 expression level. The lineage markers CD11c, CD14, CD16, CD20, CD56, CD123 and HLA-DR were combined with CD3, CD4 and CD8 staining to sort TN (Lineage^{neg} CD3^{neg}CD4^{neg}CD8^{neg}) and CD4ISP thymocytes (Lineage^{neg} CD3^{neg}CD8^{neg}CD4$^+$). γδ cells were purified as TCR-γδ$^+$CD3$^+$. Purification of B cells (CD20$^+$HLA-DR$^+$), plasmacytoid (p)DC (CD123$^+$HLA-DR^{lo}), conventional (c)DC (CD11c$^+$CD123^{neg}HLA-DR$^+$), NK (CD56$^+$/CD16$^+$CD4^{neg}CD8^{neg}CD3^{neg}) and monocyte/macrophages (CD14$^+$) was performed after CD3-expressing thymocyte depletion using EasySep™ Human CD3 Positive Selection Kit (StemCell Technologies). Purity was routinely ≥95%.

2.4. Cell cultures

The following reagents were used: anti-CD3 (clone OKT3), anti-CD28 (clone CD28.2) and mouse IgG1 mAbs, (eBioscience); Dynabeads® Human T-Activator CD3/CD28 (Invitrogen); JAK-3 inhibitor CP690550 (Axon Medchem); STAT-5 inhibitor N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (Calbiochem); anti-CD122 mAb (clone TU27, BioLegend); anti-IL-2 (clone 5334) and anti-IL-15 mAbs (clone 34593), (R&D); IL-2 (AIDS Research and reference Program, Division of AIDS, NIAID, NIH); IL-4 and IL-7 (R&D); IL-15, IL-21 and TGF-β (PeproTech). Complete RPMI and IMDM medium were made by supplementation of respective media with 10% FCS, L-glutamine, sodium pyruvate, Hepes, non-essential aminoacids, penicillin/streptomycin, 2-mercaptoethanol and gentamicin (all from Invitrogen). Total thymocytes (2×10^6) were co-cultured with primary allogeneic TECs (4×10^4) in complete RPMI medium alone or supplemented with IL-2, IL-4, IL-7, IL-15 or IL-21. For CD25^{neg}CD127^{hi}CD4SP thymocyte differentiation into FOXP3-expressing cells, sorted thymocytes (2.5×10^5 cells) were stimulated with 1 μg/ml plate-bound anti-CD3 mAb, 1 μg/ml soluble anti-CD28 mAb and 5 ng/ml TGF-β, in complete IMDM medium alone or supplemented with IL-2, IL-4, IL-7, IL-15 or IL-21, for 5 days [22]; parallel cultures consisted of thymocytes TCR-stimulated in the presence of TGF-β and IL-2, to which either IL-4, IL-7, IL-15 or IL-21 was added.

2.5. Thymic organ cultures (TOCs)

Thymic tissue was cut into ~2 mm^3 pieces and placed over Isopore membranes (Millipore) supported on Gelfoam [23,24], under the indicated culture conditions in complete RPMI medium,

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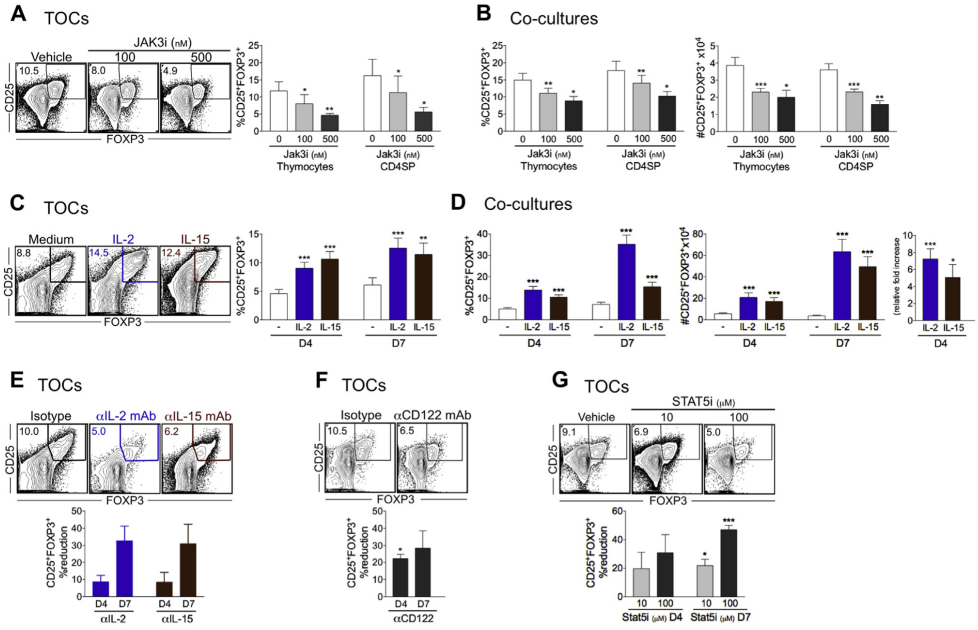

Fig. 1. The γc cytokines IL-2 and IL-15 impact on human tTreg development. (A–B) TOC and co-cultures of total thymocytes with TECs were cultured for 7 days in medium alone or supplemented with the indicated concentrations of a JAK-3 inhibitor or DMSO (vehicle): (A) Representative FACS profile of CD25 and FOXP3 expression within TOC recovered cells; graph shows frequency of $FOXP3^+CD25^+$ cells within total and CD4SP thymocytes ($n = 3$–4); (B) Frequency and number of $FOXP3^+CD25^+$ cells within total and CD4SP thymocytes in co-cultures ($n = 5$–6). (C–D) TOCs and co-cultures of total thymocytes with TECs were cultured for 7 days in medium alone or supplemented with IL-2 (10 U/ml) or IL-15 (12.5 ng/ml): (C) Representative FACS profile of CD25 and FOXP3 expression in cells recovered from TOCs at day 7; graphs depict $FOXP3^+CD25^+$ cell frequency at days 4 and 7 ($n = 10$–11); (D) Proportion and number of $CD25^+FOXP3^+$ cells recovered from co-cultures at days 4 and 7 ($n = 12$); graph on the right shows *Foxp3* mRNA levels in cells harvested at day 4 ($n = 3$). (E–G) Representative contour plots of CD25 and FOXP3 expression in cells recovered at day 7 from TOCs, in medium supplemented with: (E) anti-IL-2 (10 μg/ml), anti-IL-15 (10 μg/ml) or isotype control mAbs (anti-IgG1, 10 μg/ml) ($n = 3$–4); (F) anti-CD122 blocking (10 μg/ml) or isotype control mAbs (anti-IgG1, 10 μg/ml) ($n = 4$); (G) the indicated concentrations of an STAT-5 inhibitor or DMSO (vehicle) ($n = 4$–5); graphs show the reduction in $FOXP3^+CD25^+$ cell frequency in each test condition at days 4 and 7, in comparison to the corresponding control TOC. Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

either alone or supplemented with IL-2, IL-15, anti-IL-2 mAb, anti-IL-15 mAb, JAK-3 inhibitor, STAT-5 inhibitor and anti-CD122 mAb.

2.6. Suppression assays

The $CD25^{high}CD4SP$ populations under test were sort-purified at day 7 from co-cultures of thymocytes with TECs set in the presence of IL-2 (10 U/ml) or IL-15 (12.5 ng/ml). Control $CD25^{high}CD4$ cells and $CD25^{neg}CD4SP$ (targets) were sorted from an allogeneic thymus. To evaluate their suppressor function, each population was plated at various numbers together with 2.5×10^4 $CD25^{neg}CD4SP$ thymocytes labeled with CFSE (2.5 μM CFSE from Invitrogen, for 5 min at room temperature). Stimulation was provided by CD3/CD28 Dynabeads (bead:cell ratio 1:4, Invitrogen). Proliferation was monitored at day 4 by analyzing target cell CFSE dilution, by FACS.

2.7. RT-qPCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the Superscript III Reverse Transcriptase Kit (Invitrogen). The mRNA levels of *Foxp3*, *IL15 Ralpha chain*, *IL-2*, *IL-15* and *GAPDH* were quantified with TaqMan gene expression kits, according to the manufacturer's instructions, using a 7500 Fast Real-Time PCR System (all from Applied Biosystems). IL-2 and IL-15 expression levels in sorted thymocyte populations were determined after pre-amplification with TaqMan Preamp Master Mix (Applied Biosystems). *Bcl2* (forward: 5′-GCACCTGCAC ACCTGGAT-3′ and reverse: 5′-CCAAACTGAGCAGAGTCTTCAG-3′) and *Bim* (forward: 5′-ATGGCAAAGCAACCTTCTGATG-3′ and reverse: 5′-TCAATGCATTCTCCACACCAGG-3′) gene expression were quantified with SYBR® Green PCR Master Mix (Applied Biosystems). Results were analyzed using the ΔΔCt ($2^{-\Delta\Delta Ct}$) method.

2.8. Immunohistochemistry

Thymus pieces were preserved in 4% formaldehyde and embedded in paraffin. Deparaffinised 3 μm samples underwent antigen retrieval (Leica Buffer Ph9) by heat for 15 min. Samples were stained with the following mAb (clone and supplier in brackets): anti-IL-2 (5334, R&D), anti-IL-15 (34593, R&D), anti-CD20 (L26, Dako), anti-CD68 (PG-M1, Dako) and FOXP3 (236/E7, eBioscience). Single and double immunohistochemistry stainings were revealed by enzymatic substrate with horseradish peroxidase and alkaline phosphatase, in brown and red respectively. All slides were counterstained with Hematoxylin and mounted with Entellan (single staining) or Glycergel (double staining) from DakoPower. Bright-field images were acquired using a Leica DM 2500 Microscope equipped with a Leica Digital FireWire Camera (DFC) and HC

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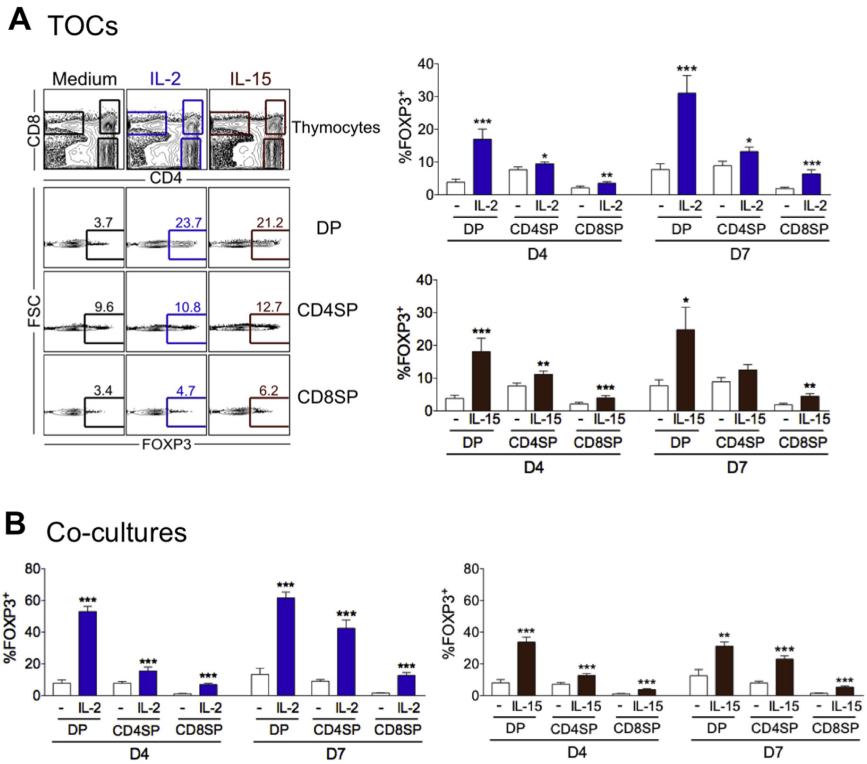

Fig. 2. IL-2 and IL-15 enhance FOXP3 expression within DP, CD8SP and CD4SP thymocytes. (A) Representative contour plots of CD4 and CD8 expression within total thymocytes, together with FOXP3$^+$ cells within gated DP, CD4SP and CD8SP thymocytes recovered at day 4 from TOCs in medium alone or supplemented with IL-2 (10 U/ml) or IL-15 (12.5 ng/ml). Graphs show the frequency of FOXP3$^+$ cells within DP, CD4SP and CD8SP thymocytes recovered from (A) TOCs ($n = 8–9$) or (B) co-cultures ($n = 12$) setup as in (A), at days 4 and 7. Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PL FLUOTAR lenses using the Leica Acquire software. FIJI software was used for scaling and Adobe Photoshop software for contrast/brightness correction.

2.9. Statistical analyses

Statistical analyses were performed with the 2-tailed Student's paired test, using GraphPad Prism v5.01 (GraphPad Software Inc.). Data were first transformed with log (base 10) to account for variability between samples before the *t* test was performed. Comparison was always done with the corresponding control thymic culture. Results are expressed as mean ± SEM. *p*-Values below 0.05 were considered significant.

3. Results

3.1. Role of γc cytokines in human tTreg development

To mimic human intrathymic T-cell development *in-vitro*, we utilized 3D thymic organ cultures (TOCs) [23,24] and 2D cultures of total thymocytes with primary thymic epithelial cells (TECs) [21], subsequently referred to as co-cultures. TECs were phenotypically characterized in terms of cytokeratin and EpCAM expression (Supplementary Fig. S1A). Consistent with T-cell differentiation, the frequency of mature CD4SP and CD8SP cells, assessed by FACS, progressively increased during the 7-day culture in both TOCs (Supplementary Fig. S1B) and co-cultures of total or CD4ISP thymocytes with TECs (data not shown and Supplementary Fig. S1C, respectively) set in medium alone.

Firstly, we assessed whether γc cytokine signaling was required for human tTreg development, by evaluating the impact of pharmacological inhibition of JAK-3, a protein tyrosine kinase that specifically associates with γc [17]. JAK-3 blockade significantly reduced the frequency and number of total CD25$^+$FOXP3$^+$ and CD25$^+$FOXP3$^+$CD4SP cells in both culture systems (Fig. 1A and B).

Next, we supplemented TOCs and co-cultures with increasing doses of IL-2, IL-4, IL-7, IL-15 and IL-21. All γc cytokines increased cell recovery and, with the exception of IL-21, augmented thymocyte survival at day 7 (Supplementary Fig. S2). However, whereas IL-4, IL-7 and IL-21 did not substantially impact upon the tTreg compartment (Supplementary Fig. S2C–E), IL-2 and IL-15 increased the frequency and number of recovered CD25$^+$FOXP3$^+$ and

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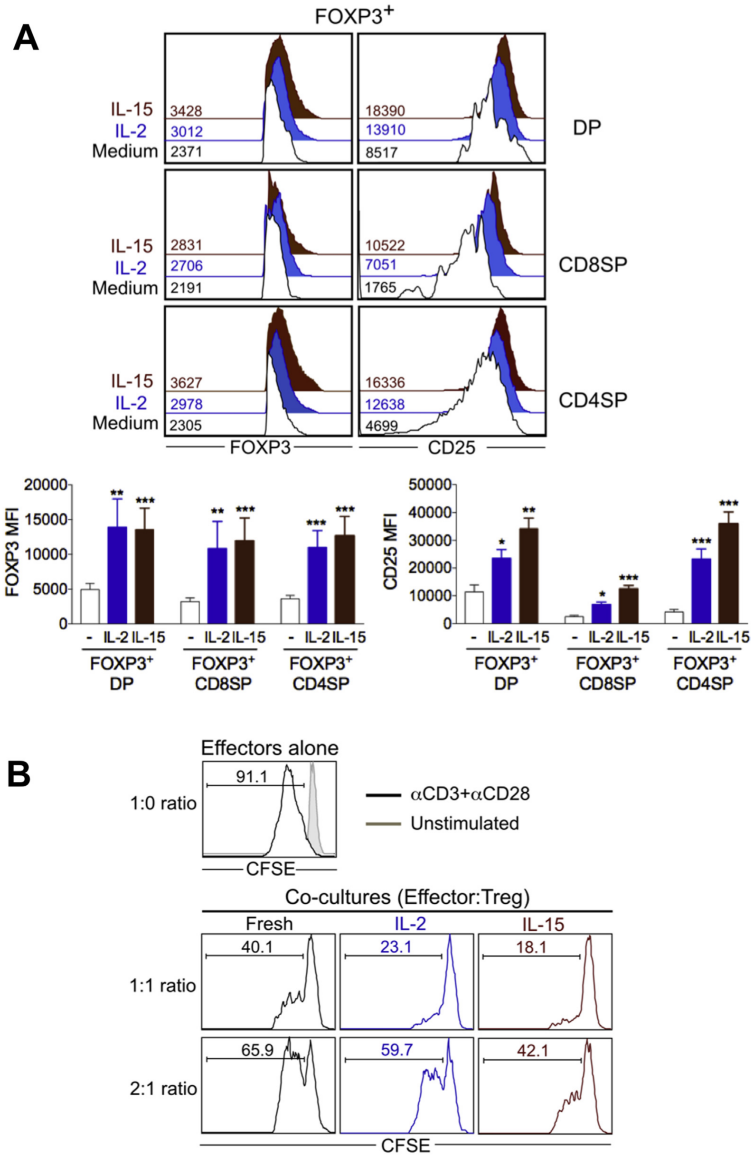

Fig. 3. FOXP3$^+$ cells accumulating in IL-2- and IL-15-supplemented co-cultures display phenotypic and functional properties of Tregs. (A) Representative histogram overlays of FOXP3 and CD25 (numbers inside histograms indicate MFI) within FOXP3$^+$DP, FOXP3$^+$CD8SP and FOXP3$^+$CD4SP cells recovered at day 7 from co-cultures of thymocytes with TECs in medium alone or supplemented with 10 U/ml IL-2 or 12.5 ng/ml IL-15; graphs show the data from different thymuses ($n = 12$). (B) Proliferation of CFSE-labeled CD25^{neg}CD4SP thymocytes unstimulated (filled lines) or stimulated (open lines) with CD3/CD28 Dynabeads (1:0 ratio; Target:Treg ratio) for 4 days either alone or in the presence of decreasing numbers of CD25^{bright}CD4SP cells (1:1 and 2:1 ratio). The CD25^{bright}CD4SP cells tested were either sort-purified from freshly-isolated thymocytes (control of suppression) or sort-purified from day 7 co-cultures of total thymocytes with TECs, cultured in the presence of exogenous 10 U/ml IL-2 or 12.5 ng/ml IL-15 ($n = 2$). Numbers correspond to the frequency of target cells that have divided at least once during the culture period. Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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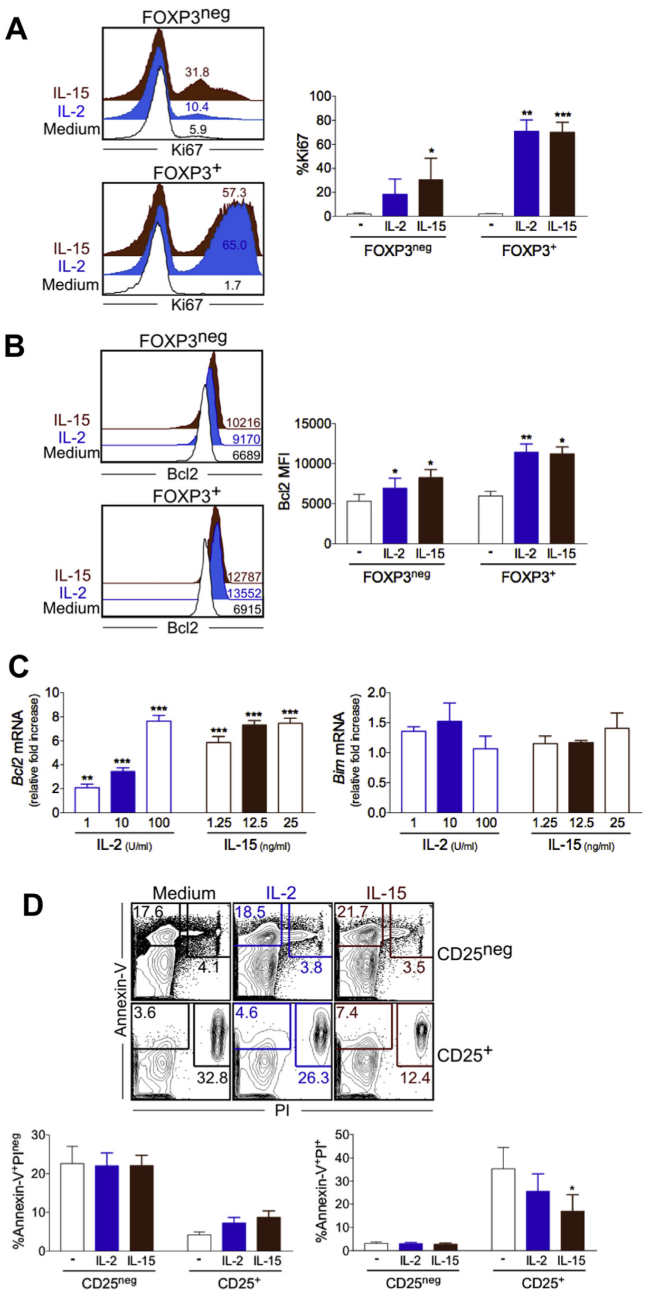

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CD25$^+$FOXP3$^+$CD4SP cells in both TOCs and co-cultures, as well as the *Foxp3* mRNA expression levels in thymocytes recovered from co-cultures at day 4 (Fig. 1C and D, Supplementary Fig. S3A and B). The impact of IL-15 on Treg frequency and number was apparently independent of IL-2, as it was unaffected by addition of a neutralizing anti-IL-2 mAb (data not shown).

A physiological role for both cytokines was further supported by the reduction in Treg frequency in TOCs upon neutralization of endogenous IL-2 or IL-15 (Fig. 1E and Supplementary Fig. S3D) and CD122 blockade (Fig. 1F and Supplementary Fig. S3E). The decrease in Tregs observed in the presence of anti-IL-2 neutralizing mAb was confirmed in the co-culture system, both in terms of frequency and absolute cell numbers (Supplementary Fig. S3C). Thus, the reduction in Tregs cannot be ascribed to a possible proliferative effect of anti-IL-2/IL-2 immune complexes on non-Treg populations [25], in agreement with the unaltered frequency of Ki67$^+$ cells (data not shown). No modulation of the Treg compartment was observed upon IL-7 neutralization (data not shown).

Signaling via IL-2/IL-15R requires the downstream transcription factor STAT-5 [17]. Addition of a pharmacological STAT-5 inhibitor to TOCs reduced Treg recovery (Fig. 1G and Supplementary Fig. S3F) further supporting the hypothesis that JAK-3/STAT-5 pathway is involved in human tTreg differentiation.

We and others have shown that in addition to CD4SP, FOXP3 is also expressed by a subset of DP and CD8SP cells in the human thymus [7–9,11]. We thus investigated the impact of IL-2 and IL-15 on these subsets, and found increased FOXP3 expression in both, upon supplementation with IL-2 and IL-15, irrespective of the culture system used (Fig. 2), as well as a consistent reduction in their frequency when we neutralized endogenous IL-2 or IL-15 (data not shown).

Overall, our data indicate that IL-2 and IL-15 are the key γc cytokines involved in human tTreg development and that their signaling largely occurs via JAK-3/STAT-5.

3.2. IL-2 and IL-15 favor the development of bona-fide Treg

We next investigated how IL-2 and IL-15 supplementation impacted on the phenotype and function of tTreg accumulating in co-cultures, and found that FOXP3$^+$DP, FOXP3$^+$CD8SP and FOXP3$^+$CD4SP thymocytes featured higher expression levels of both FOXP3 and other Treg function-associated molecules such as CD25, CTLA-4 and ICOS, as well as increased frequency of HLA-DR (Fig. 3A and Supplementary Fig. S4A and B). IL-2 and IL-15 also increased FOXP3 and CD25 expression levels within FOXP3$^+$ subsets in TOCs (data not shown).

Notably, lower levels of FOXP3 and CD25 expression were found within FOXP3$^+$ cells recovered from co-cultures and TOCs in the presence of a neutralizing anti-IL-2 or anti-IL-15 mAb (Supplementary Fig. S4C and data not shown), further supporting a physiological role for these cytokines in tTreg functional maturation.

We next evaluated the functional capacity of mature CD4SP Treg accumulating in co-cultures, using a standard *in-vitro* suppression assay [22]. CD25^{bright}CD4SP cells recovered at day 7 from IL-2- or IL-15-supplemented co-cultures inhibited the *in-vitro* proliferation of CD25^{neg}CD4SP targets, in a dose dependent manner (Fig. 3B), demonstrating they possess functional properties of *bona-fide* Treg.

3.3. IL-2 and IL-15 induce tTreg proliferation and survival

To determine mechanism(s) underlying the IL-2 and IL-15 effect on human tTreg development, we first tested whether these cytokines impacted upon the proliferation of FOXP3$^+$ cells, by assessing the frequency of Ki67 in cells recovered from co-cultures. Both cytokines considerably enhanced the proliferation of FOXP3$^+$ cells at day 4 (Fig. 4A and Supplementary Fig. S5), irrespectively of whether they were DP, CD4SP or CD8SP. Additionally, IL-2 and IL-15 increased Bcl2 expression levels within all FOXP3$^+$ subsets more efficiently than in their FOXP3^{neg} counterparts (Fig. 4B and Supplementary Fig. S5). Furthermore, whereas *Bcl2* mRNA levels increased with IL-2 and IL-15, *Bim* mRNA expression was unaffected by cytokine supplementation (Fig. 4C). Additionally, IL-2 and IL-15 consistently decreased the frequency of late apoptotic Annexin-V$^+$PI$^+$ cells within CD25$^+$ thymocytes, used here as correlate of FOXP3$^+$ cells as this readout precluded their direct labeling, recovered from co-cultures at 24 h (Fig. 4D and Supplementary Fig. S5) but had no significant effect on their CD25^{neg} counterparts.

Collectively, our data indicate that IL-2 and IL-15 promote the preferential proliferation and survival of FOXP3$^+$ cells.

3.4. IL-2 and IL-15 drive human tTreg precursor differentiation into FOXP3$^+$ cells

Notably, a significant increase in the frequency of CD25$^+$FOXP3$^+$ cells as well as in their levels of FOXP3 protein and mRNA expression was already observed at 24 h in co-cultures supplemented with IL-2 or IL-15 (Fig. 5A), a time-point at which the impact of cytokines upon proliferation was negligible (Fig. 5B). These results raised the possibility that IL-2 and IL-15 could directly drive thymocyte differentiation into tTreg.

To investigate this hypothesis, we first used a classical *in-vitro* assay for Treg differentiation in which sort-purified non-regulatory CD4SP thymocytes (sorted as CD25^{neg}CD127^{hi}CD4SP) were TCR-stimulated in the presence of TGF-β [22]. Notably, cells cultured under these conditions did not acquire FOXP3, in media without γc cytokines. However, addition of either IL-2 or IL-15 and to a lesser extent IL-7, but not IL-4 or IL-21, readily promoted FOXP3 expression. Moreover, IL-4 and IL-21 seemed to antagonize FOXP3 acquisition in the presence of TGF-β and IL-2 (Fig. 5C). Our data suggest that IL-2 and IL-15 are particularly efficient at inducing FOXP3 expression in TCR-stimulated non-regulatory human thymocytes and demonstrate that TCR/CD28 activation alone, even in the presence of TGF-β, was insufficient to promote acquisition of FOXP3 expression by these cells.

Next, we sort-purified CD127^{hi}CD4SP thymocytes according to their level of CD25 expression, and showed that CD25$^+$CD127^{hi}CD4SP cells did not require provision of TCR triggering to progress to the FOXP3$^+$ stage, a step induced equally well by IL-2 or IL-15, with approximately 40% of cells expressing FOXP3 at day 3 (Fig. 5D). Thus, CD25$^+$CD127^{hi}CD4SP cells (surrogates of CD25$^+$FOXP3^{neg}CD4SP cells) are highly enriched in tTreg precursors prone to differentiate into FOXP3$^+$ cells upon stimulation

Fig. 4. IL-2 and IL-15 favor the preferential proliferation and survival of human FOXP3$^+$ thymocytes. (A, B, D) Total thymocytes were co-cultured with TECs, in medium alone or supplemented with either IL-2 (10 U/ml) or IL-15 (12.5 ng/ml). Frequency of Ki67$^+$ cells (A) and Bcl-2 MFI (B) within FOXP3^{neg} and FOXP3$^+$ cells, at day 4 ($n = 3$–4). (C) Quantification of *Bim* and *Bcl2* mRNA levels by qPCR in total thymocytes recovered at 24 h from co-cultures in medium supplemented with the indicated concentrations of IL-2 and IL-15, in comparison to control co-cultures in medium alone ($n = 3$). (D) Frequency of Annexin-V$^+$PI^{neg} (early apoptotic) and Annexin-V$^+$PI$^+$ cells (late apoptotic) within CD25^{neg} and CD25$^+$ cells recovered at 24 h ($n = 3$). Numbers in histograms correspond to either percentage (Ki67, Annexin-V/PI) or MFI (Bcl2). Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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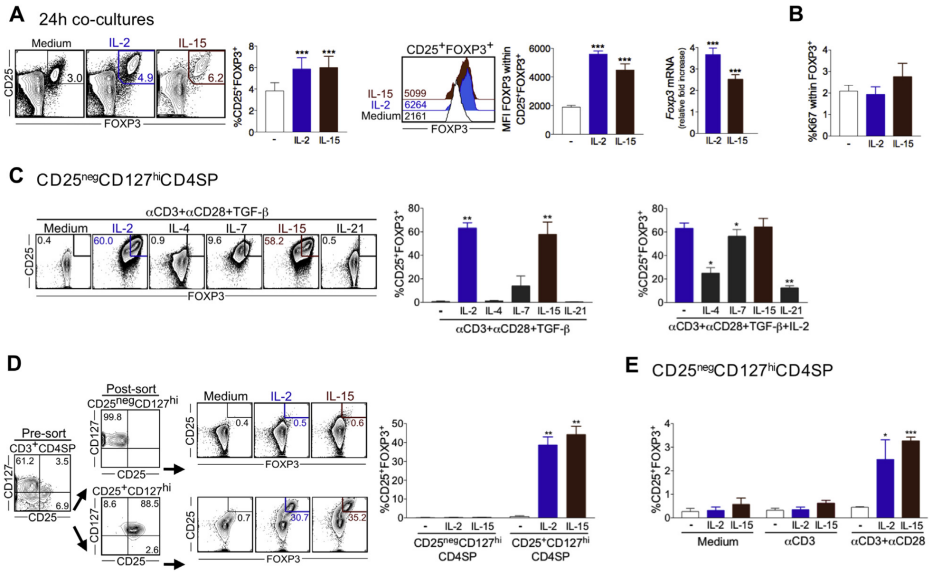

Fig. 5. IL-2 and IL-15 promote human tTreg precursor differentiation into tTregs. (A–B) 24 h co-culture of total thymocytes with TECs in medium alone or supplemented with IL-2 (10 U/ml) or IL-15 (12.5 ng/ml): (A) Representative FACS profile of FOXP3 and CD25 expression and histogram overlay of FOXP3 expression levels within $CD25^+FOXP3^+$ cells in recovered thymocytes; graphs show $CD25^+FOXP3^+$ cell frequency recovered ($n = 6$), their FOXP3 expression levels ($n = 6$), and *Foxp3* mRNA levels in recovered thymocytes ($n = 5$); (B) Frequency of Ki67 within $FOXP3^+$ cells recovered from the 24 h co-cultures ($n = 6$). (C) Sort-purified $CD25^{neg}CD127^{hi}CD4SP$ thymocytes were stimulated for 5 days with plate-bound anti-CD3 mAb, soluble anti-CD28 mAb and TGF-β, in the presence or absence of the γc cytokines IL-2 (10 U/ml), IL-4 (10 ng/ml), IL-7 (10 ng/ml), IL-15 (12.5 ng/ml) or IL-21 (25 ng/ml). Representative contour plots of CD25 and FOXP3 expression are shown, with graphs depicting the frequency of recovered $CD25^+FOXP3^+$ cells (left, $n = 3$) and after adding 10 U/ml IL-2 (right, $n = 3$). (D) Representative contour plots illustrating the sorting strategy and purity of $CD25^{neg}CD127^{hi}CD4SP$ and $CD25^+CD127^{hi}CD4SP$ cells, and their levels of CD25 and FOXP3 expression upon culture for 3 days in medium alone or supplemented with IL-2 (10 U/ml) or IL-15 (12.5 ng/ml); graph shows differentiated $CD25^+FOXP3^+$ cell frequency ($n = 3$–5). (E) Sort-purified $CD25^{neg}CD127^{hi}CD4SP$ were left in medium or stimulated overnight with plate-bound anti-CD3 mAb (1 μg/ml) alone or in combination with soluble anti-CD28 mAb (1 μg/ml), washed, and then cultured for 3 days in medium alone or supplemented with IL-2 (10 U/ml) or IL-15 (12.5 ng/ml); graphs show $CD25^+FOXP3^+$ cell frequency in each culture condition ($n = 4$). Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with IL-2 or IL-15 alone. We estimated that $CD25^+FOXP3^{neg}$ cells represent 2.6 ± 0.2% ($n = 19$) of $CD3^{hi}CD4SP$ thymocytes and observed a trend towards a direct relationship between their frequency and the proportion of $CD25^+FOXP3^+CD4SP$ cells ($r = 0.38$; $p = 0.0565$). Notably, the counterpart $CD25^{neg}CD127^{hi}CD4SP$ population lacked the capacity to differentiate into tTreg, failing to up-regulate FOXP3 upon exposure to IL-2 or IL-15 (Fig. 5D). We also found that following overnight stimulation with anti-CD3 mAb, alone or together with anti-CD28 mAb, roughly 2 and 19% of the $CD25^{neg}CD127^{hi}CD4SP$ thymocytes, respectively, acquired CD25 (with no concomitant FOXP3 expression) and that upon further exposure to IL-2 and IL-15, only cells pre-exposed to both stimuli acquired expression of FOXP3 (Fig. 5E).

Overall, our data support a 2-step model in which initial TCR and co-stimulatory signals give rise to $CD25^+FOXP3^{neg}CD4SP$ tTreg precursors that on subsequent exposure to IL-2 or IL-15 differentiate into $CD25^+FOXP3^+$ cells.

3.5. Non-overlapping pattern of IL-2 and IL-15 expression in the human thymus

There are, to our knowledge, no data regarding the pattern of expression of either IL-2 or IL-15 in the human thymus. Using immunohistochemistry, we showed that IL-2 was expressed both in the cortex and medulla, with a predominantly "patchy" distribution (Fig. 6A–D and Supplementary Fig. S6A). IL-15 presented a more diffuse pattern, mostly restricted to the medulla, although a few cytokine niches were identifiable in both cortex and medulla (Fig. 6A, C and D and Supplementary Fig. S6A). The specificity of the cytokine staining was confirmed by lack of positive labeling with the isotype control Ab (Fig. 6B), and by its loss following pre-incubation of each anti-cytokine Ab with its cytokine target (Fig. 6C). Importantly, there was a mostly non-overlapping pattern of IL-2 and IL-15 expression that was reproducibly detected in all thymuses tested (Fig. 6A and D). We also detected $FOXP3^+$ cells in the vicinity of IL-2- and IL-15-enriched areas, as assessed by single stainings performed in sequential cuts (Supplementary Fig. S6A), since technical limitations precluded co-immunostaining and double immunofluorescence for FOXP3 and cytokines. Immunohistochemistry data also suggested that IL-2 was expressed at considerably higher levels than IL-15, an observation corroborated by quantitative RT-PCR (qPCR) measurement of *IL-2* and *IL-15* mRNA expression in total thymocytes (Fig. 6E), although the putative contribution of stromal cells was not evaluated here.

We next investigated which populations within the human thymus were responsible for IL-2 and/or IL-15 secretion. qPCR analyses of sort-purified thymocyte subsets suggested that mature thymocytes were the major IL-2 source, particularly CD4SP and CD8SP, but also indicated a contribution of γδ and NK cells (Fig. 7A).

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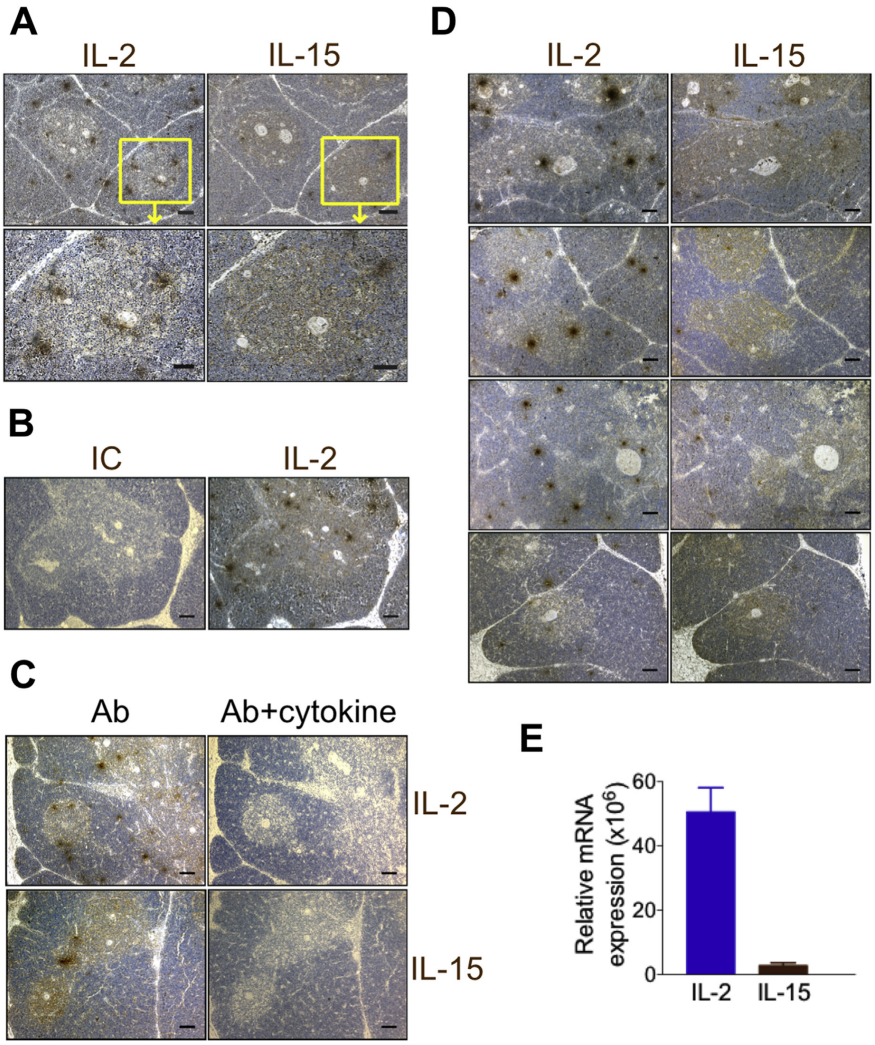

Fig. 6. IL-2 and IL-15 are expressed in non-overlapping niches in the human thymus. Illustrative immunohistochemistry staining of paraffin-embedded serial sections of human thymus with: (A) human anti-IL-2 and anti-IL-15 mAbs, with lower set of images corresponding to a zoom of the region indicated by the yellow box in the upper ones; (B) human anti-IL-2 mAb or the corresponding isotype control antibody (IC: mouse IgG1); (C) anti-IL-2 mAb or anti-IL-15 mAb, with or without pre-incubation of the antibody with its corresponding cytokine ligand, as indicated; (D) human anti-IL-2 and anti-IL-15 mAbs in 4 additional thymuses to illustrate the reproducibility of the staining pattern. Scale bars in A, 100 and 50 μm (upper and lower images, respectively); original magnification ×400. Scale bars in B, C and D correspond to 100 μm; original magnification ×100. (E) Quantification of *IL-2* and *IL-15* mRNA levels in total thymocytes, by qPCR ($n = 2$). Results are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Intracellular staining for IL-2 showed that 1.5 ± 0.3% of thymocytes ($n = 13$) produced IL-2 after PMA/ionomycin stimulation. IL-2 production was mostly confined to $CD3^+$ thymocytes (Fig. 7B), particularly CD4SP (1.8 ± 0.4%, $n = 13$) and CD8SP (4.1 ± 0.6%, $n = 13$). Additionally, a reasonable proportion of γδ (15.7 ± 4.7%, $n = 3$) and NK cells (17.9 ± 4.2%, $n = 6$) also produced IL-2 (Fig. 7B), although they only constituted 0.5 ± 0.05 and 0.2 ± 0.09% of total thymocytes, respectively. Therefore, CD4SP, CD8SP and $CD3^{bright}$DP thymocytes are the main contributors to thymic IL-2, followed by γδ and NK cells (Fig. 7C). On the other hand, most thymic IL-15 appeared to be produced by monocyte/macrophage $CD14^+$ cells, and to a lesser extent, B lymphocytes (Fig. 7D). In accordance with a

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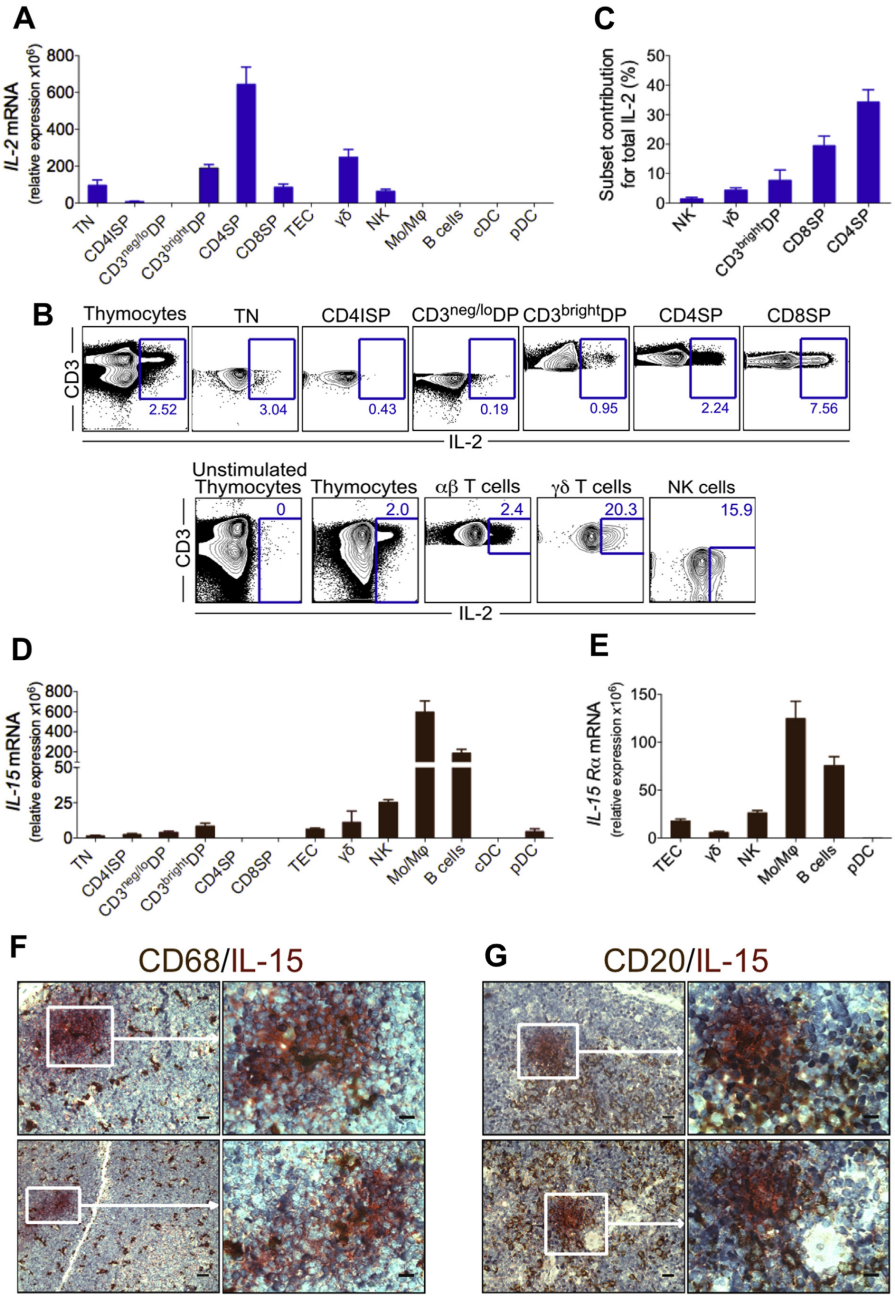

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non-overlapping pattern of IL-2 and IL-15 expression, CD4SP and CD8SP thymocytes that constituted major sources of IL-2, did not express IL-15. Moreover, whereas TECs were negative for IL-2, they readily expressed IL-15 (Fig. 7D). Taking into consideration our immunohistochemistry data, we expect that IL-15 expression in TECs to be mostly restricted to mTECs (Fig. 6A, C, D and Supplementary Fig. S6A). Since IL-15 needs to be presented in *trans* by IL-15Rα$^+$ cells to neighboring IL-2Rβ-expressing cells [26], we confirmed by qPCR that thymic populations with the potential to secrete IL-15 expressed the IL-15Rα chain (Fig. 7E).

Additionally, we performed co-immunostaining for IL-15 and the macrophage marker CD68, or the B cell marker CD20, due to the lack of a reliable anti-IL-15 mAb for flow cytometry and our inability to detect IL-15 by immunofluorescence. Immunohistochemistry data supported a contribution of both populations to thymic IL-15 (Fig. 7F and G). Indeed, macrophages and B cells were found within ~90% and 33% of the IL-15 niches, respectively. Interestingly, whereas macrophages localized in both cortex and medulla, B cells were mostly found in the medulla (Supplementary Fig. S6B). No co-localization of macrophages or B cells with IL-2 was observed (Supplementary Fig. S6C), as predicted by our qPCR data (Fig. 7A).

In summary, our data indicate that IL-2 and IL-15 are expressed in the human thymus in a mostly non-overlapping pattern, and produced by distinct populations.

4. Discussion

In the present study we revealed the key contribution of IL-2 and IL-15 to human tTreg development and characterized their mechanism of action, their pattern of expression and the populations responsible for their production in the human thymus.

This study is, to the best of our knowledge, the first that directly assesses the role of γc cytokines in human tTreg development, a still poorly defined process. Pharmacological JAK-3 blockade led, on average, to a 50% reduction in Treg frequency and number, supporting a significant contribution of γc signaling to tTreg differentiation. Of the γc cytokines tested, IL-2 and IL-15 had the strongest effects, both being capable of driving tTreg precursor differentiation into FOXP3$^+$ cells and promoting tTreg preferential expansion and survival. IL-2 and IL-15 also increased the expression level of molecules associated with effector function within all FOXP3$^+$ subsets, suggesting their involvement in tTreg functional maturation. We also established that IL-2 and IL-15 are expressed in the human thymus in a mostly non-overlapping fashion and identified mature αβ and γδ cells or monocyte/macrophages and B lymphocytes as the main producers of IL-2 or IL-15, respectively. These results suggest that in addition to TCR ligands, IL-2 and IL-15 define extra niches required for human tTreg lineage stabilization and differentiation.

Despite the differences between human and murine tTreg development, evidence also supports a requirement for γc cytokines in mice [2,3,16,17]. In fact, γC-deficient animals display a dramatic decrease in T-cells, harboring almost no Foxp3$^+$ cells [27]. Moreover, whereas IL-2-deficient animals feature a 50% reduction in tTregs [27,28], mice deficient for both IL-2 and IL-15 or for the IL-2Rβ chain bear almost none [29]. Mechanistically, while some authors suggest that IL-2 and IL-15 directly drive Foxp3 expression in mice [30], others propose these cytokines are mainly required for tTreg survival [31,32]. Here, we provide evidence that IL-2 and IL-15 are required for lineage commitment, functional programming, survival and proliferation of Tregs during their thymic development in humans. These cytokines were previously shown to enhance the proliferation and survival of human circulating Tregs as well as to increase their FOXP3 expression levels [33–35]. Our results thus suggest that the capacity of IL-2 and IL-15 to modulate the survival, phenotype and proliferation of human Tregs is acquired during their thymic differentiation, which is in agreement with their constitutive CD25 and CD122 expression. They also indicate that augmented thymic development may account for the increased Treg frequency and number observed in humans upon IL-2 therapy in the context of autoimmunity, cancer therapy, hematopoietic stem cell transplantation and HIV infection [18–20,36]. Our study also raises a note of caution regarding the proposed administration of IL-15 to boost anti-tumor immunity in humans [37], and highlights IL-21 as a safer therapeutic option.

We demonstrated that the tTreg compartment was reduced by ~50% upon STAT-5 inhibition. This dependency may explain the incapacity of IL-4 and IL-21 to positively impact on tTreg development, since neither is very efficient at activating STAT-5, utilizing instead STAT-6 or STAT-3, respectively [17]. IL-4 and IL-21 may actually antagonize the differentiation of CD25$^+$FOXP3$^+$ cells, as we observed upon TCR stimulation of CD25^{neg}CD127^{hi}CD4SP cells in presence of TGF-β and IL-2. These results are in agreement with, and extend previous reports using human thymocytes [13] and peripheral cells [38,39]. The lack of a clear positive impact of IL-7, known to efficiently activate STAT-5, on tTreg development is surprising. We observed that short-term exposure to IL-7 increased Treg frequency as well as their levels of FOXP3 and Bcl2 expression (data not shown), although less efficiently than IL-2 or IL-15. However, although IL-7 sustained the proliferation of FOXP3$^+$ cells, its positive effect on Tregs was diluted by its preferential expansion of FOXP3^{neg} cells (data not shown).

Our findings support a TCR and co-stimulation signaling requirement for CD25$^+$FOXP3^{neg}CD4SP tTreg precursor generation. We propose that, subsequently, tTreg differentiation enters a TCR/co-stimulation independent stage in which exposure to either IL-2 or IL-15 drives tTreg precursor acquisition of FOXP3 expression, with concomitant lineage stabilization and differentiation into mature tTreg. These results are compatible with a 2-step model previously proposed in mice [30], highlighting a considerable evolutionary conservation in key mechanisms governing human and mouse tTreg differentiation.

Recent data challenge the role of CD25$^+$FOXP3^{neg}CD4SP cells as a mandatory intermediate population in murine tTreg development, with an alternative pathway via CD25^{neg}FOXP3$^+$CD4SP being proposed [32,40]. We confirmed that in humans CD25^{neg}FOXP3$^+$CD4SP are present (1.6 ± 0.2% of CD4SP thymocytes, $n = 19$). In agreement with the possibility they constitute an additional population of human tTreg precursors, their FOXP3 MFI is lower than within CD25$^+$FOXP3$^+$CD4SP cells (1714 ± 152 versus 2465 ± 247, respectively, $n = 19$). Moreover, we found a direct correlation between their frequency and the proportion of CD25$^+$FOXP3$^+$CD4SP cells ($r = 0.680$; $p = 0.0007$). Unfortunately,

Fig. 7. Cellular sources of IL-2 and IL-15 in the human thymus. Quantification of *IL-2* mRNA levels by qPCR (A) in the indicated sort-purified thymocyte populations ($n = 2$–4), and representative contour plots of IL-2 expression by flow cytometry (B) following short-time PMA/Ionomycin stimulation of freshly-collected thymocytes, with unstimulated total thymocytes shown as controls ($n = 3$–13). (C) Contribution of the indicated thymocyte subsets to the thymic IL-2 pool ($n = 3$–13). Quantification of *IL-15* (D) and *IL-15-Ralpha* (E) mRNA levels by qPCR in the indicated sort-purified thymocyte populations ($n = 2$–4). Double immunohistochemistry for IL-15 and CD68 (F) or IL-15 and CD20 (G) performed in thymic sections ($n = 3$); the right-hand panel corresponds to a zoom of the indicated region, labeled in white, in the left-hand panel; scale bars, 20 μm and 10 μm in right and left panels, respectively; original magnification ×400. Results are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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tools currently available for humans do not allow direct testing of the potential of $CD25^{neg}FOXP3^{+}CD4SP$ to differentiate into mature $CD25^{+}FOXP3^{+}$ cells upon IL-2 or IL-15 stimulation. In fact, although their CD127 MFI is higher than that of $CD25^{+}FOXP3^{+}CD4SP$ cells, it is not distinctive enough to permit their isolation. New tools are also required to assess the TCR repertoire of $CD25^{+}FOXP3^{neg}$, $CD25^{neg}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$ cells within $CD3^{hi}CD4SP$ thymocytes in order to provide a characterization of tTreg precursors and establish a temporal framework for human tTreg development.

Given the dependency of tTreg differentiation on IL-2 or IL-15, it was critical to identify their thymic source. We found that IL-2 and IL-15 are expressed in the human thymus in distinct locations, as reported in mice [41]. Notably, enriched areas of IL-2 and IL-15 production designated here as cytokine niches, were present in the human thymus. A similar pattern of IL-2 secretion was previously reported in human tonsils [42] and in murine thymus [43]. We expect these niches, likely resulting from post-secretion binding of cytokine to extracellular matrix components [44], to locally increase cytokine availability and prolong their *in-vivo* half-life. Of physiological relevance, we show that tTregs are found in close proximity to IL-2- and IL-15-producing cells.

We estimated that ~1.5% of human thymocytes had the potential to secrete IL-2 and identified mature αβ and γδ thymocytes as its main producers, a phenomenon conserved in the murine thymus [45]. We propose that the main IL-15 sources are monocyte/macrophages and B lymphocytes, populations shown in humans to produce IL-15 in the periphery [46–48], as well as TECs, which in mice secrete IL-15 [49]. Interestingly, the majority of human $CD25^{bright}CD4SP$ cells express CCR8, migrating in response to CCL1/I-309 secreted by macrophages [50]. Moreover, B lymphocytes were recently shown to select tTreg in mice [51]. Another important issue for future studies will be the comparison of the TCR repertoire of tTregs that differentiate in presence of IL-2 or IL-15 and the possible role of cells that produce these cytokines in mediating tTreg selection.

Our results support the possibility of the therapeutic manipulation of tTreg by targeting IL-2 and/or IL-15 pathways, and, in this way, potentially help control autoimmune manifestations. In fact, a growing interest in the use of low dose IL-2 in humans to selectively expand Treg has resulted from the promising data reported in two clinical trials, one in the context of HCV-induced vasculitis [52] and the other in chronic GVHD [53]. In both trials, IL-2 treatment was shown to be safe, associated with a marked increase in the proportion and number of total Tregs that correlated with clinical improvement, and had no obvious impact upon conventional CD4 T cells. Encouraging preliminary results using low dose IL-2 to expand Tregs were also recently obtained in Type I diabetes [54] and alopecia areata patients [55]. Furthermore, several clinical trials exploring IL-2 administration in diverse autoimmune disease settings are currently ongoing. Importantly, in agreement with our hypothesis that IL-2 impinges on the thymus, low dose IL-2 in the context of GVHD was shown to increase tTreg export [20]. We therefore propose that the impact of IL-2 or IL-15 upon human tTreg development and the requirement for efficient thymic function should be evaluated in clinical trials involving these cytokines.

5. Conclusions

Collectively our data suggest that following human tTreg precursor generation, subsequent exposure to either IL-2 or IL-15 drives their final differentiation into $FOXP3^{+}$ cells. These cytokines then act by enhancing tTreg survival and proliferation as well as by fostering their functional maturation. The physiological role of these cytokines is further supported by our demonstration of their production in the human thymus in a non-overlapping pattern by distinct cell populations. Ultimately, we expect that the ability to increase tTreg output via administration of IL-2 or IL-15 constitutes a novel approach for the management of autoimmunity.

Author contributions

IC and AES designed research; IC, VNS, ARP, CM, AIP, HNC and RBF performed research; IC, VNS, ARP, CM, AIP, HNC and RBF analyzed data; and IC, RBF and AES wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2014.11.002.

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