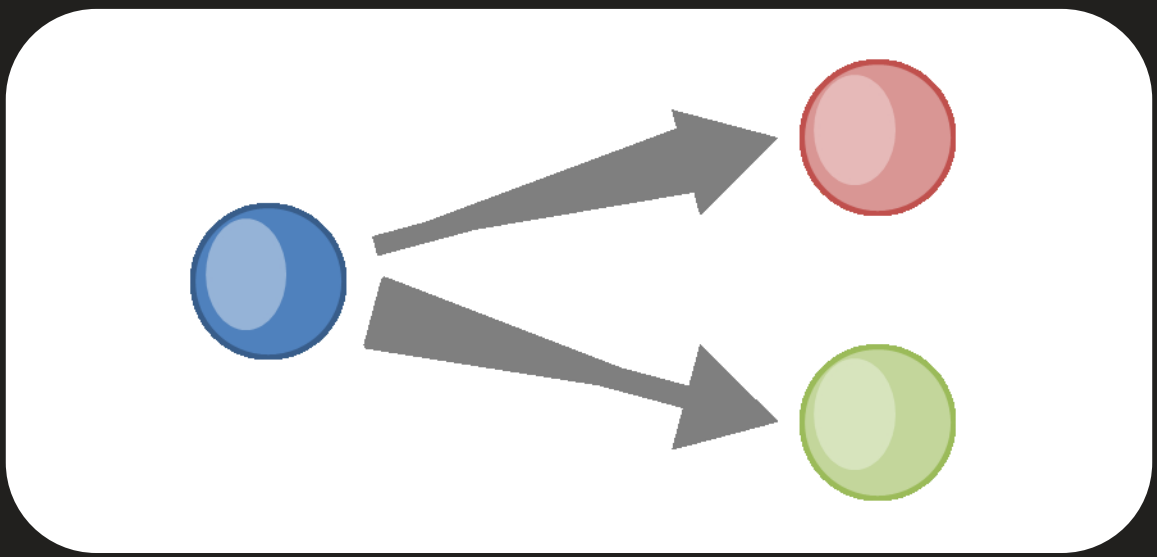
T cell Maturation and Regulatory T Cell Differentiation:

From the Thymus to the Periphery

Ricardo de Sousa e Paiva



Dissertation presented to obtain the Ph.D degree in Immunology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
October, 2012

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

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Instituto Gulbenkian de Ciência

Under the supervision of Dr. Jocelyne Demengeot

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Knowledge Creation

“A boy became a man when he killed his first full-grown buck. From an early age his father, uncles and grandfather taught him the ways of hunting. The hunter is required to follow traditions that insure good hunting and unselfish sharing of the harvest.”

Washoe’s Boy's Rite-of-Passage

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Abbreviations used

Ag – Antigen

Ab - Antibody

AIRE – Autoimmune regulator transcription factor

APC – Antigen presenting cell

biaLN – Brachial, inguinal and axillary lymph nodes

BM – Bone marrow

BMDC – Bone marrow derived in vitro differentiated dendritic cells

CD4 SP – CD4 single positive ($CD4^+$ $CD8^-$) cells

cTEC or CEC – Cortical thymic epithelial cell

DC – Dendritic cells

DN – Double negative ($CD4^-CD8^-$) cells

dn – Dominant negative

DP – Double positive ($CD4^+CD8^+$) cells

EAE – Experimental autoimmune encephalomyelitis

FACS – Fluorescence activated cell sorting

Foxp3 – Forkhead Box P3 transcription factor

GFP – Green fluorescent protein

IL – Interleukin

IRES – Internal ribosome entry site

KO – Knock out (genetically based gene deficiency)

LIL – Large intestine lymphocytes

LIP – Lymphopenia induced proliferation

LN – Lymph nodes

MBP – Myelin basic protein

MHC – Major histocompatibility complex

mTEC or MEC – Medullary thymic epithelial cell

pTreg – Peripheral Treg

PTX – Pertussis toxin

RA – Retinoic acid

Rag – Recombination activating gene

RTE – Recent Thymic emigrant

Sf - Scurfy

SPL – Spleen

Tconv – Non regulatory T cell

TGF – Transforming growth factor

TCR – T cell receptor

TEC – Thymic epithelial cell

Tg – Transgenic or transgene

TgTg – Homozygote anti-MBP TCR transgenic mice

Th – Helper T cell

THY – Thymus or thymocytes

TREC – TCR rearrangement excision circles

Treg – Regulatory T cells

tTreg – Thymic Treg

TxT – Thymectomized or thymectomy

T/R^{-} – Monoclonal anti-myelin basic protein TCR-specific transgenic RAG-deficient mice

WT – Wild type

Acknowledgments

I would like to start by thanking the people that were responsible for my stay at this amazing place made of amazing science and people that is the Instituto Gulbenkian de Ciência. First, I thank Constantin who gave me the opportunity to do my undergrad internship and was the person who got my attention to the matters of immunology. Through great discussions and teaching it was my pre-doctoral training and made me feel I really wanted to do Science. Then, I would also like to thank the Programa Gulbenkian de Doutoramento interview committee, Professor Antóno Coutinho, Doctor Moisés Mallo and Doctor Henrique Teotónio for believing I deserved to be part of the Doctoral Program and with that giving me the opportunity to do my PhD studies at the IGC.

I am deeply thankful to Jocelyne for having taken me in the Lab and accepting to be my supervisor. Irrespective of small frictions at some points you were responsible for keeping me on the right track and I feel you taught me what Science is all about. From those endless afternoons where we discussed ideas, plans, questions and concepts I learnt so much I cannot name it all, but I feel the most valuable thing I learnt was how to think as an immunologist.

I would also like to thank everyone that passed by the Lab during my stay. In particular to Lisa and Santiago for teaching me most of what I know to do in the bench, immunology related, but also for invaluable discussions, with its fair those of positivism and critical assessment. To Andreia, for being a serious motor on everything I was doing. Albeit with a way of addressing matters that could be much more amenable, I believe you always push in the right direction. To Catarina, for the night shift presence, all the help with whatever I asked for and for the infinite patience on my worst days, thank you. To Marie, for always being on the best mood possible, for showing everyone how productive one can be, and for the nice weekends where my

(or shall I say our) attempts to stand on a board on a wave, despite being deep winter and my feet freezing so much I couldn't feel them, were never that successful. Anyway it was always great fun and I will try to remind you not only as a friend, but in many ways as a role model. To Elodie, with whom, despite being more reserved, I always got good laughs and enjoyable moments. To Rosa, for always being available to help with whatever was asked and to fulfill last minute requests. Also, for all the trust and sharing of personal experiences, it was a pleasure to learn from you. Not to forget, Leonor, for the positive vibes regarding my project, João for babysitting me enough when I joined the lab and for invaluable help when I was still learning. To Thiago Carvalho for the endless high quality humor and critical thinking on every subject. To Inês, Ola and all other visitors, who always treated me with respect and turned my stay at the Lab a richer experience.

I could not thank enough to Manuel and Rui not only to make my work easier, but for the friendship that we built during these years. While football and work were the main connections our friendship extends beyond those affairs. I met many people in my stay at the IGC and I cannot name all that had an impact, from João and Carlos for interesting conversations about Science and all other things, to Martina and Nadja for the endless energy and fun emanating from the terror duo, to everyone who took part in the events I was involved with. I had a lot of fun thanks to you guys.

Last but not least, I would like to thank my family who are upstream of these years. Aos meus Pais, pelo apoio incondicional, mas não acrítico, às minhas decisões. Em particular à minha Mãe pela paciência, tolerância e compreensão aparentemente todas de forma infinita ao longo de toda a minha vida. À minha irmã Cláudia por ter partilhado a casa comigo sem tornar a experiencia muito dolorosa o que me permitiu fazer o meu trabalho. E à Tânia por ter lá estado sempre que precisei.

Thank you all.

Summary

Immunological tolerance, that is, the failure to mount an immune response to an otherwise immunogenic molecule, is one of the fundamental questions in immunology. The fact that lymphocytes express antigen receptors that are generated randomly and have the potential to recognize any conceivable antigen, adds another puzzle to the physiology of immunological tolerance. The other side of the coin, the general absence of immune responses to self antigens, is ensured by a tight regulation and several selection steps during T and B cell differentiation. One of these processes is the differentiation of regulatory T cells (Treg). While developing in the thymus, T cell clones bearing receptors with high affinity/avidity to antigens present at the time of differentiation may be eliminated by apoptosis or, alternatively, express Foxp3 and become Treg. Treg are key players in the regulation of immunological tolerance since humans and mice with complete loss of function variants of this gene develop fatal autoimmune conditions early in life. Importantly, peripheral $CD4^{+}Foxp3^{-}$ T cells have recently been demonstrated to acquire Foxp3 expression upon stimulation in a non-inflammatory environment. Thus, understanding how Treg develop and function may be expected to guide future therapeutic approaches.

The focus of this thesis is therefore to address rules and factors that affect Treg differentiation, in particular cell intrinsic properties related to T cell ontogeny. Making use of several transgenic (Tg) mouse strains, we addressed: I) the source of Treg specific to peripherally administered antigen in an inflammatory setting; II) the susceptibility of T cells at different states of maturation to upregulate Foxp3; and III) the function and stability of Treg developed from peripheral cells.

It is established that peripheral antigens, either blood born or transported by dendritic cells, can impact on thymic selection leading to deletion or Treg differentiation in clones that recognize such antigens. However, it was not

known if peripheral antigens can also contribute to these processes in inflammatory settings. Using TCR Tg mice that recognize either self or exogenous antigens we showed that Treg can develop in the thymus after peripheral Ag immunization with a strong adjuvant and demonstrated that the thymus was essential to achieve a tolerant state. In the self reactive TCR Tg strain, Treg were also found to be essential to prevent the spontaneous autoimmune disease that develops in untreated mice. We further demonstrated that thymocytes, unlike peripheral T cells, are refractory to IL-6, a cytokine that inhibits Foxp3 induction in mature cells.

We then assessed the capacity of immature cells to upregulate Foxp3 outside the thymus. A small fraction of naïve Foxp3$^-$ CD4 T cells upregulates Foxp3 when adoptively transferred to lymphopenic recipients. Using this experimental system we report that CD4 Foxp3$^-$ SP thymocytes differentiate to Treg more efficiently than peripheral naive CD4 Foxp3$^-$ cells. We then showed that this results from an intrinsic property of the cell that does not require the presence of the thymus in recipient mice. By varying the number of recent thymic emigrants (RTE) in the transferred cells by thymectomizing donor mice or by cell sorting, we demonstrated that RTE are the main source of Treg differentiated during lymphopenia induced proliferation (LIP). Last, by manipulating available TGFβ or the sensitivity of T cells to TGFβ, we found that immature, but not mature, cells are able to differentiate into Treg when TGFβ availability is limited.

Finally, we assessed the function and stability of Treg differentiated from peripheral cells *in vivo*, and once again using the lymphopenia induced proliferation model, we demonstrated that upregulation of Foxp3 by peripheral cells limits LIP. Then, by isolating Foxp3$^+$ developed during LIP (iTreg) and performing secondary adoptive transfer together with fresh Foxp3$^-$ cells, we found that few cells from iTreg origin were recovered. Nevertheless, these cells kept Foxp3 expression showing that Treg derived

in vivo from peripheral cells are stable. The low recovery of iTreg could be due to a requirement of factors secreted by the activated T cells that were present when they differentiated in the first transfer. In concordance with this hypothesis we found that *in vivo* derived iTreg upregulate the IFN-γ receptor which matches the dominant Th1 polarization associated with LIP, consistent with the recently described notion of Treg plasticity.

In conclusion, the work in this thesis shows that T cells lose the potential to become Treg as they age in a cell intrinsic manner that also applies to extrathymic Treg differentiation. This new piece of information suggests that therapeutic interventions aiming at generating tolerance through Treg will have a higher chance of success if administered at the earliest age possible.

Título

A Maturação das Células T e a Diferenciação de Células T Reguladoras: Do Timo à Periferia

Resumo

A Tolerância Imunológica, ou seja, a ausência de uma resposta imunitária a moléculas de outro modo imunogénicas, é uma das questões fundamentais em imunologia. O facto de os linfócitos expressarem recetores de antigénio que são gerados aleatoriamente com o potencial de reconhecerem qualquer antigénio concebível acrescenta um quebra-cabeças formidável à fisiologia da tolerância imunológica. No entanto, a ausência de resposta imunitária contra antigénios do próprio organismo é garantida por uma forte regulação e vários passos de seleção no processo de diferenciação dos linfócitos T e B. Um destes processos é a diferenciação de células T reguladoras (Treg). Ao maturarem no timo, clones de células T que apresentam recetores com alta afinidade/avidez para antigénios presentes no momento são induzidas a entrar em apoptose ou, a expressarem o fator de transcrição Foxp3 e assim diferenciarem-se em Treg. O fato de Humanos e ratinhos com uma deficiência completa da proteína Foxp3 desenvolverem, rapidamente após o nascimento, condições fatais de origem autoimune, ilustra o carácter essencial das Treg. Mais recentemente foi descrito que células já exportadas do timo também apresentam capacidade de expressar Foxp3 quando estimuladas em condições não inflamatórias. Desta forma, é esperado que a elucidação da fisiologia das Treg possa guiar aplicações terapêuticas futuras.

O foco desta tese é estudar regras e fatores que afetam a diferenciação das Treg, em particular propriedades intrínsecas das células T relacionadas com a sua ontogenia. Usando várias estirpes de ratinhos transgénicos (Tg), estudámos: I) a origem das Treg diferenciadas contra antigénios periféricos

em condições de inflamação; II) a suscetibilidade de células T em diferentes estados de maturação para serem induzidas a expressar Foxp3; e III) a função e estabilidade das Treg diferenciadas a partir de células isoladas da periferia.

Encontra-se estabelecido que antigénios da periferia, quer em circulação na corrente sanguínea ou transportados por células dendríticas, podem contribuir para a diversidade antigénica no timo, levando à eliminação ou à diferenciação para Treg de clones que os reconhecem. No entanto, não se encontrava clarificado se os antigénios periféricos podem participar nos mesmos processos durante reações inflamatórias. Através do uso de ratinhos que expressam recetores transgénicos reativos quer com antigénios endógenos quer exógenos, mostrámos que a diferenciação de Treg pode ocorrer no timo após imunização com um adjuvante que gera uma forte reação inflamatória. Demonstrámos também que o timo era essencial para se atingir um estado de tolerância imunológica. Nas experiencias em ratinhos em que o recetor transgénico reconhece um auto-antigénio demonstrámos que as Treg são essenciais para se obter uma proteção da doença autoimune que se desenvolve de forma espontânea nesta estirpe. Demonstrámos ainda que os timócitos, ao contrário das células periféricas, são refratários ao efeito da IL-6, uma citoquina que inibe a expressão de Foxp3 em células maturas.

De seguida, estudámos a capacidade das células T imaturas de expressar Foxp3 fora do timo. Uma pequena fração de células T Foxp3$^-$ isoladas da periferia passa a expressar Foxp3 quando as células são transferidas para ratinhos linfopénicos. Usando este sistema experimental, observámos que os timócitos CD4 SP Foxp3$^-$ são mais eficazes a diferenciarem-se em Treg do que as células CD4 Foxp3$^-$ isoladas da periferia. Demonstrámos ainda que esta propriedade é intrínseca das células imaturas e é independente da presença do timo nos ratinhos recipientes. Variando a quantidade de células

que foram exportadas do timo recentemente (RTE – do inglês Recent Thymic Emigrants) na população de células transferidas, quer ao timectomizar os dadores ou por isolamento seletivo das células RTE e das células já residentes na periferia, demonstrámos que as células RTE são a principal fonte de Treg que se diferencia durante a expansão induzida por linfopenia. Por último, através da manipulação da quantidade de TGFβ disponível ou a sensitividade das células ao TGFβ, estabelecemos que as células imaturas, ao contrário das imaturas, têm a capacidade de se diferenciar em Treg quando os níveis de TGFβ são limitados.

Finalmente, testámos a função e estabilidade das Treg diferenciadas *in vivo* a partir de células T isoladas da periferia. Usando novamente o sistema de transferência de células para ratinhos linfopénicos, observámos que a diferenciação de Treg a partir de células T Foxp3$^-$ controla a expansão da população global de células T transferidas. Por outro lado, ao isolar as Treg diferenciadas a partir de células T periféricas durante a sua expansão em recipientes linfopénicos e transferindo-as de novo com uma nova população de células Foxp3$^-$ isoladas de fresco, observámos que poucas células da população que era Foxp3$^+$ eram recuperadas. No entanto, as células recuperadas mantiveram a expressão de Foxp3, o que indica representarem uma linhagem de Treg estável. O baixo número de Treg recuperado pode ser causado por uma necessidade de receber sinais das células T ativadas durante a expansão em linfopenia, o que ocorre concomitantemente com diferenciação das Treg. De acordo com esta hipótese, verificámos que as Treg diferenciadas durante a expansão em linfopenia aumentam o nível do recetor para IFNγ, o que é concordante com a polarização dominante das células transferidas para o tipo Th1, e é também consistente com a noção recente de plasticidade das Treg.

Em conclusão, o trabalho apresentado nesta tese demonstra que as células T perdem o potencial para se diferenciar em Treg à medida que envelhecem

de uma forma intrínseca e que se aplica à diferenciação de Treg fora do timo. Este fato sugere que aplicações terapêuticas com o objetivo de estabelecer tolerância imunológica através da diferenciação de Treg terão uma maior probabilidade de sucesso se forem postas em prática em pacientes o mais jovens possível.

Chapter 1

Introduction

This chapter is intended to provide enough background, as it existed at the time this doctoral work was initiated, to frame the hypotheses put forward by then. This thesis concerns the study of T cells, specifically the differentiation and function of a subpopulation of this lymphocyte subset known to provide powerful regulation in a broad collection of settings, the Foxp3 expressing regulatory T cells (Treg). As such, the results presented in later chapters require an introduction on the origin and development of the concept of immunological tolerance, T cell differentiation and basic notions on regulatory T cell physiology.

1.1 Immunological Tolerance: An historical note

The development of a theory of Immunity has been attributed to Elie (Ilya) Metchnikoff in the end of the 19th century early 20th century (1), however Immunology has its foundations in the applications of the first vaccines and its examination through the lens of the scientific method performed by Edward Jenner in the end of the 18th century (2). The early vaccines in the western world were actually only the systematic study, reporting and application of a behavior based on centuries of empiric knowledge. There is historical evidence supporting that inoculation was already applied throughout the globe centuries before the first applications by Jenner. The development of the crude forms of immunization are predicted to have been put in practice after sporadic observations that individuals that had survived an often fatal infectious disease had became protected from contracting it a second time (3). In fact, the understanding of vaccines started almost a century later after the introduction of vaccination itself (4). With the mounting interest in microbiology and the germ theory of disease championed by Louis Pasteur (5), the initial explorations into the working of the immune system by Metchnikoff (1), Jules Bordet (6), Paul Ehrlich (7) and Robert Koch (8, 9), Immunology, as the study of the protection mechanisms

organisms possess against invading foreign bodies, gained sufficient momentum to become a field of research by itself. While the separation of immunology from microbiology has in many aspects never taken place, early reports and conceptual dwellings of the founding fathers of immunology fell upon the possible reactivity against self. Of note, Ehrlich put forward the concept of *horror autotoxicus*, which proposed, without offering an explanation, that the organism could not produce pathogenic anti self toxins (10). This concept remained an unchallenged dogma despite experimental evidence indicating the existence of auto immune diseases and immunology concerned the study of protection from pathogens and little attention was given to explore the possibility of self reactivity.

While Metchnikoff exposed his interpretation of natural and acquired immunity (1) (which have later been developed to the notions of innate and adaptive immunity) he was also in the middle of a scientific riddle that was another foundation of immunology. He supported the exclusive cellular nature of immunity as opposed to the humoral theory of immunity, of which Ehrlich was a fierce supporter. Later, reconciliating experiments showing a synergistic effect of blood derived factors with cellular processes to eliminate pathogens led to the attribution of the 1908 Nobel Prize in Physiology or Medicine to both Metchnikoff and Ehrlich (11, 12). While the cellular theory of immunity was based solely in phagocytes the humoral theory was based in anti toxins and the components that allow efficient cellular lysis and toxin neutralization to take place.

Retrospectively, the discovery of antibody production could be seen as the foundational lead from which modern immunology got its start. The facts that an animal could be immunized with an extract of any pathogen and even with synthetic molecules (13), and that antibodies would be produced on demand, without being detected beforehand, opened a wide array of questions. Notably, regarding the identification of the antibodies' source and the mechanisms by which they are formed. With the identification of B cells,

or at least lymphocytes, as antibody producing cells, the focus turned to the questions linked with the ability to mount a response with specificity to any given antigen. At this point two competing hypothesis dominated the conceptual framework. One was the instructive mechanism, which implied that the antigen would itself shape the antibodies with a subsequent auto induction to produce more of the same (14). The other, was the elective model, in which the pre-existence of a very diverse set of antibodies allows that one recognizing the antigen would always be found, and this interaction would then stimulate the production of more of the same antibody. The cornerstone of this debate can be considered the model developed by Niels Jerne and Macfarland Burnet of the clonal selection theory (15, 16), built upon the early elective theories (7). In this model it was proposed that the antibody producing cells would be the selected unit. A great number of antibody producing cells would be generated and upon encounter with the antigen the ones producing appropriate antibodies would be selected to produce the amounts required to neutralize antigen. The confirmation that each cell only produces one antibody (17) and the clarification of the mechanism of generation of antibody diversity, by somatic rearrangement of B cell receptor genes (18-20), set the field to understand how clones were produced and how a functional repertoire is achieved. With the fact that the immune system has indeed the ability to generate a virtual infinite set of receptors came the question of how is the system set not to attack self, that is, immunological self tolerance.

However, the concept of Immunological tolerance had been already put forward, again by Burnet, inspired by the description of Owen and colleagues that non-identical calf twins which undergo anastomosis during fetal development are immunologically unresponsive to blood from each other while mounting an immune response to an unrelated third party blood donor (21). Burnet proposed that there would be a time window when the organism is developing for the establishment of tolerance to foreign antigens

(22). The concepts underlying Burnets theory were then formally demonstrated by Peter Medawar and colleagues (23), and both Burnet and Medawar shared the 1960 Nobel Prize in Physiology or Medicine for the discovery of acquired immunological tolerance.

Later came developments to the theory for the mechanistic basis of acquired tolerance, from a developmental time window (22, 23) and experiments addressing recessive and dominant regulation modes have been clarifying the processes behind the self/non-self discrimination and anti-self immune reactivity regulation and deregulation.

Joshua Lederberg put forward a theory in line with Burnet's proposition. According to the Lederberg model, cells would first differentiate in a state which allowed their disabling in case of self-reactivity (24). While Burnet proposed a developmental time window, that is, tolerance could only be attained at young age, Lederberg transformed this into a cell age, rather than an organism age, interpretation in which recently made cells would have this property, which would obviously be more frequent in fetal and neonatal life. At this point, the theories concerned the production of antibodies and the antibody forming cells. While a role for the thymus to establish functional immunity was reported shortly after, the existence of T cells and the variable nature of T cell receptors came only some three decades later (25-27). Also later came the clarification of the existence of MHC recognition and compatibility (28) which added a new piece to the puzzle of immunological tolerance.

With the knowledge about T cells building up, a seminal collection of experiments was performed by Le Douarin and colleagues paving the way to the demonstration of the existence of regulatory T cells. The ground breaking experiments consisted in the transplantation of thymic epithelium from quail to chick embryos and showed that only the thymic epithelium allowed for lifelong tolerance to a wing transplanted shortly after birth,

allowing a quail wing to not be rejected and fully develop (29). The fact that the wing transplant was performed during neonatal life, together with earlier work showing that neonatal thymectomy led to autoimmune disease (30), led to the reformulation of Burnet's theory to include an essential requirement for Thymic T cell selection to achieve a fully functional tolerant T cell compartment. Later work applying a similar approach to the mouse model organism showed that the thymic epithelium requirement to achieve tolerance across MHC incompatible donors was also true in mice (31). It is nevertheless worthwhile mentioning that the tolerance put in check in the thymic epithelium systems is related to MHC alloreactivity, which may involve a specific mode of T cell activation rather than tolerance to a novel antigen presented in the MHC in which T cells are selected.

The strong suggestion of a thymic derived dominant mode of tolerance started a race to identify the regulatory cells. This search was based in surface sub-phenotyping and cell isolation coupled to functional assays for tolerance. This endeavor is extensively reviewed by Shimon Sakaguchi in (32) and culminated in the identification of CD25 positive (33), and later Foxp3 expressing (34, 35), CD4 T cells as the long sought after Treg subset.

Even after close to one century of research, immunological tolerance is not fully understood. As a good example of our ignorance is the lack of understanding for the etiology and of efficient treatment for the majority of autoimmune diseases. Likewise, the management of uncontrolled immune responses to innocuous agents as allergies is also still rather inefficient.

1.2 T cell ontogeny: Thymocytes and Recent Thymic Emigrants

T cells when stimulated through the TCR in a proinflammatory microenvironment differentiate to an effector phenotype that may consist in a direct cytotoxic activity (for CD8 T cells) or to become a helper T cell (CD4 T cells). As such T cells can be direct effectors in an immune response or orchestrate and amplify the response of other cells according to cues received from the antigen presenting cells and microenvironment (36-39). On the other hand, and as introduced in the section above, T cells are also essential actors to attain immunological self tolerance, and the focus of this thesis, regulatory T cells, are particularly important in that function.

When a microorganism invades the host, cells from the innate immune system get activated, either by sensing the pathogen directly or by sensing alteration in tissue integrity. Such cells, like macrophages, neutrophils, and dendritic cells provide an early immune response, which is an initial barrier to confine and destroy pathogens. On the other hand, these cell subsets are the most common cells to initiate an inflammatory response that leads to the participation of the adaptive arm of the immune system (40, 41). Antigen presenting cells phagocyte and digest the microorganisms while migrating to the LN where they present peptides from the digested material to T cells. T cells that recognize processed antigens presented in the context of MHC molecules are activated and mobilized to initiate the adaptive phase of the immune response. CD4 T cells recognize their cognate antigens presented in the context of MHC-II (loads mainly Ags from extracellular origin) and are able to interact with B cells that also recognize Ags from the same environment and give rise to a humoral immune response. At the same time CD4 helper T cells can also potentiate CD8 T cell responses and influence the phenotype of other cells of the immune system. On the other hand, CD8 T cells recognize their cognate Ags when presented in the context of MHC-I (mainly loaded with peptides of intracellular origin) and mount a cytotoxic immune response (42).

Antigen receptors are generated in each clone by re-arranging one of several V (D) and J gene segments, with extra nucleotides being added and/or deletions taking place in the junction between each segment in the recombination events. This mechanism offers the possibility to generate TCRs that can recognize any conceivable antigen. However, the rather non-templated generation of Antigen receptors leads to the generation of non functional TCRs and TCRs that recognize self derived Ags. As a consequence, T cells have to undergo a stringent selection and maturation program to ensure the peripheral, ready to respond, naïve T cells are competent to scan MHC molecules in search for foreign antigens while presenting minimal anti-self reactivity (43, 44).

For T cells, this “education” process takes place in the thymus (Fig. 1). The immunological role of the Thymus was first reported by Jacques Miller in 1961 (27), who also later reported the requirement for the presence of both thymus and bone marrow derived cells to obtain efficient antibody production eventually leading to the identification of B and T lymphocytes (45). Since the early progenitors of T cells reside in the bone marrow, T cell progenitors have to migrate to the thymus to differentiate into T cells. Common Lymphoid Progenitors (Lin^- IL-$7R\alpha^+$ Thy-1^- Sca-1^{lo} c-Kit^{lo}) migrate through the blood stream and enter at the Thymus at cortico-medullary junction (46). In the first steps of thymocyte maturation, cells go through several stages denominated double negative (DN – due to the lack of expression of CD4 and CD8) which can be subdivided from DN1 to DN4 by the expression of CD44 and CD25. The progressive differentiation of Thymocytes to T cells takes place while cells migrate from the initial entry point, to the sub capsular zone of the thymic cortex, and later to the medulla passing through the cortico-medullary junction (46). DN1, or Early T cell progenitors (ETP), are characterized by expressing CD44 but not CD25, and retain some potential to become dendritic cells, macrophages, Natural killer cells and B cells. While differentiation to these lineages is restrained by the thymic

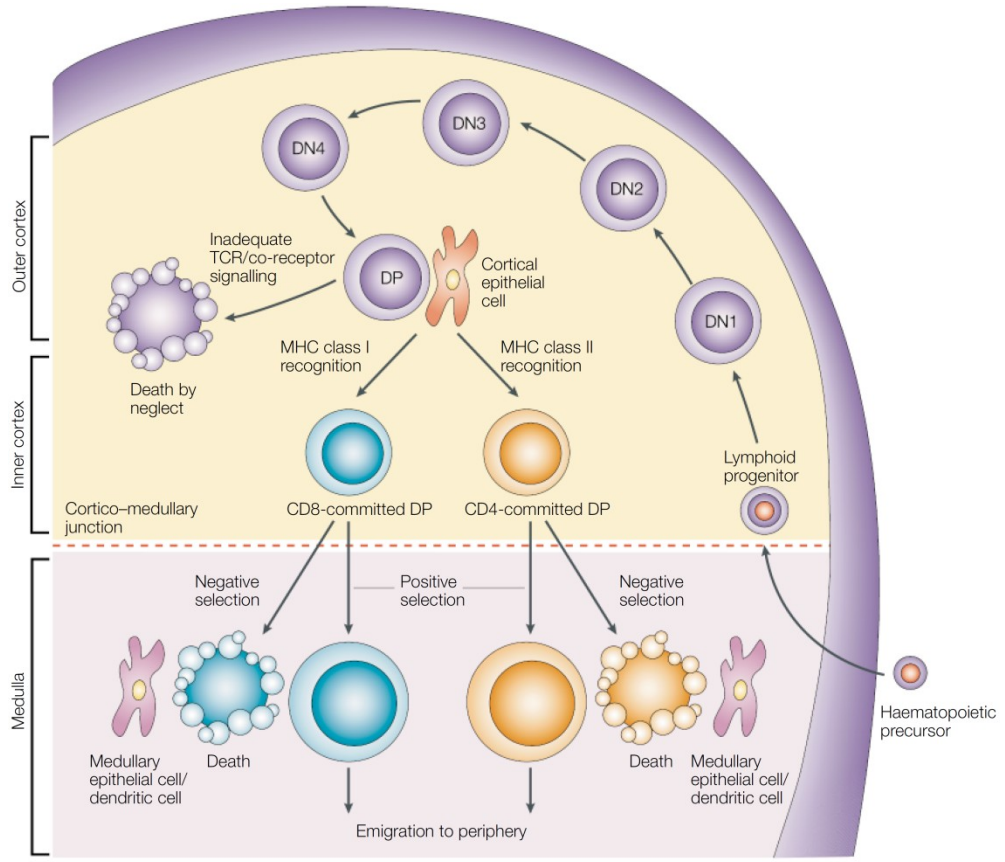

Figure 1. Overview of T cell differentiation in the thymus. Haematopoietic precursors migrate from the bone marrow through the blood stream and enter the thymus in the cortico-medullary junction. After receiving T cell determining signals the progenitor undergoes several steps of maturation. First, it upregulates CD44 and enters the Double Negative 1 stage (DN1 – $CD4^-CD8^-$), or early T cell precursor (ETP), then after receiving further T cell driving signals it progresses to the DN2 stage, or pro-T cell, where it upregulates CD25 expression becoming $CD44^+CD25^+$. Between DN2 and DN3, pre-T, where thymocytes downregulate CD44 becoming $CD44^-CD25^+$, RAG1 and RAG2 are expressed and β chain locus of the TCR is rearranged. After successful pairing with the pre-T alpha thymocytes progress to the DN4 ($CD44^-CD25^-$) and later to the DP stage where RAG1 and RAG2 are re-expressed and the α chain locus is recombined. At this stage cells that do not generate a functional receptor die by neglect due to the lack of the essential survival signal provided by a functional TCR and its interaction with MHC. The cells that

generate a functional TCR are probed for interaction with MHC-I and MHC-II and become committed to the CD8 or CD4 lineage, accordingly to the respective MHC with which the TCR forms stable contacts. This is positive selection step and occurs through interactions with the cortical thymic epithelial cells (cTECs). After succesfull positive selection DP thymocytes cross the cortico-medullary junction to the medulla where they interact with DCs and with medullary thymic epithelial cells (mTEC) and undergo negative selection. This selection step consists in elimination of clones that present TCRs with high affinity/avidity for the presented peptide-MHC complexes. Concomitantly, cells that interact with the MHC-II with high affinity/avidity but below deletion threshold are diverted to the Treg pool. Adapted from (47).

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microenvironment, Notch 1 signaling together with transcription factors Runx1, Gata-3 and E box proteins enforce a T cell lineage commitment by inducing T lineage gene expression (48). After receiving T cell driving signals, DN1 cells progress to the DN2 stage, or pro-T cell. This stage is characterized by CD25 and CD44 expression and marks the loss of the potential to differentiate to other lineages. At the late DN2 and early DN3 ($CD44^{-}CD25^{+}$) stages, cells upregulate the enzymes responsible for the rearrangement of antigen receptors, the recombination activating genes 1 and 2 (RAG1 and RAG 2), that, for the case of cells that are directed to the $\alpha\beta$ lineage, rearrange the TCR beta chain. The clones with the generated gene segments at this stage undergo the β chain selection, which is a test for the ability to pair with the pre-Tα chain and be expressed at the cell surface (48, 49). At this point the precursors are at the DN3 stage, or early pre-T, and only the clones with a valid TCRβ progress to the late DN3 and then to DN4 ($CD25^{-}CD44^{-}$), or late pre-T, stages. After a proliferative burst cells progress to the Double Positive stage (DP – $CD4^{+}CD8^{+}$), RAG1 and RAG2 are re-expressed and the TCRα chain locus recombined (43). At this stage, cells are found close to the cortico-medullary junction and it is here,

and through the interaction with cortical thymic epithelial cells, that positive selection takes place. Clones need to receive survival signals through the generated TCR otherwise they undergo programmed cell death, a process denominated “death by neglect” (50). After a full αβTCR molecule is expressed at the cell surface, MHC-II or MHC-I valid interaction lead to the commitment of the clones to the respective CD4 or CD8 lineage. DP thymocytes migrate then through the cortico-medullary junction to the medulla where they encounter DCs and medullary thymic epithelial cells (46). The interactions with these cell subsets undertake the negative selection checkpoint in which clones presenting TCRs with high avidity/affinity for the presented antigens are deleted. In the same stage clones that present TCRs that interact with peptide-MHC with affinities/avidities just below the threshold for deletion are diverted to the Treg lineage (51, 52). These last steps are the essential tolerance determining mechanisms inside the thymus. Deletion of autoreactive thymocytes prevents the majority of self reactive T cell clones from being exported to the periphery (recessive tolerance), while the positive selection of clones with string interaction with peptide-MHC ascertains peripheral recognition of Antigens and dominant tolerance over T cell clones that escape deletion.

The positive and negative selection of generated α and β chains of TCR involves the interaction with peptide loaded MHCs. Therefore the efficient generation of a functional peripheral pool of T cells by these processes depends to a large extent in the diversity and amount of peptides available in the thymus. On one hand, efficient positive selection ensures that T cells, once in the periphery, will receive low TCR signaling essential for their survival. On the other hand, the diversity of antigens is essential for the deletion of auto-reactive clones preventing them from reaching the periphery. Since Treg are also selected based on the interaction of their TCR with peptide-MHC, the diversity of antigen is equally important to

generate a Treg pool that can recognize enough peripheral antigens to ensure self-tolerance.

The thymus architecture and cellular composition is known to be correlated with the establishment of a functional T cell compartment, and its deregulation is usually accompanied by the development of a dysfunctional T cell peripheral population (53, 54). However, the antigenic relationship between the thymus and the periphery is not fully elucidated. Establishing a functional T cell repertoire is tied to the efficient selection of thymocytes and the later incorporation of nascent T cells in the peripheral pool. As such, the intra and extra thymic T cell maturation are key points to be clarified in order to understand how the T cell repertoire develops and to indicate ways it may be manipulated with therapeutic goals (55). Antigen diversity in the thymus is determined by AIRE driven ectopic expression of peripheral tissue antigens in medullary thymic epithelial cells (56), uptake of blood borne antigens (57) and by antigen carried by immigrating DC (58, 59).

Ectopic expression of endogenous antigens is mainly driven by the autoimmune regulator (AIRE). AIRE is expressed in medullary thymic epithelial cells and is thought to randomly facilitate ectopic gene expression. AIRE is essential for immune self-tolerance as it the multi-organ manifestations that develop in humans and mice that cannot express a functional protein (56). Intriguingly, AIRE is only essential during the first days of life which indicates that deletion after the neonatal period has a much less preponderant factor than in the initial steps of building a peripheral T cell pool (60). The thymus is partially isolated from the circulatory system by a thymus-blood barrier (61). However, this barrier, while preventing the passage of high molecular weight molecules, is permeable to low molecular weight compounds such as free peptides. Last, it has been shown that DCs from the skin are able to travel into the thymus carrying antigens that can mediate negative selection and induce Treg (62).

Despite being known that the antigenic landscape in the thymus has some cross talk to the periphery the full characterization of the dynamics and constraints of such antigen exchange is still lacking. In particular, we address in a later chapter if peripheral antigen could also access the thymus in conditions of pro-inflammatory immunization.

In physiological conditions most T cells come through the intrathymic differentiation process able to correctly interact with the host MHC. The interaction of selected TCRs allows T cells to scan MHC-peptide complexes in search for foreign antigens and, in the absence of foreign antigens, to receive low intensity TCR signaling that allows them to survive and be ready to mount an efficient response (63, 64). The clones that are on the higher end of self reactivity, but have escaped negative selection and induction of Foxp3 expression, are controlled by Treg, even if after differentiating to the naturally activated/memory pool (65-67). However, when T cells are exported from the thymus they are not yet fully mature. Recent thymic emigrants (RTE) display specific phenotypic characteristics for which the molecular basis is largely unknown (55).

It is known that TCR signaling threshold is increased in DP thymocytes when compared to peripheral T cells. Mainly, the amount of antigen to induce deletion of double positive thymocytes is much lower (calculated to be about 100 fold less) than the amount required to induce an effector T cell response. The decrease in the threshold of TCR signaling to elicit a response in T cells when compared to deletion ensures a minimally self reactive T cell repertoire is established in the periphery (66, 68). However, the differences in TCR signaling thresholds in later stages of T cell maturation are not fully characterized, particularly after the CD4/CD8 lineage commitment. Thymocytes do display a maturation dependent TCR sensitivity tuning. The changes in TCR signaling response correlates with differential inhibitory co-receptor expression, signaling pathways triggered glycosylation of cell surface molecules and specific microRNA expression. These

characteristics eventually become indistinguishable from peripheral T cells as the cells are exported and incorporate the peripheral pool (69-74).

However, the study of the steps of maturation that take place outside the thymus is still poorly understood, including the molecular basis that rules the final maturation of nascent T cells as they incorporate the resident peripheral T cell pool (55). While all T cells could be referred to as recent thymic emigrants, since it is a question on how long ago the cells differentiated and were exported, several studies have indicated a window of around 3 weeks were phenotypic characteristics that discriminate RTE from resident T cells can still be detected. The recent advances of RTE understanding have been manly led by Pamela Fink research group. The fast pace of new findings was made possible by the innovative use of a mouse line that expresses a green fluorescent protein (GFP) driven by the RAG2 promoter (75) to follow RTE. In this mouse line, thymocytes start to express the reporter while in the DN stage and achieve high levels of GFP in all DP thymocytes, in concordance with the expected RAG expression. In the periphery, more than 50% CD4 T cells are positive for GFP at 5-6 weeks of age. This percentage decreases to background levels by 4 weeks after thymectomy (76). The aforementioned results confirm that this mouse model provides an accurate tool to identify and isolate RTEs. This is a big step from the previous experimental systems available, such as intrathymic injection or thymus graft, which implied heavy manipulation of mice or cells with the inherent potential to create artifacts. Using this experimental system it has been clearly established that RTE undergo phenotypic changes that occur progressively and are concomitantly to the loss of GFP signal. In the periphery, it is possible to discriminate 3 populations according to GFP expression, a GFP^{hi} (calculated to be T cells exported in the previous week), GFP^{lo} (exported between 2-3 weeks) and GFP^{-} (peripheral resident T cells) (76). The study of the 3 populations revealed a change in both surface phenotype and functional readouts. After thymic export, RTE progressively

downregulate CD24, Thy1 and CD3, rapidly loose CD69 expression and progressively upregulate Qa-2, CD28, CD45RB and IL7-Rα. Functionally, RTE display an intermediate dependence on IL-2 in *in vitro* proliferation assays, that is, lower dependence than CD4SP thymocytes but higher than peripheral resident mature cells. RTE also show a slightly impaired upregulation of CD25 upon in vitro activation (76). Later work has shown that RTEs are outcompeted by peripheral resident mature cells in lymphoreplete recipients, an effect that can be reverted by forced expression of the IL7-Rα or expression of the anti apoptotic protein Bcl-2. However, when in lymphopenic hosts RTE are then able to repopulate the peripheral pool as well as mature cells, which is concordant with a limiting niche for RTE when tested in lymphoreplete recipients (77). Fink group has also reported that RTE display a bias to differentiate to a Th2 against Th1 effector T cell polarization (78), require contact with lymphoid organs to mature (79), but while interaction with MHC shapes the RTE TCR repertoire, it does not induce phenotypic maturation. However, the maturation of RTE to a mature naïve phenotype seems to be dependant of an yet unidentified dendritic cell derived cue (80).

In the case of Humans, RTE have been mainly studied through the detection of the excision circles that are a byproduct of TCR rearrangement (TCR rearrangement excision circles – TRECs). Since TRECs do not undergo replication with cell divisions, they are diluted as cells proliferate. As a consequence, recently exported cells that have undergone fewer cell divisions than peripheral resident cells are enriched in TRECs. In any case, just after the first cell division one of the daughter cells will not carry the TREC which makes this method highly unreliable from the beginning. Together with the fact that identification of TRECs does not allow for cell isolation, this method offers limited value except for the information of how many thymocytes gave rise to the cells for which the TREC content is being measured. However, CD31 expression has been more recently identified as

a marker that positively discriminates RTE. And, while this marker is apparently not identifying all RTEs, the inclusion of PTK7 positive cells has recently improved the capacity of researcher to more accurately isolate Human RTEs. In general Human RTEs have been reported to display a similar phenotype as mouse RTEs. They display poor proliferation and produce low amounts of IL-2 and IFNγ upon *in vitro* stimulation and their survival is highly dependent on IL-7 (81).

In healthy conditions the peripheral number of T cells is remarkably stable (82). The maintenance of peripheral T cell numbers and TCR diversity is a maintained by the incorporation of newly formed T cells into the resident pool and by proliferation of the resident cells. Replenishment of the peripheral T cell pool by proliferation leads to the decrease of T cell diversity, due to not all clones proliferating equally and stochastic survivability/proliferation. On the other hand, incorporation of newly differentiate cells is essential to maintain T cell diversity which is required for an efficient immune response to be mounted against pathogens encountered for the first time (82).

Several experimental approaches have shown that T cells have several niches which are occupied and competed for, by respective cellular subsets (82). By performing thymic grafts under the kidney capsule of euthymic recipients it has been demonstrated that the number of cells in the periphery of grafted recipients increases accordingly to the expected contribution of extra thymic output (83). The increase, while being indexed to the number of grafts, reached a plateau illustrated by the number of cells being unaltered irrespective of recipients being analyzed 8 or 16 weeks after grafting. After thymic removal T cell numbers decayed again to normal levels showing that the increase of cell number when the graft was present was exclusively derived from the extra production of recent thymic emigrants. Calculations regarding the cell numbers obtained were concordant with RTEs being

exempted from competing with the mature resident T cells peripheral for about 3 weeks.

In contrast activated/memory T cells have been shown to depend on cytokine signaling alone and to not require TCR signaling to survive (82). The activated/memory T cell pool less stringent requirements for survival, than the requirements for naïve T cells, guarantees the maintenance of immunological memory. On the other hand, the timely determined, less stringent niche for nascent T cells ensures that the pool of naïve T cells can be continuously replenished by recruiting RTEs in that way maintaining TCR diversity, guaranteeing an higher chance of an effective primary response in the future.

However, thymic function is sometimes reduced, either due to age dependent thymic involution, thymectomy or severe inflammation. When thymic output is diminished the lack of replenishment of the naïve T cell pool diminishes the competition for the available niches. As naïve T cells proliferate in response to increased cytokine availability and a decrease in clonal competition for MHC-peptide signaling, they acquire an activated/memory phenotype. Initial proliferation by the resident cells masks the lack of incorporation of new T cells into the pool. However, the proliferative potential of cells will eventually decrease, and the enrichment in activated/memory T cells leads to an increase in Treg which results in a contracted naïve T cell pool (82).

Naïve and activated/memory T cell niches are mainly determined by clonal competition for peptide-MHC accessibility and/or cytokine signaling (84, 85), while the factors conditioning RTE survival and maturation are largely unknown. Similarly to the determination of peripheral T cell numbers, the thymus also has mechanisms that determine the number of thymocytes at any given moment and the amount of T cells exported. The thymic architecture is highly dependent on the interaction between thymocytes and

stromal cells and IL-7 has a prominent role in intrathymic homeostasis (86, 87).

Another classical system related to T cell maturation is the newborn immune system. After birth, the T cell pool is exclusively composed of recently differentiated T cells and the innate immune system displays lower activation levels, taking about 3 weeks in mice to become fully immunocompetent (88, 89). As introduced in the previous section, the particular response of newborns to antigenic challenges is one of the original propositions for the establishment of tolerance. In the same line of thought as the concepts behind the particular characteristics of RTE, recent reports have identified other developmental determined phenotypes of cell intrinsic nature. One report has shown that neonatal RTEs are more responsive than adult RTEs in respect to Th1/Th2 cytokine secretion and proliferative response to IL-7 alone (90). On the other hand, it has been shown that the susceptibility of thymocytes to upregulate Foxp3 decreases with age (91). This is a finding closely related to the concepts addressed in the following chapters of this thesis.

The classical role of the thymus, in producing correctly selected functional T cells, and the less commonly studied contribution of RTE export, need to be fully clarified so that the thymic activity may one day be harnessed for clinical applications. The clarification of the intrathymic antigenic landscape and its interconnection with the peripheral sources of antigen has great potential to vaccine development, be it to generate protective immunity or to establish tolerance. Still on the thymus role, understanding the particular characteristics of nascent T cells is an important missing link to understand immune responses at young age. At the same time, it is possible that some RTE specific properties may open opportunities for new and more efficient therapeutic applications.

1.3 Regulatory T cells: Differentiation and Function

T cells have a central role not only in orchestrating adaptive immune responses but also as its regulators. While T cells can differentiate to several different phenotypes that direct the immune response they can also adopt immunoregulatory transcriptional programs that limit immune responses and maintain immune homeostasis. Several T cells with regulatory properties exist, such as Tr1 (92) and Th3 (93), and even Th17 when differentiated in particular microenvironment (94, 95). However, the T cell subset that has been most studied and shown to be essential for immune self tolerance is the Foxp3 expressing regulatory T cells. As mentioned above, the work presented in this thesis is focused on the study of the later subset, and as such, they are the focus of this introductory section.

The research focus on Treg has mainly been concerned in accurately identifying Treg, their differentiation mechanisms and functions. The vast amount of knowledge harnessed, mainly with the use of the mouse as a model organism, have now taken the field to invest in exploring potential clinical applications for Treg. However, Treg differentiation, function and physiology in general are still not fully understood. It is likely that key features of this T cell subset that may prove invaluable to guarantee the safety and efficiency of Treg based therapies, are still to be fully characterized.

As introduced in the first section of this chapter, following early indications of a thymus derived component for immune tolerance (29, 30), regulatory T cells were identified as Foxp3 expressing CD4 T cells (34, 35). The real frenzy in the regulatory T cell field started with the identification of the high affinity IL-2 receptor alpha chain (CD25) as a marker for regulatory T cells. In the seminal paper by Shimon Sakaguchi *et al.* (33), peripheral cells of a WT mouse, either unfractionated or after CD25 expressing cells being depleted, were transferred to nude recipient mice, which lack a thymus and

therefore all T cells. When recipients were analyzed 3 months later the authors found that depletion of $CD25^+$ cells from the transferred population resulted in multiorgan auto-immune attack. This effect could be prevented if $CD25^+$ cells were re-introduced into the mixture, clearly showing the regulatory properties of this subset. Since this was the smallest subset of T cells, 5-15 % of CD4 T cells, identified to date that presented this regulatory property, CD25 became the gold standard to identify and isolate regulatory T cells. With the possibility to isolate Treg and its conventional counterpart the field regained credibility lost due to past misguided directions and started to advance at a fast pace.

CD25 maintained its position as the best marker to identify Treg until 2003 when two independent groups identified the Foxp3 as the master transcription factor for Treg differentiation and function. This finding was fueled by the identification of mutations in the Foxp3 gene in the Scurfy mouse and in IPEX patients which develop severe multiorgan autoimmune disease at early age. Hori *et al* showed that forced expression of Foxp3 in conventional T cells would turn them regulatory while generating phenotypic characteristics identical to the ones found in isolated $CD25^+$ Treg (34). Simultaneously, Fontenot *et al.* generated a conditional Foxp3 KO and a similar ectopic expression system which resulted in similar findings (35). Moreover, CD25 expressing CD4 T cells express high amounts of Foxp3 while CD25 negative counterpart express very little, confirming the specificity of this transcription factor to identify Treg and endow them with regulatory properties. With this two works Foxp3 became the new marker to identify Treg.

However, it was known that activated T cells upregulate CD25 expression and this was taken as a possible source of confounding factors for the study of Treg. Despite the majority of $CD25^+$ cells isolated from naïve mice being $Foxp3^+$ (>90-95%), the potential for discrepancies between CD25 and Foxp3 expression called for new methods that allowed the use of Foxp3 as an

identification marker for Treg in standard cellular immunology assays. Since Foxp3 is a transcription factor it is found inside the cells and not in its surface. As a consequence, its labeling with antibodies requires the cells to be permeabilized, which impedes the isolation of live cells.

The strategy that was put in practice to allow for the isolation of live Foxp3 expressing cells was the generation of genetically modified mouse lines expressing fluorescent proteins together with Foxp3. Two reporter mice were developed in parallel. Fontenot *et al.* developed a GFP-Foxp3 fusion reporter while Wan *et al.* generated an IRES-mRFP reporter, both by knocking in the designed constructs into the Foxp3 gene (96, 97). These new tools allowed FACS sorting of live Foxp3 expressing cells, as well as their negative counterpart, to very high purity. With this new capacity, Treg experiments became cleaner and it was possible to clarify the doubts that had been casted upon Treg data based on the not so high purity of Foxp3 populations, isolated through CD25 discrimination. While, the use of genetic based reporter systems have failed to refute any claim made from the work performed using the CD25 as a marker to isolate Treg, the high purity of cell populations fractionated according to Foxp3 expression has allowed the execution of experiments for which results using CD25 would have been less clear or even un-interpretable. The development of fluorescent based Foxp3 expression reporters set the road to the actual state of the art in Treg identification and isolation.

One of the fundamental questions about Treg is their differentiation mechanism(s). Soon after the identification of CD25 as a marker to identify and isolate Treg (33), it was described that $CD25^+$ cells could be found in the thymus within the CD4 SP subset (98). At the same time, the fact that, if Treg are subtracted from a peripheral T cell pool that is transferred to syngenic T lymphopenic recipients, an uncontrolled response takes place (33), strongly undermined the notion that Treg could differentiate from non Treg cells after thymic export. Added up with the original observations of a

thymic derived tolerance, the paradigm of an exclusive thymic origin for Tregs settled in.

With the experimental confirmation that Treg originated in the thymus from developing thymocytes, two models regarding the mechanism by which Foxp3 expression is induced dominated the conceptual framework. One hypothesis was, or is, that some thymocytes randomly turn on the Treg genetic program somewhere between positive and negative selection and later undergo TCR based selection to confirm the Treg fate (99). This is the so called stochastic model. Alternatively, the instructive model proposes that the TCR reactivity is the inducing signal that turns Foxp3 expression on. In this case clones receiving TCR signals close to negative selection but below the threshold of deletion would be diverted to the Treg lineage (100).

The collection of data found in the literature indicates that the instructive model is likely to be the correct one. First, the identification of $Foxp3^+$ cells in monoclonal CD4 derived TCR Tg mice requires the concomitant presence of the Ag in the thymus (101-103). In its absence Foxp3 is not expressed nor a Treg phenotype observed. If the mice are not in a Rag deficient background Treg do appear irrespective of TCR specificity (104). Work with TCR Tg mice in a Rag competent setup may have blurred the conclusion for the need of the cognate antigen, mainly by giving the impression that Treg may develop in its absence. The presence of Treg in these conditions seemed to indicate a stochastic/elective model of Treg differentiation. However, it was later clarified that Foxp3 is exclusively expressed in clones that rearrange the endogenous alpha chain that pairs with the beta chain from the transgenic TCR, therefore not recognizing the same antigen as the full TCR Tg receptor but rather other antigen present in the thymus (105). Furthermore, the fact that anti-nonself TCR Tg T cells, Rag competent but Foxp3 deficient, eventually develop multiorgan autoimmune disease that parallels the one observed in scurfy mice, whereas the Rag deficient do not, has reinforced the notion that the Treg differentiated in Rag competent TCR Tg systems are

indeed recognizing a self antigen (106, 107). This collection of data strengthened the notion of the antigen being required in the thymus for Treg differentiate and supported the view of a mostly anti-self Treg repertoire.

Nevertheless, a more concrete confirmation came only more recently with the use of Treg derived TCR Tg mouse lines (108-110). To evaluate the Treg differentiation dynamics several groups set to develop TCR Tg mice in which the TCR had been extracted from a Treg. Given that the early body of work on this issue clearly indicated that Treg are mainly self reactive, selected in the thymus and with high affinity/avidity to antigens present there, it was predicted that Treg would readily be identified in the thymus of such TCR Tg mice. Two main outcomes were expected, either Treg would develop at very high frequencies, as it is often observed in TCR Tg mice that also express the cognate Ag in the thymus through a second Tg, or they would be limited to frequencies similar to what Is observed in WT mice. However, for the utmost surprise, monoclonal TCR Tg lines displayed very little Treg in the thymus and peripheral lymphoid organs (108-110). To test for possible effects of the TCR Tg authors performed BM chimeras with WT mice and intrathymic transfer of thymocytes to WT recipients. In these conditions, Treg promptly developed. Moreover, through titrated combinations of WT and TCR Tg progenitors it was observed that the frequency of $Foxp3^+$ cells within the TCR Tg cells was inversely correlated with the frequency of TCR Tg cells within the total population. Basically, the less TCR Tg cells in the total thymocytes pool, the higher the frequency of $Foxp3^+$ cells differentiated, reaching up to almost 100%. Actually, each group developed several TCR Tg lines and found differences in the kinetics of $Foxp3^+$ enrichment showing that TCR specificity imposes a quantitative limit on each clone's representation in the Treg pool. This limit is likely to be a product of the strength of TCR affinity and the amount of available antigen, but a contribution of other factors to determining this niche has not been excluded. Despite these modern approaches, there were older reports

already indicating a limited niche for Treg differentiation for a particular TCR (111). In this work it was described that increasing the amount of intrathymic cognate antigen for a particular TCR Tg led to an increase of Foxp3$^+$ cells but just to a certain extent, and that after hitting a plateau of a maximum number, there was only an increase in frequency as a result of increased deletion. In summary, these findings established that there are limited niches for Treg differentiation.

The reports mentioned above regarding the requirement of Ag to obtain Treg clearly indicate that Treg are anti-self. Another classical experimental system contributing to this notion was the anti MBP TCR Tg mice. When in a RAG deficient background T cells direct an immune response against the central nervous system and the mice develop EAE (112). However, when in a Rag competent background disease does develop (105). The later work showed that Rag expression allowed the rearrangement of the endogenous TCR alpha chain that in some instances could pair with the transgenic TCR beta chain and lead to new specificities that included Treg. More importantly this work was also among the first to demonstrate that Treg can regulate T cells known beforehand to be specific for a self Ag, and that its absence can lead to TCR targeted autoimmunity (105).

In summary, the fact that Treg differentiate in the thymus upon interaction with cognate antigen sealed the long sought after demonstration for dominant tolerance orchestrated by self recognition in T cell differentiation and function (100). At the same time, data like the one produced in the study of Treg derived TCR Tg also demonstrated that TCR specificities that can incorporate the Treg pool, even if doing so close to 100% in a given condition, also have a chance to escape both, the incorporation of the Treg pool and elimination by deletion. This apparently depends on the functional clonal size of its specificity and the competition for a limited niche for Treg differentiation. Treg guarantees immunological tolerance by the recognition of self antigen to control immune responses, and therefore does so by

assertion, in contrast, deletion is a mechanism that achieves tolerance by ignorance, a required but insufficient mechanism.

A later turnaround to the established concept of exclusively thymus derived Treg origin was the demonstration by Wanjun Chen and colleagues that $CD25^-$ T cells isolated from the peripheral lymphoid organs of adult mice could be induced to upregulate CD25 and gain regulatory activity through the action of TCR stimulation in the presence of TGFβ (113). The potential of Tconv cells to upregulate Foxp3 after thymic export represented a serious shake of the pillars of thymic Treg fundamentalists. This mechanism opened the potential to differentiate Treg to exogenous antigens with the inherent potential risk of tolerance induction against pathogens or even full immune paralysis. Irrespective of such theoretical worries the field rapidly progressed to demonstrate several protocols by which it was possible to induce the differentiation of Treg in the periphery.

The differentiation of Treg from $CD45RB^{hi}$ $CD25^-$ CD4 T cells isolated from the periphery of naïve WT mice was rapidly shown to also take place *in vivo* upon adoptive transfer to lymphopenic hosts and involving upregulation of Foxp3 expression (114). The conversion of polyclonal WT naïve T cells to Treg was later also shown to take place in lymphoreplete recipients (115). Apostolou and von Boehmer reported that slow delivery of low amounts of antigen, through the use of an implanted osmotic pump, also lead to Treg and tolerance (116). This was shown to be accompanied by the induction of a regulatory T cell phenotype in TCR Tg recipients, euthymic or thymectomized, as well as in adoptively transferred monoclonal TCR transgenic T cells recognizing the infused antigen. Tolerance was also achieved in implanted WT Balb/c mice. Treg generated through Ag delivered by the pump were capable of controlling *in vitro* proliferation, *in vivo* recall responses to the infused antigen, and diabetes progression in a system of insulin driven Ag Tg plus TCR Tg T cell transfer. Moreover, this work showed that increasing the amount of antigen delivered led to an inhibition of Treg

differentiation, which indicates that the Ag as to be presented in a sub immunogenic condition and that TCR signaling for peripheral Treg induction is likely to follow its own rules when in comparison to the high stimulation required for thymic Treg differentiation. Cobbold *et al.* demonstrated that anti-CD4 blockade together with antigen stimulation could lead to Treg induction. The authors also showed that tolerance to skin grafts achieved through anti-CD4 administration was dependent on TGFβ and led to long lasting Foxp3 expressing CD4 Treg that accumulated in the grafted tissue (117). The induction of CD4 T cell mediated dominant tolerance through manipulation of co-stimulation was later extended to antibodies against other molecules, such as CD154 (118), CD45RB (119) and others (120). Mucida *et al*. showed that oral delivery of Ag led to the differentiation of Treg in a TCR-BCR double Tg mouse line, which are devoid of Treg if left unmanipulated (121). Developed Treg suppressed asthmatic reactions, as measured by cell number in bronchoalveolarlavage and immunoglobulin levels in sera, as well as the differentiation of effector/memory T cells upon i.p. immunization. In this setting tolerance induction and Foxp3 expression were shown to be dependent on TGFβ but independent of IL-10. Kretschmer *et al.* reported that Ag targeting to DCs leads to Treg differentiation (122). In their system, adoptive transfer of monoclonal TCR Tg T cells reactive to hemagglutinin A (HA) to lymphoreplete recipients together with HA coupled to anti-DEC antibody administration led to *de novo* generation of Foxp3 regulatory T cells. Treg generation was more efficient when low dose antigen was administered and no DC maturation signal was delivered. Once again Treg induction was demonstrated to be TGFβ dependent. Surprisingly, if TCR Tg T cells could not express IL-2 there was an enrichment of Foxp3 expressing cells in comparison to the IL2 competent counterpart. This collection of methods represents the main mechanisms described so far for the induction of regulatory T cells from T cells harvested from secondary lymphoid organs, that is, from cells that after thymic selection and maturation were not part of the Treg pool.

Notably, experimental evidence that peripheral cells could be recruited to the Treg pool had been produced earlier (123). In particular, a study by Modigliani and colleagues indicated that RTE are licensed to be “educated” into the Treg pool by pre-existing Treg. This evidence was nevertheless of indirect nature as this conclusion was inferred from observing that the timing of T cell reconstitution was inversely correlated with the capacity to induce T cell based regulation on newly formed cells, without Treg being directly addressed. Based on this finding and other reports indicating that T cells could be tolerized in the periphery, it was proposed that Treg differentiation in the periphery could be a particular case of the Lederberg model in which recently made T cells would have a maturation determined window of opportunity to be influenced to become Treg by other Treg (100).

The fact that Treg can differentiate from both the thymus and periphery provides a reconciliatory explanation for initial studies where Treg TCR repertoire was shown to display some degree of overlap with Tconv repertoire, and even TCRs that could equally well recognize exogenous antigens (124, 125). It is nowadays clear that the peripheral Treg may contain Treg that recognize non self antigens and the Tconv pool may recognize self. These antagonistic reactivities are maintained at equilibrium where pathological autoimmunity is a rather rare event and immune responsiveness against invading pathogens the norm.

Regardless of the many experimental systems already described that lead to Foxp3 expression in peripheral T cells it is not possible to accurately determine the contribution of peripherally differentiated Treg to the pool of Treg found at steady state in healthy unmanipulated hosts. Two main angles have been taken in Treg research addressing this shortcoming. First the study of the rules determining both thymic and peripheral Treg induction, and second, the comparison of Treg found at steady state in naïve mice with Treg differentiated from peripheral Tconv cells. Studying Treg differentiated by different methods or harvested from different organs, searching for ways

to discriminate extrathymic from intrathymic Treg has mainly brought about a notion of Treg heterogeneity. Furthermore, the several studies indicating differences between Treg of thymic and peripheral origin have been shown to be true only for particular settings and to not apply to iTreg induced by some of the methods. Helios expression was one of such markers, expression of which was proposed as a potential marker to identify Treg originating in the thymus. However, Treg differentiated from naïve T cells only lack Helios expression when obtained through the classical *in vitro* protocol while they do upregulate Helios if differentiated *in vivo* (126, 127). Apart from this false positive, other markers have only been found to be partially enriched in one or the other subset. Thus the unequivocal identification of Treg origin in naïve mice is still not possible. In general, iTreg present the prototypical Treg surface phenotype, expressing high levels of CD25, CTLA4 and GITR (113, 114). Other molecules such as Helios, KLRG and Nrp-1 are up or down regulated in different conditions and while sometimes discriminating iTreg from nTreg its expression level does not offer a valid prediction for iTreg differentiated in all conditions.

On the other hand it is becoming clear that Treg have the potential to further adopt specific phenotypes according to its origin or conditioning. The potential heterogeneity of Treg was well captured in a work by Feuerer *et al.* where Treg from the thymus or periphery of WT naïve mice were compared to Treg differentiated from naïve T cells *in vitro* and *in vivo* through several protocols. This report identified a core Treg transcriptional profile and several additional genes upregulated or downregulated in Treg from several of the conditions studied (128).

Studies addressing the mechanisms ruling Treg differentiation have identified T cell activation, IL-2 and TGFβ as the main factors that lead to Foxp3 expression and the ensuing regulatory activity in T cells. The

molecular details of the requirement for these signals and the pathways that are activated or inhibited leading to Foxp3 expression have been extensively characterized.

There are several proofs that indicate TCR signaling is essential for Treg differentiation. First, the requirement for Antigen presence in TCR Tg mouse lines for the differentiation of Tregs, as already mentioned above, offers the most striking evidence (101-103). More recently, Moran *et al.* using a reporter for Nur77, which expression levels follow the intensity of TCR signaling, have shown that Treg display signs of having suffered higher TCR stimulation than positively select Tconv cells, as had been postulated and indicated by TCR Tg systems (129), again reinforcing the requirement for Ag recognition with high affinity/avidity. On the other hand, B7/CD28 co-stimulation has also been reported has being essential for the differentiation of Treg (130, 131). Thus, T cell activation through the TCR together with co-stimulatory signals is essential for the induction of Foxp3 expression.

TCR and CD28 induce several downstream pathways that have been shown essential for Treg differentiation, the most notable being the NF-κB pathway. Regarding Treg differentiation, T cell activation delivers essential signals through PKC-θ, CARMA1, Bcl-10 and Malt-1 which eventually lead to c-Rel activation and its translocation to the nucleus (132-136). CARMA-1, Bcl-10 and Malt-1 are denominated the CBL complex and have been shown to be involved in the expression of Foxp3. In a study to elucidate the role of this complex, Molinero *et al.* found that CARMA1 is absolutely required for the expression of Foxp3 (132). Using a CARMA1 KO mouse line they showed that the inability to signal through the CBL complex led to a cell intrinsic defect in Treg generation. This defect was not rescued by the expression of a STAT5 constitutively active transgene, showing that the defect was not linked to the lack of IL-2 signaling which is also caused by the defect in the CBL complex.

C-Rel is a member of the NF-kB family that is involved in the response to T cell activation. Ruan *et al.* and Long *et al.* reported that c-Rel deficient mice have dramatically reduced Treg, both in the thymus and the periphery (137, 138). It was also reported that the Treg deficiency in c-Rel KO mice is a cell intrinsic defect and that it can also be detected in TGFβ mediated Foxp3 induction in naïve T cells *in vitro*. Long et al. focused in demonstrating that artificially increasing NF-kB activity rescued the Treg deficiency in other mutant mouse lines in pathways involved in NF-kB activation, showing that NF-kB activity through c-Rel was indeed the essential signal driving Foxp3 expression. On the other hand, Ruan et al. extensively characterized the thymic and *in vitro* Treg differentiation impairment in c-Rel deficient mice, which occurs in a cell intrinsic manner. In this last report the authors also demonstrated the assembly of a "c-Rel enhaceossome" composed of c-Rel, p65, NFAT, Smad and CREB in the Foxp3 promoter and enhancer regions. Visekruna *et al.* revealed that c-Rel was also relevant in Treg differentiation through the regulation of IL-2 production, a cell extrinsic effect having impact on the induction of Foxp3 in naïve T cells (139). Last Deenick *et al.* confirmed that c-Rel and not NF-kB1 (p50) was the main NF-kB effector molecule leading to Foxp3 expression (140). These studies established that c-Rel translocation is an essential, non redundant, mechanism leading to Foxp3 expression.

T cell activation for the induction of Foxp3 expression requires not only TCR signaling but also co-stimulation. T cells need to receive signals through CD28 to activate the NFAT-AP-1 pathway. It has been shown that NFAT cooperates with Smad3 to induce Foxp3 induction in peripheral T cells (141). These factors bind to a conserved nucleotide sequence in an enhancer close to Foxp3 and promote epigenetic changes that allow Foxp3 expression.

As an apparent exception, it has been reported that $CD4^+CD25^+GITR^+Foxp3^-$ peripheral T cells and thymocytes become $Foxp3^+$

under the sole action of cytokines (142-144). However, it was shown that the cells had to get the activation signal to get this Treg permissive phenotype which then allows the action of cytokines, mainly IL-2, to induce Foxp3 expression. This proposed mechanism implies a two step model for Treg induction, in which cells are first activated and later a cytokine signal directing them to the Treg lineage (145).

The other essential factor for Treg induction, as for any other T cell polarization mechanism, is the milieu surrounding the T cells receiving the TCR activation signals introduced above. The main cytokines that lead to Foxp3 expression are TGFβ and IL-2, with retinoic acid enhancing the process. The essential role of TGFβ in immune homeostasis was clearly demonstrated by several transgenic mouse models tampering with the ability of cells to sense TGFβ signals (146). Regarding Treg differentiation, the fact that TGFβ signaling deficient mouse models still display Treg in the thymus led to the notion that TGFβ was not required for thymic Treg differentiation (147). However, later work demonstrated that IL-2 was amplifying a very small proportion of Treg that differentiated when TGFβ signaling was abolished (148). For extrathymic Treg, it is clear that TGFβ is absolutely required. Molecularly the main mediators of TGFβ signaling are SMAD2 and SMAD3, which have some degree of redundancy so that Treg differentiation is only prevented by the absence of both (149). This and other works have shown that TGFβ is a central molecule allowing the expression of Foxp3.

The other essential factor is IL-2. IL-2 and IL-2Rα deficient mice suffer from T cell mediated autoimmune disease, but, both mouse models display thymic Treg (150). However, the concomitant abolishment of IL-2Rα and the common gamma chain cytokine receptor leads to a dramatic reduction of Treg (143, 150), indicating a redundant role of other cytokines signaling through the common gamma chain. In a study following the requirement for common gamma chain receptor dependent signaling it was found that IL-

2Rβ and IL-7Rα double KO mice present a nearly complete absence of thymic Foxp3$^+$ cells demonstrating that IL-7 is the major redundant factor leading to Foxp3 expression in the absence of IL-2 signaling (151). The requirement of common gamma chain cytokines is translated through the requirement of STAT5 signaling, of which, regarding Treg differentiation, IL-2 seems to be the more potent inducer (143). In contrast to thymic Treg differentiation, *in vitro* induction of Foxp3 by the action of T cell activation and TGFβ is strictly dependent on IL-2 and other common gamma chain binding cytokines, such as IL-7 and IL-15 cannot rescue IL-2 signaling deficiency (152). The fact that IL-7 and IL-15 cannot compensate for the lack of IL-2 is not clear but indicates that a pathway other than STAT5 phosphorilation is triggered by IL-2.

On the opposite side, the cellular milieu can also convey inhibitory signals that prevent Foxp3 induction in the presence of TGFβ and IL-2. The cytokines with stronger inhibitory effect known to date are IL-6 and IL-4. When T cells are activated in the presence of IL-6 and TGFβ the cells do not upregulate Foxp3 expression and are instead polarized to an IL-17 secreting (153). IL-6 induces STAT3 and reinforces expression of IL-21 and IL-23R, which, in the presence of TGFβ, lead to RORγt expression and the establishment of a Th17 phenotype (154). In turn, IL-4 together with TGFβ lead to an IL-9 secreting helper T cell pathway, again preventing Foxp3 expression (155, 156). This pathway has been recently described and is yet ill characterized. Inhibition of Treg differentiation from naïve T cells can also be caused by activated/memory T cells which readily secrete pro-inflammatory cytokines upon activation. In this scenario, retinoic acid has been reported to relieve naïve T cells from this inhibitory effect. Retinoic acid and TGFβ enrichment in the gastrointestinal tract seem to be accountable for the increased Treg differentiation that has been reported to be associated with that anatomical location as well as being another factor in the TGFβ determined Treg-Th17 crossroad (157-159).

Apart from the essential T cell stimulation and favorable cytokine milieu required for Treg differentiation there have been other pathways not traditionally related with T cell polarization that have been implicated. In particular pathways originally linked with responses to different metabolic states such as the FoxO1 and mTOR. In summary, Treg differentiation is determined by the right intensity of T cell activation and the microenvironment in which the cells are laying.

Other important aspect of Treg physiology is the maintenance of the suppressor phenotype and the potential sub-phenotypes required for Treg activity in different scenarios. T cell polarization as a terminal differentiation process has been recently disputed and several pieces of evidence have led to a concept of phenotypic plasticity, a novel concept in the T cell field. For helper T cells, this plasticity has been found between Th17 - Th1 and Th2 - Th9 phenotypes. In the case of Treg, plasticity has been addressed through the possibility of Treg to lose Foxp3 expression and through the establishment of diverse gene expression programs specific for the conditions in which Treg differentiate or are later embedded.

Early work by Tung *et al.* had shown that T cell based self tolerance is only maintained if the antigens remain present (160). This was demonstrated by testing tolerance of female mice to ovaries after having been or not ovariectomized. The experiments performed in this work revealed that 3-7 days after the organ is removed the immune system looses the capacity to tolerate the same organ from a naïve donor once it is re-implanted. While the fate of the cells responsible for tolerance in this system was not addressed, it was clearly demonstrated the need for the presence of the antigen for the maintenance of tolerance. In light of the actual knowledge about antigen specific immune tolerance, Treg are prime candidates to mediate this phenomenon, which implies Treg require continuous TCR signaling to be maintained or to maintain their regulatory phenotype.

More recently it has been shown that Treg can lose Foxp3 expression, while surviving and becoming T cells with pathogenic potential (161-163). Given the enrichment in self reactivities of high affinity/avidity in the Treg pool, the evaluation of the potential harm resulting in autoimmune targeting from the exchange of the regulatory activity for an effector one is rather straightforward. Besides the requirement for TCR signaling, these works have established IL-2 and TGFβ signaling as another requirement for Treg to maintain Foxp3 expression and its regulatory activity.

Using a Foxp3 driven lineage tracer Zhou *et al.* reported that in a naïve mouse a small population of cells that have ever expressed Foxp3 downregulate its expression (163). Similarly in this work and in the report by Duarte *et al.* it was also established that Treg loose Foxp3 expression when transferred to lymphopenic recipients (161, 163). The later report described that the Treg population transferred tends to be divided in half of Treg and Tconv after 4 weeks in this system. The loss of Foxp3 expression may be avoided if Tconv cells are co-transferred or if exogenous IL-2 is provided. These results are in accordance to earlier works showing that the amount of Treg is indexed to the amount of naïve T cells, likely through the IL-2 secreted by the non regulatory T cells (82). In parallel Komatsu *et al.* showed that loss of Foxp3 expression could also be prevented by sustained TGFβ signaling (162). Curiously, the main signals that lead to the maintenance of the Foxp3 expression in Treg are also the crucial ones required for its induction.

The other facet of Treg plasticity, that is, the adoption of different phenotypes according to the milieu they are inserted in, was mainly explored using genetically modified mouse models where Treg were made deficient in several transcription factors known to be relevant for Tconv cell phenotypes. These include T-bet, IRF4, and Stat3 (164-166). What these works have established is that Treg need to turn on a transcriptional program dependent on the same transcription factors that control Tconv polarization.

Accordingly, mice in which Treg are deficient in T-bet develop uncontrolled Th1 responses (164), if deficient in IRF-4 a Th2 response (165) and if Treg are deficient in Stat3 a Th17 response (166). While this is a very recent concept, the evidence produced so far clearly supports the notion that Treg heterogeneity extends beyond the initial differentiation pathways leading to Foxp3 expression.

Treg encompass 5-10% of peripheral T cells but provide a powerful and diverse collection of regulatory activities. The strongest demonstrations of the essential role of Treg are the autoimmune conditions that develop in both mice and humans harboring non-functional Foxp3 alleles (167-169). While original works about T cell based tolerance addressed mainly reactivity against non self antigens, when Treg were first identified they were readily shown to be essential for immunological self-tolerance (33). Nevertheless, older models of tolerance were readily revisited applying new tools that allowed the demonstration of a central role for Treg in many different scenarios.

In the scope of regulating anti-self responses, Treg were shown to maintain self reactive T cell clones in check preventing systemic autoimmunity, but also to be involved in controlling responses targeted at specific organs, such as the pancreas in mouse models of Type I Diabetes (130). However, Treg are also able to regulate T cell responses to non self antigens. In physiological conditions and in the absence of external manipulations, they are responsible to control the immune activity against commensal microorganisms or innocuous environmental antigens at mucosal interfaces, such as the intestine and lungs (170-172). On the other hand, manipulations leading to Treg differentiation can lead to tolerance against allogeneic grafts or prevention of allergic reactions (33, 117).

Treg are also able to regulate responses in scenarios where the target is composed of both self and non-self antigens. Good examples of this

condition are the control of responses against the fetus in pregnancy (where maternal and paternal antigens are both present) and responses against neo-self antigens expressed by tumors (where new antigens may be present but are embedded in a collection of self antigens shared with healthy tissues) (173, 174).

Another important role of Treg is the setting of T cell homeostatic states, such as controlling the extent of immune responses and immune pathology caused by exacerbated immune responses (175), or simply control the size of the peripheral pool of activated/memory CD4 T cells (65). More important for the work presented in the later chapters is the known ability of Treg to control the expansion of naïve T cells in lymphopenic recipients. Transfer of naïve T cells into T and B cell deficient recipients leads to inflammatory bowel disease which can be prevented by co-transfer of Treg (176).

The study of Treg regulation in the several contexts presented above has demonstrated that Treg exert their regulatory activity through cell contact dependent and independent mechanisms. The cell contact dependent mechanisms entail the engagement of surface molecules, such as CTLA-4, ICOS (177) and CD103, as well as manipulation of soluble factors, of which TGFβ, IL-10 and IL-2 are the best studied. CTLA-4 delivers inhibitory signals by engaging CD80/CD86 on APCs while at the same time outcompeting CD28, which conveys the opposite effect. Mice with Treg specific deletion of CTLA-4 develop a lymphoproliferative multiorgan autoimmune disorder without displaying major abnormalities in thymic Treg differentiation (178). This last finding demonstrates that CTLA-4 is essential for Treg activity but not for Treg differentiation. Notably, CTLA-4 expression is itself upregulated by Foxp3. It is worth mentioning that Foxp3 is not only required for Treg differentiation but its sustained expression is essential for Treg activity (179). On the other hand, ICOS (177) and CD103 (180) endow Treg with particular adhesion properties that allow proper cellular interactions and migration, which are essential for regulation to take place. Treg can also directly kill

target cells through the action of granzyme B or perforin thus preventing those cells from participating in immune responses (181, 182).

The humoral side of Treg regulation has been best characterized in terms of Treg production of TGFβ and IL-10, as well as the consumption of IL-2. The first two are hallmark cytokines that dampen immune reactions or direct them to reactions that have lower intrinsic host damage potential (183). On the other hand, IL-2 is involved in T cell proliferation and activation and its consumption by Treg limits the amplitude of T cell reactions (184). Other mechanisms of Treg based suppression have also been reported, such as the induction of IDO expression in APCs leading to the degradation of tryptophan and the generation of an immunosuppressive milieu (185) and their participation in the of the CD39-CD73 extracellular enzymatic reaction that leads to the immunosuppressive mediator adenosine (186).

Of the above mediators of Treg activity, CTLA-4, TGFβ and IL-2 can be considered core mechanisms, given that mice with deficiencies in each pathway lead to pathological conditions that parallel the ones observed in Foxp3 deficient mice (124, 146, 178, 187). In spite of the diverse set of mechanisms already found to be used by Treg, it is likely that more pathways are yet to be unraveled, especially given the diverse set of immune activities that Treg have been reported to regulate.

The intense interest that Treg have earned comes from the indispensable role they play in immune tolerance and in the regulatory activity exerted in many different immune processes. Together with the shortcomings that still exist in the clinic to deal with most autoimmune diseases as well as allergic and asthmatic pathologies, Treg are expected to provide the solutions of the future in this area.

At the same time the mystery of immune tolerance is still alive and the mechanisms behind the way a healthy immune system is able to recognize and eliminate invading pathogens while sparing the host from this attack is

not fully understood. For this question to be answered and therapeutic applications to be developed it is required that the understanding of Treg differentiation and physiology to be further characterized. In this work we addressed intrathymic generation of Treg in the scope of the origin of antigen, extrathymic Treg in relation to the susceptibility of conventional T cells to become regulatory and the function and phenotype of this Treg generated outside the thymus. We believe these findings bring important notions that are relevant for both challenges mentioned above, the understanding of immune tolerance in health and for gathering knowledge that may help design safe and efficient therapies.

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Chapter 2

Intrathymic Differentiation of Adaptive Foxp3^{+} Regulatory T cells Upon Peripheral Proinflammatory Immunization

Chapter 2 Preliminary Notes

The author of the thesis designed, planed and performed the experiments presented in Figure 2.3 and participated in the preparation of the reply to the reviewers for publication. The main work was performed by Santiago Zeleney with the participation of the remaining authors. Analysis and interpretation of the results were divided by all authors.

Adapted from the work published in the Journal of Immunology:

Zelenay, S., M.L. Bergman, R.S. Paiva, A.C. Lino, A.C. Martins, J.H. Duarte, M.F. Moraes-Fontes, A.M. Bilate, J.J. Lafaille, and J. Demengeot. Cutting edge: Intrathymic differentiation of adaptive Foxp3+ regulatory T cells upon peripheral proinflammatory immunization. *J Immunol* 185:3829-3833.

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Abstract

Thymocytes differentiate into $CD4^+$ $Foxp3^+$ regulatory T cells (Treg) upon interaction between their TCR and peptide-MHC-II complexes locally expressed in the thymus. Conversion of naïve $CD4^+$ T cells into Treg can additionally take place in the periphery under non-inflammatory conditions of Ag encounter. Here, making use of TCR transgenic models naturally devoid of $Foxp3^+$ cells, we report *de novo* generation of Treg upon a single footpad injection of Ag mixed with a classic proinflammatory adjuvant. Abrupt Treg differentiation upon immunization occurred intrathymically and was essential for robust tolerance induction in a mouse model of spontaneous encephalomyelitis. This phenomenon could be attributed to a specific feature of thymocytes that, in contrast to mature peripheral $CD4^+$ T cells, were insensitive to the inhibitory effects of IL-6 on the induction of Foxp3 expression. Our findings uncover a pathway for Treg generation with major implications for immunity and tolerance induction.

Introduction

Thymocytes expressing TCRs specific for self-Ags presented in the thymus die through the process of negative selection (1) or differentiate into $Foxp3^+$ regulatory T cells (Treg) (2). Auto-reactive thymocytes escaping these selection events are exported to peripheral organs and tissues, where Treg control their activation and pathogenic potential (2). In addition to thymic production of 'natural' Treg, peripheral $CD4^+$ T cells can differentiate into 'adaptive' (or induced) $Foxp3^+$ cells in a TGF-β-dependent manner upon Ag encounter in absence of inflammation *in vivo* (3, 4). For example, peripheral Treg conversion has been observed following administration of free Ag by osmotic pumps (5), by the oral route (6) or by specifically targeting the Ag to dendritic cells (DC) in absence of adjuvants (7). Proinflammatory and/or effector Th cell cytokines, such as IL-6, IFN-γ or IL-4 inhibit peripheral Treg conversion and promote instead the differentiation into IL-17, IFN-γ or IL-4 producing effector Th cells (8-10).

Negative selection and Treg differentiation in the thymus are believed to be restricted to T cells interacting with Ags expressed locally, including many tissue-specific proteins ectopically expressed by medullary thymic epithelial cells (11, 12). However, recent studies indicate that abundant blood-borne Ags and peripheral Ag-loaded DC commonly reach the thymus (13, 14) and participate not only to negative selection but also to differentiation of $Foxp3^+$ cells (15). Yet, it is not known whether intrathymic differentiation of Treg occurs following peripheral administration of Ag mixed with standard adjuvants and whether this process is affected by ongoing inflammation. Clarifying these issues is essential to determine the full dynamic and outcome of immune responses to self and non-self peripheral Ags.

Here, using monoclonal TCR transgenic (Tg) mice naturally devoid of $Foxp3^+$ cells, we show that Ags mixed with CFA and administered by a single footpad injection can reach the thymus and locally promote the

differentiation of Ag-specific thymocytes into $Foxp3^+$ cells, despite the inflammatory conditions. This event could be attributed to a specific feature of thymocytes that, in contrast to peripheral mature $CD4^+$ T cells, lack surface IL-6-receptor expression and are thus refractory to the inhibitory effects of IL-6 on the differentiation of $Foxp3^+$ cells. Our findings reveal a previously unrecognized mechanism by which the thymus may establish dominant tolerance to self and non-self Ags during the course of an immune response.

Results and Discussion

Immunization promotes tolerance induction through differentiation of Treg

Anti-myelin basic protein (MBP) TCR-specific transgenic RAG-deficient mice (T/R^-) or homozygote for the Tg-TCR (TgTg) spontaneously develop severe progressive experimental autoimmune encephalomyelitis (EAE) by 2 months of age and succumb in their third month of life (17). As these mice are naturally devoid of $Foxp3^+$ cells, they offer an ideal system to test protocols that may induce the *de novo* generation of Treg. Administration of the nominal Ag together with IFA has been shown to prevent EAE occurrence in T/R^- mice, presumably through the induction of T cell anergy (18). We first tested whether immunization protocols known to induce various degrees of inflammation would differentially affect disease onset and progression in T/R^- mice. A single footpad injection of 100 μg of the agonist N-terminal Ac1-17 MBP peptide (pMBP) mixed with IFA or CFA protected T/R^- for at least 3 months, while administration of CFA alone did not alter the course of the disease (Fig. 2.1*A*). Protection from EAE associated with the emergence of $Foxp3^+$ cells, readily detectable in PBL as early as 6 days post-injection and undetectable in control animals (Fig. 2.1*B,C*). $Foxp3^+$ cells reached ~14% of $CD4^+$ T cells by 6 weeks post-immunization and remained at ≥ 4% for an additional 6-7 weeks. Full EAE protection lasted 3 months, after which immunized mice developed a chronic and mild disease that coincided with a significant decline in peripheral $Foxp3^+$ cell frequency. Renewed injection of CFA-pMBP 3 months after the first immunization prolonged protection to more than 7 months of age. As CFA is a more potent adjuvant than IFA, it was selected to further analyze the function and origin of $Foxp3^+$ cells generated upon proinflammatory immunization.

A

Genotype	Treatment	n	EAE incidence (%)	Survival (%)
T/R$^-$	None	>40	100	0
	CFA-PBS	4	100	0
	IFA-pMBP	6	0	100
	CFA-pMBP	24	0	100
	CFA-pMBP + PTX	6	100	0
TgTg	None	>40	100	0
	CFA-pMBP	4	0	100
TgTg^{scurfy}	CFA-pMBP	5	100	0

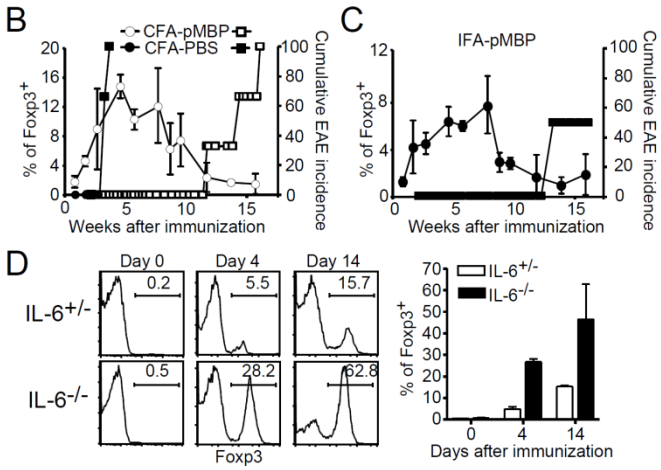

Figure 2.1. CFA-peptide immunization promotes tolerance mediated by *de novo* generated Foxp3$^+$ Treg. *A,* For each protocol, treatment of mice was initiated at one month of age and mice were scored for EAE at least twice per week, until three months of age. Data represent pooled results of 2 to 8 independent experiments. n refers to number of mice. *B,C,* 1-month-old T/R$^-$ mice were immunized with CFA-PBS, CFA-pMBP (*B*) or with IFA-pMBP (*C*). Percentage of Foxp3$^+$ cells in a CD4$^+$ cell gate in PBL (mean ± s.d, n=3 per group, circles) and cumulative EAE incidence (squares) along time. *D,* 1-month-old TgTg IL-6$^{+/-}$ or IL-6$^{-/-}$ mice were immunized with CFA-pMBP into the footpad and their draining LN analyzed 4 or 14 days later. Representative histogram for Foxp3 and percentage of Foxp3$^+$cells measured in a CD4$^+$ cell gate. n=2-6 in each group. Data are representative of two independent experiments.

Foxp3$^+$ cells emerging upon CFA-pMBP immunization were phenotypically and functionally *bona fide* Treg. About 60% of them expressed CD25, and most CD25$^+$ cells were Foxp3$^+$, GITR$^+$ and CD103$^+$. They efficiently

suppressed the proliferation of conventional $CD4^+$ $CD25^-$ cells *in vitro* and displayed regulatory activity *in vivo,* as shown by adoptive transfer experiments (Supplemental Fig. S2.1). Finally, immunization did not prevent nor delayed severe EAE in TgTg bearing a Foxp3 null mutation ($Foxp3^{scurfy}$) (Fig. 2.1*A*) formally demonstrating that dominant tolerance through *de novo* induction of $Foxp3^+$ Treg, and not T cell anergy (18), is the mechanism of disease prevention following immunization of T/R^- mice.

Concomitant differentiation of effector T helper cells and Treg upon proinflammatory immunization

The above observations needed to be reconciled with the notion that CFA is an adjuvant used to induce EAE in WT mice. Our immunization regimen promoted T cell expansion, activation and differentiation into IFN-γ and IL-17-producing cells (Supplemental Fig. S2.2), all readily detectable by day 4 post-injection and concomitant with $Foxp3^+$ cell emergence. Protocols for efficient induction of EAE in WT animals commonly rely on the coadministration of pertussis toxin (PTX) and the immunogen. Consistently, administration of PTX abrogated the protective effect of CFA-pMBP (Fig. 2.1*A*), amplified cellular expansion, increased the number of IFN-γ-producing cells and reduced both $Foxp3^+$ cell frequency and number (Supplemental Fig. S2.2). We conclude that while CFA-pMBP promotes Th1-, Th17- and Treg-differentiation, PTX inhibits the induction, migration and/or expansion of $Foxp3^+$ cells, a role reminiscent of its effect on Treg survival and function (19).

As expected, CFA administration also provoked rapid and vigorous production of innate cytokines including IL-6 (not shown). Intriguingly, this cytokine has been shown to play a key role in preventing peripheral Treg conversion, notably upon CFA administration (8). Consistent with this notion, immunized $IL\text{-}6^{-/-}$ TgTg mice, displayed a 3 to 5 fold increased frequency of

peripheral Foxp3$^+$ cells when compared to control IL-6$^{+/-}$ TgTg mice similarly treated (Fig. 2.1*D*). As unimmunized IL-6$^{-/-}$ and IL-6$^{+/-}$ TgTg mice were devoid of Foxp3$^+$ cells, we conclude, as previously shown (8), that inflammation driven by IL-6 interfered with Treg conversion. Collectively, our results suggest that a subset of T cells in IL-6-competent mice is insensitive to the inhibitory effect of IL-6 and consequently can convert to Treg despite ongoing inflammation.

Peripheral immunization induces intrathymic differentiation of Ag-specific Treg

We next examined whether Foxp3$^+$ cell differentiation in immunized T/R$^-$ mice occurred in the thymus. Remarkably, kinetic analysis in T/R$^-$ mice immunized with a single footpad injection of CFA-pMBP revealed that Foxp3$^+$ cells represented ~12% of CD4$^+$ single-positive T (CD4SP) cells in the thymus by day 3 post-immunization (Fig. 2.2*A*-*C*). Noteworthy, Foxp3$^+$ cells were not detectable in LNs before day 4 excluding the possibility that thymic Foxp3$^+$ cells represented circulating peripherally differentiated Treg (Fig. 2.2*A*). Despite an increasing frequency of thymic Treg during 7 days post-immunization, the total number of Foxp3$^+$ thymocytes was highest at day 3 and gradually decreased following the reduction in total CD4SP. In contrast, peripheral Foxp3$^+$ cell frequency increased at least until day 14 post-immunization (Fig. 2.2*B,C*). Intrathymic differentiation of Treg following footpad immunization was Ag-specific and not a singularity of anti-MBP Tg mice, or of small synthetic self-peptides, as similar results were obtained upon CFA-peptide or CFA-protein, but not CFA-PBS, administration to anti-OVA DO11.10 RAG1$^{-/-}$ TCR-Tg mice (Supplemental Fig. S2.3 and S2.4). Together, these results demonstrate that not only blood-borne (15), but also peripheral Ags administered subcutaneously in a stable

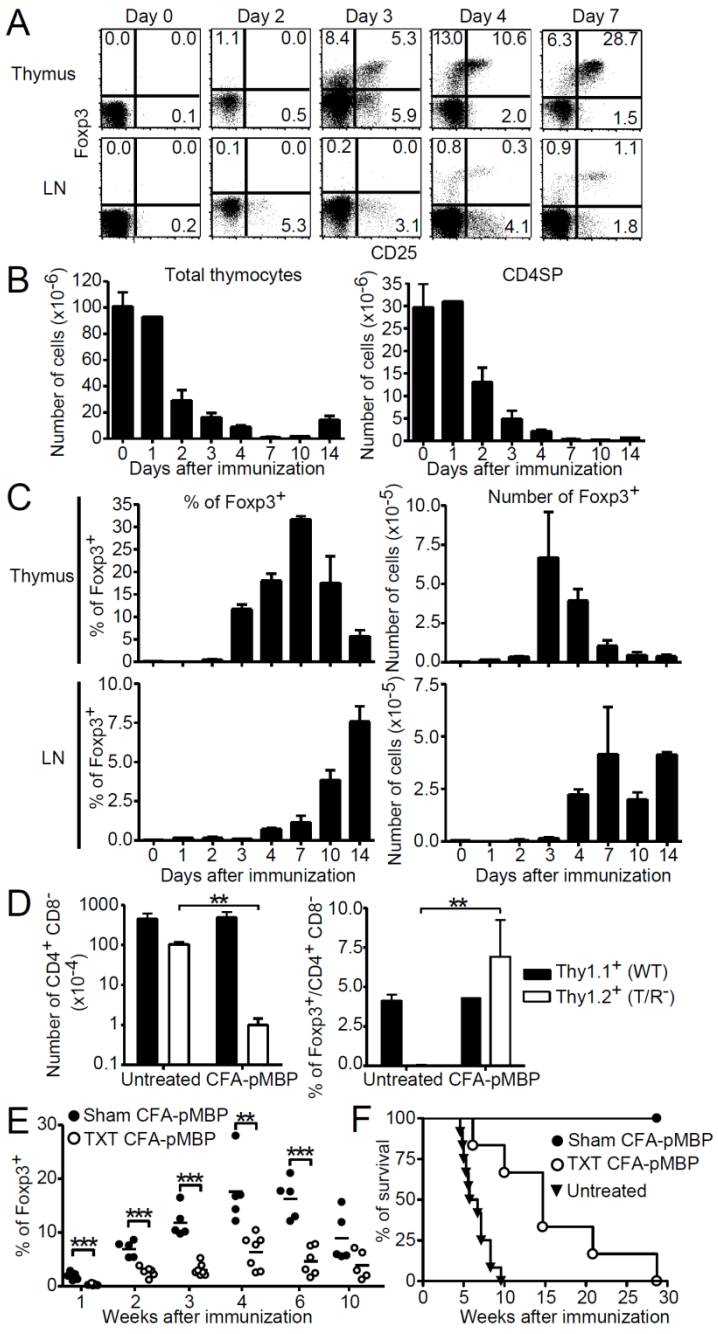

Figure 2.2. Peripheral proinflammatory immunization leads to abrupt Ag-specific Treg differentiation in the thymus. *A-C*, 1-month-old T/R$^-$ mice were immunized with CFA-pMBP into the footpad and thymus and LN were analyzed at different time points. *A*, Representative dot-plot for Foxp3 versus CD25 inside a

$CD4^+$ $CD8^-$ gate from the thymus (upper panel) or from LN (lower panel). *B,* Number of total thymocytes (left) and CD4SP thymocytes (right). *C*, Percentage of $Foxp3^+$ cells among CD4SP thymocytes or $CD4^+$ LN cells (left) and number of $Foxp3^+$ CD4SP thymocytes or $Foxp3^+$ $CD4^+$ LN cells (right). *B* and *C* are pooled kinetic analysis from five experiments with 2 to 12 mice in each time point. *D*, Lethally irradiated C57BL/10.PL-*Thy1.1* mice were reconstituted with a 9:1 mixture of BM cells from $Thy1.2^+$ T/R^- mice and $Thy1.1^+$ WT mice. Chimeric mice were untreated (n=3) or immunized into the footpad with CFA-pMBP (n=3) 2 months after reconstitution and analyzed 4 days later. Number of CD4SP thymocytes (left) and percentage of $Foxp3^+$ cells among CD4SP cells (right). Data are representative of three independent experiments, Student's t test. *E,F,* One group of 1-month-old euthymic T/R^- mice was left untreated (triangles, n=12) while both TXT (open circles, n=7) and sham-TXT (closed circles, n=5) were immunized with CFA-pMBP 3 days post-surgery. *E,* Percentage of $Foxp3^+$ cells in a $CD4^+$ cell gate from PBL over time, Student's t test. *F,* Percentage of survival over time ($p<0.01$: untreated versus TXT CFA-pMBP, and $p<0.01$: TXT CFA-pMBP versus Sham CFA-pMBP; logrank test).

water-in-oil emulsion, can enter the thymus and be presented locally to promote both deletion and Treg differentiation.

We next ascertained that Ag-specific Treg differentiate in the thymus following immunization of mice bearing a polyclonal repertoire of lymphocytes. BM from T/R^- ($Thy1.2^+$) and WT ($Thy1.1^+$) mice were co-injected into lethally irradiated WT ($Thy1.1^+$) animals. These mixed BM chimeras were immunized with CFA-pMBP at 2 months post-reconstitution when they contained 5-10% of Tg CD4SP cells. Analysis of their thymi 4 days post-immunization revealed both negative selection and Treg differentiation of Ag-specific CD4SP thymocytes, as indicated by a 100-fold reduction in number and the emergence of ~7% $Foxp3^+$ cells among $Thy1.2^+$ cells. Importantly, among the WT polyclonal $Thy1.1^+$ cells, neither thymocyte number nor $Foxp3^+$ cell frequency were affected by immunization (Fig.

2.2*D*). These results demonstrate that intrathymic Treg differentiation upon peripheral proinflammatory immunization is restricted to Ag-specific T cells and can occur in the context of a normal polyclonal repertoire of lymphocytes.

To directly evaluate the contribution of the thymus to tolerance induction and to the accumulation of peripheral Treg upon immunization, healthy 4-week-old T/R$^-$ mice were thymectomized (TXT) and immunized with CFA-pMBP 3 days later. Strikingly, Foxp3$^+$ cells were undetectable in peripheral blood of TXT mice for the first 2 weeks post-immunization. In addition, Treg frequency was significantly lower in TXT than in euthymic mice at any time point of the 10-week-long kinetic (Fig. 2.2*E*). These results are in agreement with our kinetic analysis above, indicating that early and efficient differentiation of Treg is restricted to the thymus. Nevertheless, immunization delayed disease progression in TXT mice, possibly as a result of peripheral generation of functional Foxp3$^+$ cells. However, while all TXT T/R$^-$ mice succumbed to EAE by 30 weeks after immunization, at this same time, euthymic animals were alive and only 40% of them showed signs of mild disease (score $\leq$ 2) (Fig. 2.2F). Together, our data demonstrate that the thymus, most likely due to intrathymic Treg differentiation, is essential for induction of potent and long lasting tolerance.

Immature CD4$^+$ single-positive thymocytes are refractory to IL-6-mediated inhibition of Foxp3 induction

Taken together the above results indicated that intrathymic Treg differentiation, in contrast to peripheral, is insensitive to the inhibitory effects of inflammation. We next tested whether these differences could be attributed to intrinsic features of developing T cells by performing *in vitro* Treg differentiation assays. As expected, addition of IL-6 to T/R$^-$ peripheral CD4$^+$ T cells inhibited by ~70% the generation of Foxp3$^+$ cells induced by

TGF-β (Fig. 2.3*A*). However, this inhibition was significantly lower (~40%) for CD4SP thymocytes. Similar results were obtained when testing WT $CD4^+$ $CD8^-$ $Foxp3^-$ cells isolated from $Foxp3^{GFP}$ knock-in mice (Fig. 2.3*B*,*C*). Strikingly, purified HSA^{high} $Foxp3^-$ CD4SP thymocytes, purged of recirculating and more mature cells (e.g. (16)), were totally refractory to the inhibitory effect of IL-6. The sensitivity of each cell subset to IL-6 directly correlated with the level of both surface IL-6Rα expression (Fig. 2.3*D*) and proximal IL-6R signalling, determined by intracellular phosphorylated-Stat3 detection upon IL-6 exposure (Fig. 2.3*E*). Together, these results indicate that newly formed T cells are insensitive to IL-6 mediated inhibition of Foxp3 induction due to their very limited responsiveness to this cytokine. These findings are in agreement with the notion that maturation stage is one key factor controlling the predispostion of T cells towards Treg differentiation (20) and provides a molecular basis to our observation that *de novo* generation of Treg takes place in the thymus rather than in the periphery upon proinflammatory immunization.

In conclusion, we show that a single footpad injection of Ags mixed with a highly inflammatory adjuvant promotes the *de novo* generation of protective Ag-specific Treg. This unexpected finding is explained with the demonstration that vigorous Treg differentiation happened intrathymically early post-immunization. The latter phenomenon occurred despite the elevated levels of inflammatory mediators in circulation, notably IL-6, due to, most likely, the specific resistance of newly formed T cells to the inhibitory effects of IL-6 on Treg differentiation.

An essential role of the thymus in ensuring induced peripheral tolerance has been occasionally reported, and mostly attributed to a property of recent thymic emigrants (e.g. (21)). In view of our findings, it is tempting to speculate that immature T cells exported to the periphery may be the

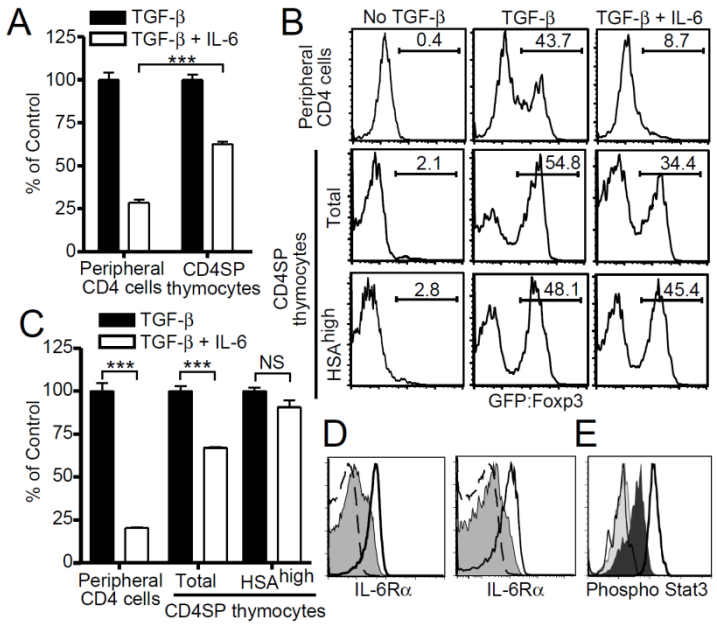

Figure 2.3. Immature CD4SP thymocytes are refractory to IL-6-mediated inhibition of Foxp3 induction. *A,* $CD4^+$ $CD8^-$ thymocytes or $CD4^+$ LN cells were isolated from T/R^- mice and cultured with plate-bound anti-CD3, anti-CD28, TGF-β ± IL-6. Percentage of $Foxp3^+$ cells with TGF-β + IL-6 relative to control with only TGF-β. *B,C,* $CD4^+$ GFP^- LN cells and either total $CD4^+$ $CD8^-$ GFP^- or HSA^{high} thymocytes were isolated from $Foxp3^{GFP}$ mice and cultured as in *A* plus IL-2. *B,* Representative histogram of GFP-expression in $CD4^+$ T cells. *C,* Percentage of GFP^+ cells with TGF-β + IL-6 relative to control with only TGF-β. Data are representative of three independent experiments. *D,* Representative histograms of surface IL-6Rα expression in gated $CD4^+$ GFP^- cells from $Foxp3^{GFP}$ mice. Left, $CD4^+$ LN cells (thick-solid histogram) compared to CD4SP thymocytes (grey-filled histogram). Right, immature HSA^{high} (grey-filled histogram) compared to mature HSA^{low} (thick-solid histogram) CD4SP thymocytes. In both cases, $CD4^+$ $CD8^+$ thymocytes serve as negative control (dashed histogram). *E,* Representative histograms of intracellular phospho-Stat3 staining in untreated or IL-6-stimulated $CD4^+$ GFP^- cells purified from $Foxp3^{GFP}$ mice. Untreated and IL-6-stimulated LN cells (thin-solid and thick-solid histograms, respectively) are compared to untreated and IL-6-stimulated immature HSA^{high} CD4SP thymocytes (light-grey and dark-grey filled histograms, respectively).

preferential precursors of peripherally differentiated Treg. A recent study reported increased Treg frequency in the thymus of non-Tg mice upon induction of EAE by immunization, a phenomenon associated with elevated intrathymic IL-7 expression (22). These thymic Treg allowed disease remission suggesting that they were immunogen specific. Together, this work and ours, indicate that intrathymic Treg differentiation upon peripheral immunization is a robust event, due to both microenvironment modifications and, as we demonstrate here, thymocyte-intrinsic properties.

Tolerance induction to peripheral Ags that gain access to the thymus may be essential not only to purge the repertoire of self-reactive T cells specific to Ags that are not expressed by thymic APCs (13) but also to broaden the repertoire of Foxp3$^+$ Treg assuring robust dominant tolerance to peripheral tissues. In addition, our evidence that intrathymic differentiation of Treg can take place under strong and systemic inflammatory conditions uncover a potential mechanism for the emergence of pathogen-specific Treg during infections, provided pathogen-derived Ags gain access to the thymus. Noteworthy, microbe-specific Treg have been shown to be essential for protection against a secondary challenge to the microbe (23). Thus, specific Treg differentiation upon Ag access to the thymus may play an essential role not only in the establishment of self-tolerance but also for immunity to reinfection. Finally, our observations have major implications for the design of vaccines for prevention or treatment of infectious diseases, cancer or autoimmunity.

Material and methods

Mice.

C57BL/10.PL, C57BL/10.PL-*Thy1.1*, C57BL/10.PL RAG1$^{-/-}$-MBP-TCR transgenic (referred to as T/R^{-}), C57BL/6, and Foxp3^{GFP} reporter knock-in mice were bred at the Instituto Gulbenkian de Ciência Animal Facility. Foxp3^{scurfy}, IL-6$^{-/-}$, IL-6$^{+/-}$, a WT homozygote anti-MBP TCR Tg C57BL/10.PL mice were bred at the Skirball Institute Central Animal Facility, New York University Medical Center. Mouse experimental protocols were approved by the Institutional Ethical Committee and the Portuguese Veterinary General Division. Foxp3^{GFP} knock-in mice (16) were kindly provided by A. Rudensky (U. Washington, Seattle, WA, USA).

Immunizations, EAE scoring and chimera generation

For immunizations, mice received 100 µl (50 µl in each foot pad) of peptide or protein emulsified in CFA (Difco readymade CFA). When indicated pertussis toxin (List Biological Laboratories) was administrated i.v. in two doses of 200 ng at a 1-day-interval. EAE was monitored at previously described (17). For adoptive transfer, purified cells suspended in 100 µl of PBS were injected into the retro-orbital plexus. For mixed bone marrow (BM) chimeras, recipient mice were lethally irradiated (900 rads) and reconstituted the following day with T-cell-depleted BM cells.

Cell purification and analysis

Cell suspensions from spleen, blood, thymus or LN (popliteal, inguinal, axilary, mesenteric and brachial) were incubated with a saturating amount of Fc-block (anti-CD16/CD32) before staining. Nuclear Foxp3 was detected following manufacturer (ebioscience) instructions. For intracellular cytokine

staining, cells were stimulated for 4h with PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml, Calbiochem). Brefeldin A (10 µg/ml, Sigma) was added for the last 2 h of stimulation. Data were acquired on a FACS Calibur or Aria (BD) and analyzed inside a lymphocyte gate with CellQuest (BD) and Flowjo (Tri Star Inc.) softwares. Cell purification was performed using Aria or MoFlo high speed cell sorters (Cytomation Inc.).

Cell culture

Foxp3-induction assays: cells were plated at $2.5x10^4$ cell/well in flat-bottom 96-well plates with 3 µg/ml plate-bound anti-CD3 mAb, 1 µg/ml soluble anti-CD28 mAb (e-bioscience), ~10 U/ml IL-2 (X63-IL-2 supernatant), 0.2 ng/ml TGF-β1 and 20 ng/ml IL-6 (R&D). Cultures were set in triplicates in a final volume of 200 µl for 72h. Stat3-phosphorylation assays: cells were stimulated for 15 min with 100 ng/ml IL-6 and stained with anti-phospho Stat3-AlexaFluor 647 Ab (4/P-Stat3, BD) following manufacturer instructions.

Statistical analysis

Statistical significance was determined using the two-tailed Student's t test and the logrank test. $P<0.05$ was considered significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Acknowledgements

This work was supported by the Fundação para a Ciência e Tecnologia, Portugal and the Programa Operacional Ciência e Inovação with the co-participation of the Fundo Comunitario Europeu (FEDER) and the EU-FP7–NAIMIT consortium. We thank R. M. Santos for antibody preparation, A. Perez and R. Gardner for operating the cell sorter and M. Rebelo for mouse colony management. We are most grateful to A. Coutinho and T. L. Carvalho for constructive discussions during the development of this work and to C. Reis e Sousa for critical reading of the manuscript.

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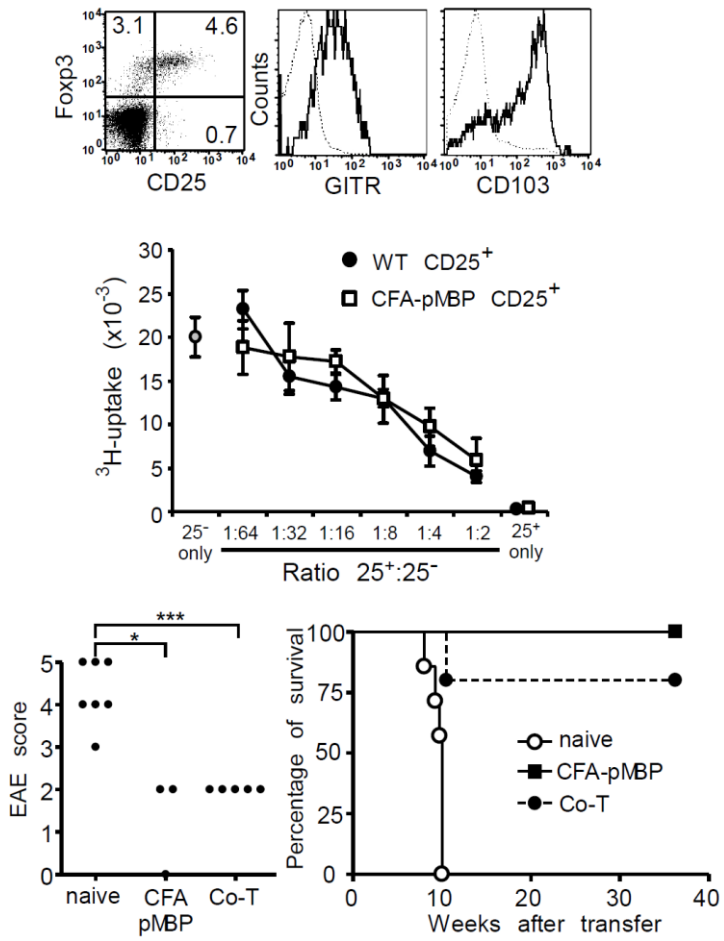
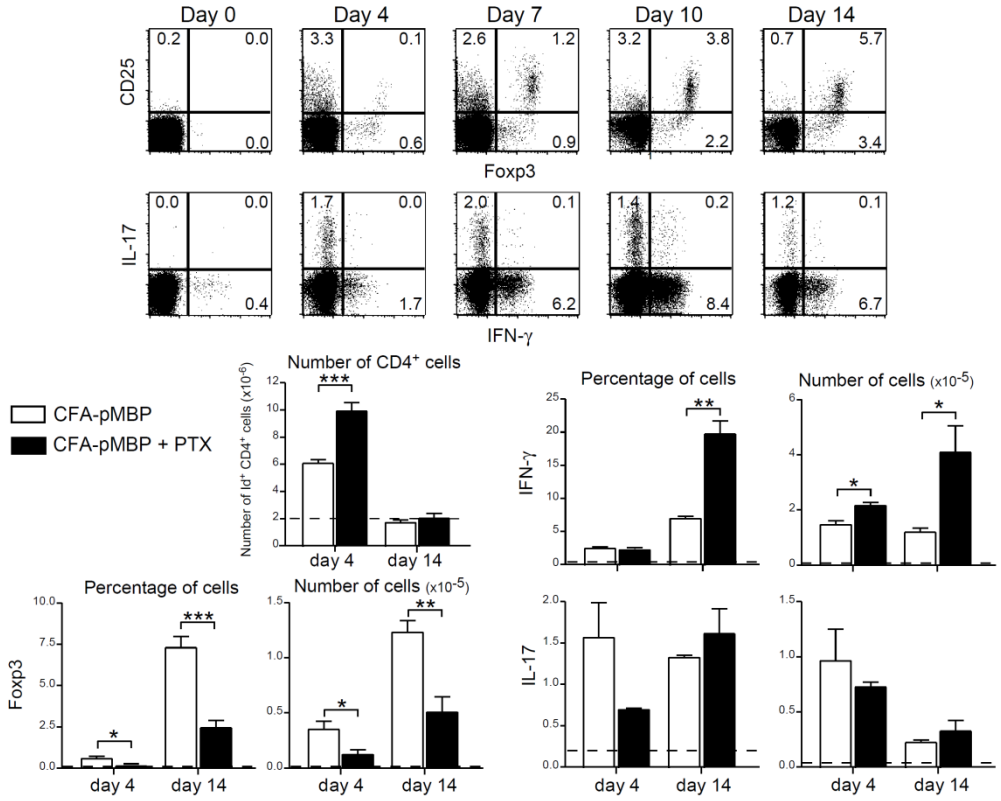
Appendix to Chapter 2 – Supplementary Figures

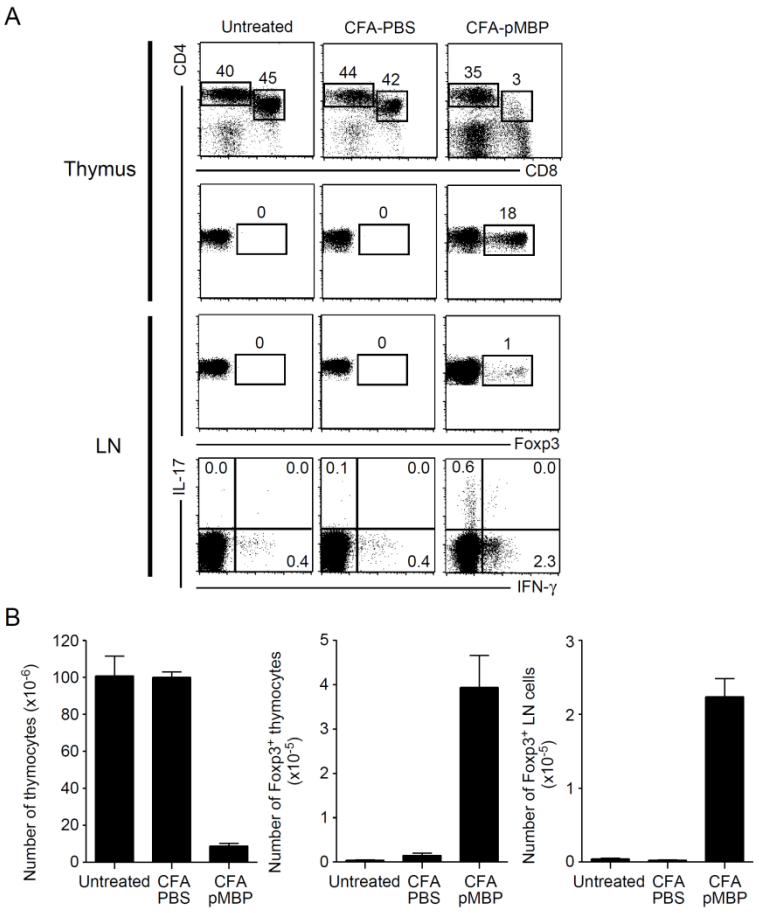


Supplemental Figure S2.1. Foxp3$^+$ cells emerging upon CFA-pMBP immunization resemble bona fide Treg. Upper panel: 1-month-old T/R$^-$ mice were immunized with CFA-pMBP. LN cells were analyzed 14 days after immunization. Representative dot- plot for Foxp3 versus CD25 within CD4$^+$ cells and histograms for GITR and CD103 within CD4$^+$ CD25$^+$ cells (solid line) or CD4$^+$ CD25$^-$ cells (dotted line). Middle panel: CD4$^+$ CD25$^+$ cells from CFA-pMBP-treated T/R$^-$ mice 14 days post-immunization (open squares) or from naïve WT mice (filled circles) were plated at different ratios with CD4$^+$ CD25$^-$ cells from WT mice at 2.5x10^4 cell/well in U-bottom 96-well plates for 72 h together with 10^5 irradiated (30 Gy) splenocytes as APC and 1 µg/ml anti-CD3 mAb (clone 145.2C11; home-made). Cultures were set in triplicates in a final volume of 200 µl. Proliferation was monitored by addition of [^{3}H] thymidine for the last 6 h of culture. Lower panels: RAG1$^{-/-}$ mice received 2.5x10^5

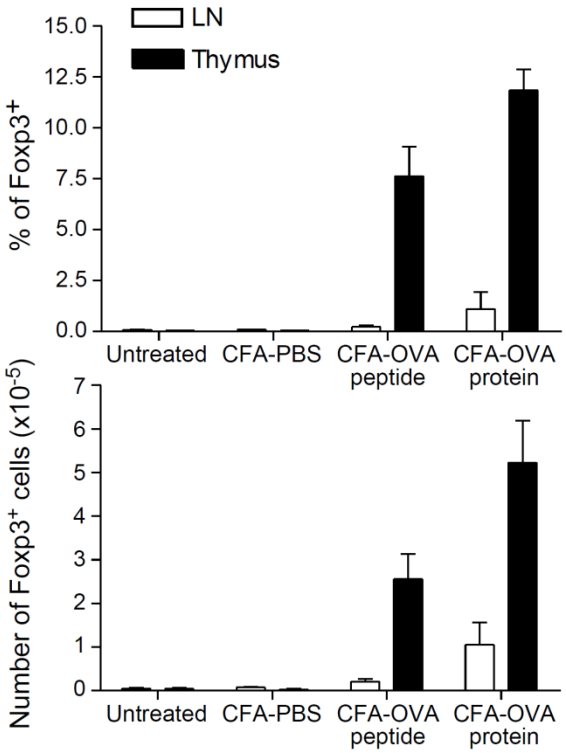
$CD4^+$ cells isolated from either 1-month-old T/R^- mice (naive, n=7), or from CFA-pMBP-immunized T/R^- mice 60 days after immunization (CFA-pMBP, n=3) or $2.5x10^5$ $CD4^+$ cells of each population (Co-T, n=5). EAE score of each mouse 40 days after transfer ($p<0.05$: naïve versus CFA-pMBP, and $p<0.01$: naïve versus Co-T; Pearson's Chi- square exact test). Right, Percentage of survival over time ($p<0.05$: naïve versus CFA- pMBP, and $p<0.01$: naïve versus Co-T; logrank test).



Supplemental Figure S2.2. Simultaneous differentiation of Treg and effector T helper cells upon proinflammatory immunization. 1-month-old T/R$^-$ mice were immunized with CFA-pMBP (white bars) or CFA-pMBP and PTX (black bars, PTX on day 0 and 2) and their draining LN analyzed at different time points after immunization. A, Representative dot-plot for CD25 versus Foxp3 and IL-17 versus IFN-y within CD4$^+$ T cells. B, Number of total CD4$^+$ T cells and percentage and number of Foxp3$^+$ cells, IFN-y- and IL-17-producing cells measured in a CD4$^+$ gate. The dotted line corresponds to untreated mice. Data are representative of three independent experiments with n=3 per group.



Supplemental Figure S2.3. No noticeable difference between untreated and CFA-PBS injected mice. 1-month-old T/R$^-$ mice were either untreated or immunized with CFA-PBS or CFA-pMBP into the footpad and thymus and LN were analyzed 4 days later. A, Representative dot-plots for CD4 versus CD8 inside a live lymphocyte gate, for CD4 versus Foxp3 inside a CD4$^+$ CD8$^-$ gate from the thymus or from draining LN and for IL-17 versus IFN-y from draining LN. B, Number of total thymocytes, Foxp3$^+$ thymocytes or Foxp3$^+$ LN cells.



Supplemental Figure S2.4. Intrathymic Foxp3^{+} cell differentiation in DO11.10 RAG1$^{-/-}$ mice upon CFA-OVA peptide or whole OVA protein immunization. 6-week-old DO11.10 RAG1$^{-/-}$ were either untreated or injected with CFA mixed with PBS or 100 μg of OVA peptide or 10 mg of OVA protein into the footpad and analyzed 4 days later. Percentage (upper panel) and number (lower panel) of Foxp3^{+} cells among CD4^{+} CD8^{-} thymocytes or LN cells, n=2-6 in each group.

Chapter 3

Recent Thymic Emigrants are precursors of peripheral regulatory T cells in mice and humans

Chapter 3 preliminary notes

The author of the thesis designed and planed the experiments together with the supervisor Dr. Jocelyne Demengeot. The author also participated in all the experiments presented, except for Figure 3.7, with the participation of the co-authors mentioned in the beginning of the chapter for particular sections. The author also prepared the figures. Analysis and interpretation of the results were performed by the author and the supervisor Dr. Jocelyne Demengeot.

Recent Thymic Emigrants are precursors of peripheral regulatory T cells in mice and humans

by

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RSP, ACL, MLB, SZ, IC, JD, designed the mouse research.

RSP, ACL, MLB, SZ, performed mouse experiments,

RSP, ACL, MLB, SZ and JD analyzed and interpreted the mouse data

AES, Contributed to the provision of human samples

IC, designed and performed the experiments on human tissues

IC, AES and JD, analyzed and interpreted the human data

RSP, IC and JD wrote the manuscript

Abstract

Most Foxp3^{+} CD4 regulatory T cells (Treg) precursors are newly formed thymocytes that acquire Foxp3 expression in the thymus. However, differentiation of Treg can also occur in the periphery. What limits this second layer of Treg production in physiological conditions remains to be understood. In this work, we tested the hypothesis that, similarly to thymic Treg, the precursors of peripheral Treg are immature T cells. We evidence that CD4^{+}CD8^{-}Foxp3^{-} thymocytes and recent thymic emigrants (RTE), contrarily to peripheral naïve mature cells, efficiently differentiate into Treg upon transfer into lymphopenic mice. By varying donor and recipient mice as well as conducting *ex vivo* assays, we document that the preferential conversion of newly formed T cells does not require intrathymic pre-activation, is cell-intrinsic and correlates with low and high sensitivity to natural inhibitors and inducers of Foxp3 expression, such as IL-6 and TGF-β, respectively. Finally, *ex vivo* analysis of human thymocytes and peripheral blood T cells revealed that human RTE and newly developed T cells share an increased potential to acquire a FOXP3^{bright} CD25^{high} Treg phenotype. Our findings indicating that RTE are the preferential precursors of Treg differentiated in the periphery should guide the design of Treg based therapies.

Introduction

Foxp3 expressing CD4 T cells (Treg) are antigen specific suppressors cells that prevent autoimmunity and allergy, dampen protective responses and restrict associated immunopathologies (1). While never directly demonstrated, it is predicted that tight regulation of Treg production and expansion is required to prevent immune paralysis. Treg were first shown to differentiate in the thymus from newly developed thymocytes upon TCR engagement by antigen expressed and/or presented locally (2). Such a developmental time and space defined by the thymus, would suffice to explain Treg restricted domain of competence once reaching the periphery. Hence, the thymic Treg (tTreg) reactive repertoire is necessarily restricted to self-antigens, including peripheral tissue and organ specific gene products expressed promiscuously by thymic epithelial cells (3), and to peripheral antigens accessing the thymus (4, 5). It is now well documented that Treg can differentiate in the periphery (pTreg), in both therapeutic and physiologic settings. Antigen specific pTreg were reported upon subcutaneous administration of peptides (6) or exposure to intestinal antigens either from food (7) or microbiota (8). Peripheral *de novo* differentiation of Treg was also documented during pregnancy (9), and upon lymphopenia (8). Hence, pTreg differentiation may complement tTreg self-reactive repertoire by including reactivities against those self-antigens not expressed in the thymus but it also recruits clones reactive to fully foreign antigens. What limits extrathymic Treg differentiation in physiological conditions, such that protective immune responses are effective, remains to be elucidated. In turn, this knowledge would help to improve the development of therapies that aim at enhancing or inhibiting *de novo* Treg differentiation, as in autoimmune diseases and transplantation or cancer, respectively.

In the last few years, several studies have aimed at establishing the differences between tTreg and pTreg, phenotypically and functionally as well as concerning the signals required for their differentiation. When taken

together, these works do not provide a consensual picture. Hence, expression of various membrane and nuclear molecules tentatively subdivided tTreg and pTreg in some, but not other experimental settings (8, 10, 11). Epigenetic marks, notably CpG methylation at the Foxp3 regulatory sequences were shown to dissociate tTreg from *in vitro* induced Treg but not from pTreg (12). Controversies also arose as whether TGF-β is required for Foxp3 induction solely in peripheral cells, notably through engagement of the Smad3 motif in the CNS1 regulatory sequence 5' of the coding region (13-16). Overall, tTreg and pTreg share many features, among which a similar two-step process for their differentiation, from TCR to IL-2 signaling (17-19). As these signals are abundant in the periphery, physiological limitation of pTreg production should rely on other features.

In this work we hypothesized that the precursors of pTreg are limiting and that similarly to tTreg, their progenitors are recently developed T cells. Adoptive transfer experiments in lymphopenic mice indicated that thymocytes as well as recent thymic emigrants (RTE) readily acquire a Treg phenotype in the periphery. In contrast, very few peripheral mature resident naïve cells, purged of RTE by either thymectomy or cell sorting, converted to $Foxp3^+$ Treg in this assay. T cell maturation stage also conditioned *in vitro* induced Foxp3 expression, both in mice and humans. The decreased susceptibility of mature cells to acquire a Treg phenotype was further correlated with their increased sensitivity to inhibitors, such as IL-6, and decreased sensitivity to facilitators such as TGF-β. Together our results show that T cell maturation stage conditions *de novo* Foxp3 expression and that RTE are the preferential precursors of Treg differentiated in the periphery. These findings provide a rationale for limited pTreg in physiology and raise specific concerns for the success of pTreg based therapies.

Results

Newly developed CD4 T cells are enriched in precursors of regulatory T cells

To directly test whether maturation stage conditions CD4 T cell susceptibility to acquire Foxp3 expression *in vivo*, we first compared newly formed single positive (SP) CD4 thymocytes with peripheral CD4 cells isolated from lymph nodes (LN) in assays of lymphopenia induced Treg differentiation (8). The two cell subsets were isolated from Foxp3-GFP reporter mice (20), purified as GFP^- and separately injected i.v. into T cell deficient animals ($TCR\beta^{-/-}$). As donor and recipient mice were raised in strict SPF conditions, adoptive transfer did not result in noticeable pathology for at least 8 weeks (not shown). Cellular analysis was performed at 4 weeks post-transfer and donor cells were identified as $CD4^+$ $TCR\beta^+$ (Fig. 3.1). As described earlier (8), a small but readily detectable fraction of LN cells acquired Foxp3 expression, representing a maximum of 2.5 % of the recovered cells. Strikingly, this frequency was 4 to 10 fold increased in recipients of thymocytes, in all lymphoid organs analyzed, including those draining the intestine (Fig. 3.1A, B). Skin-draining LN, such as brachial, inguinal and axillary, analyzed either pooled or separately (not shown) appeared as a preferential site for $Foxp3^+$ cell differentiation and/or accumulation. Importantly, thymocytes expanded less than LN cells in these organs but equally well in other sites (Fig. 3.1C), and yet invariably gave rise to more $Foxp3^+$ cells (see Fig. S3.1 for a pool of 6 independent experiments). We next tested the conversion efficiency of thymocytes and LN cells upon adoptive transfer into WT mice rendered mildly lymphopenic the day before injection (Fig. S3.1). In this setting, resident host lymphocytes limited the expansion of the donor cells that represented 7% of total CD4 lymphocytes in spleen and LN at the time of analysis. Yet, donor thymocytes showed increased conversion into Treg when compared to donor mature cells, a difference noticeable already in blood at one week post-transfer. We conclude that thymocytes are remarkably more susceptible than peripheral cells to convert into a Treg

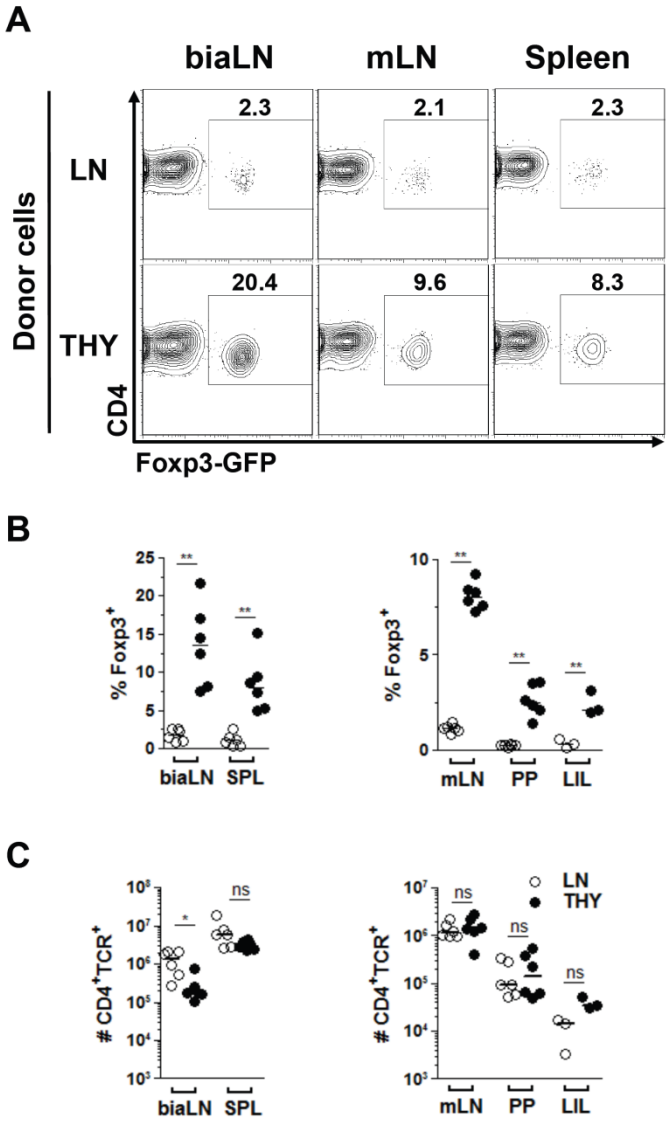

Figure 3.1. Thymocytes are enriched in precursors of Treg that differentiate in the periphery upon lymphopenia. TCRβ$^{-/-}$ mice received i.v. 3x10^5 CD4$^+$CD8$^-$ Foxp3$^-$ cells purified from either thymi or pooled lymph nodes isolated from Foxp3-GFP reporter mice and analyzed 4 weeks later by FACS. **A)** Representative analysis of Foxp3 expression in gated CD4$^+$TCR$^+$ lymphocytes from the indicated organs in mice recipient of either LN cells or thymocytes (THY). **B, C)** frequency of Foxp3$^+$ cells within CD4$^+$TCR$^+$ lymphocytes (B) and number of CD4$^+$TCR$^+$ cells (C) recovered in a single experiment, representative of seven independent repeats.

Each dot represents one mouse. White and black circles, correspond to LN and Thy as donor cells, respectively. Sp: spleen, biaLN: pooled branchial, inguinal and axillary LN, mLN: pooled mesenteric LN, PP: pooled payer patches, LIL: large intestine lamina propria and intraepithelial lymphocytes. Note the different scales in left and right panels. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, Mann-Whitney test. LIL data in panel (B) p value of 0.0059, t test.

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phenotype and that this feature is not associated with the extent of T cell proliferation. We next ascertained that our results did not owe to trivial cell sorting artifacts, by conducting voluntary contamination experiments (Fig. S3.1). Thymocyte and LN preparations (Thy1.2 Foxp3-GFP$^-$) were each mixed at 99:1 with Thy1.1 Foxp3-GFP$^+$ cells purified from the corresponding organs before adoptive transfer. Analysis of the recipient mice 4 weeks post-transfer revealed that the majority of the recovered Foxp3$^+$ cells derived from the originally Foxp3$^-$ cell subset, whether thymocytes or LN cells. As expected, thymocytes gave rise to higher frequencies of Treg than LN cells in these experiments as well as in the single transfers above. Together, these results establish that thymocytes are specifically prone to undergo true *de novo* differentiation into a Foxp3$^+$ phenotype once reaching the periphery.

Peripheral Treg derived from thymocytes and LN cells are functionally and phenotypically indistinguishable

Several surface markers have been proposed to discriminate Treg that were generated in the thymus or induced in the periphery. We tested whether Foxp3$^+$ cells originated from thymocytes or LN cells in the experiments above share similar phenotypes. Thymic and peripheral cells from unmanipulated WT mice served as references. An additional control consisted of *in vivo* expanded Treg, obtained from TCRβ$^{-/-}$ mice that had

received 4 weeks earlier a mixture of Thy1.2 Foxp3$^+$ and Thy1.1 Foxp3$^-$ cells isolated from LN of unmanipulated WT mice. Pair-wise analysis of the surface markers CD103 and KLRG1 or GITR and CD25 revealed no differences between Foxp3$^+$ cells that differentiated from LN cells or thymocytes upon lymphopenia (Fig. S3.2). Both shared a phenotype resembling the previously described iTreg, i.e. enriched in CD103$^+$KLRG1$^+$ (10) and GITR$^+$CD25$^+$ (11), clearly distinguishable from thymic and peripheral Treg at steady state, but strikingly similar to *in vivo* expanded Treg. Analysis of Helios and Nrp1 expression also did not discriminate LN or thymocyte derived Treg in our adoptive transfers (Fig. 3.2A,B). However, both cell subsets displayed a phenotype similar to that of thymic Treg, namely Helios$^+$ with a clear Nrp1$^-$ subpopulation (11), that was only residual in Treg found in the periphery, whether or not expanded *in vivo*. Finally, a classical proliferation assay confirmed that both thymocyte and LN derived Foxp3$^+$ cells are suppressors (Fig. 3.2C). Together, these analyses indicate that Foxp3$^+$ cells developed from thymocyte or LN cells in lymphopenic hosts are remarkably similar to *bona fide* Treg.

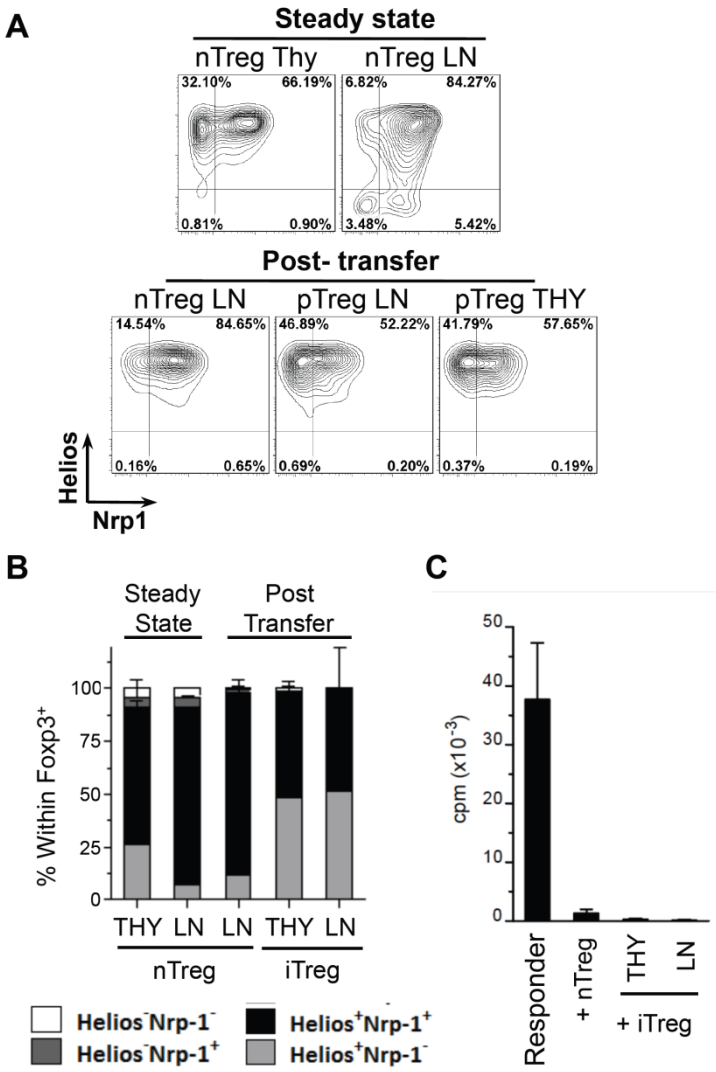

Figure 3.2. Thymocytes and LN cells converted into Treg upon lymphopenia are functionally and phenotypically indistinguishable. **A, B)** Phenotypic analysis of Foxp3$^+$ cells from the spleens of animals treated as in Fig. 3.1A for Helios and Nrp1. Representative FACS staining (A) and distribution of each subset (B) gated on Foxp3$^+$ CD4$^+$ TCR$^+$ cells. Three populations of Natural Treg served as control: Foxp3$^+$ thymic and peripheral from unmanipulated mice (nTreg steady state, upper part), Thy1.2 Foxp3$^+$ peripheral cells from TCR$\beta^{-/-}$ recipient mice that received 4 weeks earlier a mixture of Thy1.1 Foxp3$^-$ and Thy1.2 Foxp3$^+$ LN cells at a 9:1 ratio (nTreg post-transfer, bottom left). THY, thymocytes; LN, lymph nodes; nTreg, natural

Treg; pTreg, Treg differentiated in the periphery. **C)** Standard suppression assay performed with naïve Foxp3$^-$ cells as responders (R) and CD4$^+$Foxp3$^+$ Treg. Treg were either natural Treg sorted from pooled LN of Foxp3-gfp animals (nTreg) or *in vivo* differentiated Treg sorted from recipient of either thymocytes (THY) or LN transfers as in Figure 3.1A.

Thymocytes enhanced susceptibility to differentiate into Treg is a cell intrinsic property

We next tested whether the differential capacity of thymocyte and peripheral cells to acquire Foxp3 expression was controlled by environmental factors. The cell surface marker analysis above left open the possibility that *de novo* differentiation of Treg in lymphopenic hosts relied on donor cell recirculation to the thymus and that thymocytes would be more competent at this migration. We challenged this scenario by repeating the adoptive transfer experiments using athymic mice as recipients (Fig. 3.3A). Transfer of thymocytes or LN cells into nude-Foxn1$^{-/-}$ animals reproduced the results obtained in TCRβ$^{-/-}$ recipients, excluding a contribution of the thymus. Secretion of pro-inflammatory cytokines by activated T cells has been proposed to limit naïve T cell conversion into Treg in other systems (21). It was conceivable that LN preparations, enriched in naturally activated cells when compared to thymocytes, would produce more of these inhibitory factors upon lymphopenia induced proliferation (LIP). We first tested whether purging LN preparations from naturally activated cells would enhance their capacity to acquire Foxp3 expression. However, adoptive transfers of peripheral CD4$^+$ Foxp3$^-$ cells either unfractionated or enriched in naïve cells (CD45RB^{hi}) resulted in a similar low frequencies of Foxp3$^+$ cells (Fig. 3.3B). It was still possible that naïve peripheral cells produce more inhibitory factors

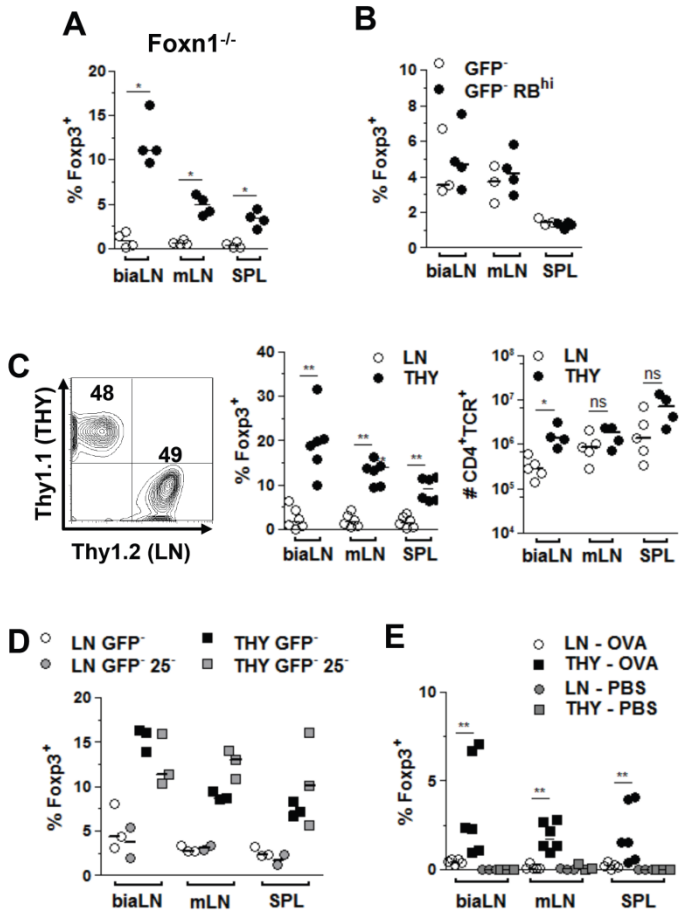

Figure 3.3. Enhanced susceptibility of thymocytes to differentiate into Treg *in vivo* is a cell intrinsic property. Donor cell preparation, adoptive transfer and analysis were performed as in Fig.1 with the following alterations: **A)** Recipients were athymic nude-Foxn1$^{-/-}$ mice. **B)** Donor cells were CD4^{+}Foxp3^{-} from LN either total (GFP^{-}) or purged of activated cells by selecting CD45RB^{high} cells (see Fig. S3.3). **C)** LN and Thymocytes CD4^{+}CD8^{-} Foxp3^{-} donor cells were isolated from Thy1.1 and Thy1.2 Foxp3GFP mice, respectively, and co-injected at a 1:1 ratio (left) into TCRβ$^{-/-}$ animals. Analyses were performed by gating the recovered cells on Thy1.1^{+} (LN) or Thy1.2^{+} (THY). **D)** Donor cells were CD4^{+}Foxp3^{-} from LN or thymocytes either total (GFP^{-}) or purged of potential pre-committed CD25^{+} cells (GFP^{-}25^{-}), as illustrated in Fig. S3.3. **E)** Donor thymocytes and LN cells were isolated from OTII Foxp3^{GFP} Rag2$^{-/-}$ animals. TCRβ$^{-/-}$ recipient mice received 0.1 mg ovalbumin or PBS i.v. the day before and 5 days after adoptive transfer. Analysis

was performed at day 8. Shown is one out of at least 2 experiments for each condition.

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than thymocytes upon LIP. We tested this hypothesis by performing co-transfers of thymocytes and LN cells at a 1:1 ratio, each prepared from Thy1.1 and Thy1.2 congenic donors, respectively (Fig. 3.3C). In these experiments, as in single transfer assays, recovered cells displayed a 5 to 10 fold higher frequency of $Foxp3^+$ cells when originating from thymocytes vs LN preparations, despite similar expansion. These results exclude specific modifications of environmental factors upon LIP as an explanation for thymocytes and peripheral cells' differential conversion to Treg.

As the thymus is the site of natural Treg differentiation, it was plausible that our thymocyte preparations were enriched in pre-committed $Foxp3^-$ Treg and these could explain an apparent cell population property. Expression of CD25 by $Foxp3^-$ cells has been proposed to indicate an early step along the Treg differentiation pathway resulting from TCR triggering, both in the thymus and in the periphery (17-19). However, depleting or not $CD25^+$ cells from thymocyte and LN cell preparations before adoptive transfer did not modify the frequency of recovered Treg from either donor population (Fig. 3.3D). To further exclude antigen triggered pre-committed Treg in our thymocyte preparations we performed adoptive transfer of monoclonal TCR transgenic cells specific for the foreign antigen ovalbumin (OVA), never exposed to their nominal antigen. Recipients $TCR\beta^{-/-}$ mice were injected i.v. with either PBS or endotoxin free OVA protein the day before and 5 days after cell infusion. Strikingly, antigen-dependent induction of Foxp3 expression was efficient in mice that received thymocytes but barely detectable in animal recipients of LN cells (Fig. 3.3E). These results indicated that neither intrathymic pre-selection nor specific repertoire

features suffice to explain the increased susceptibility of thymocytes to differentiate into Treg. From this set of experiments, we conclude that cell intrinsic properties account for the differential susceptibility of thymocytes and LN cells to acquire Foxp3 expression.

Recent thymic emigrants are the precursors of Treg differentiated upon lymphopenia

We show above that thymocytes are specifically prone to undergo true *de novo* differentiation into Treg once exposed to the periphery. We next tested whether naturally exported thymocytes share similar features (Fig. 3.4). $CD4^+$SP cells naturally exit the thymus before full maturation, and complete their differentiation in the periphery (22). Consistent with their intermediate maturation stage, recent thymic emigrants (RTE) can be identified through their intermediate level of expression for CD24, CD45RB or Qa-2 (22) when compared to thymocytes and peripheral mature cells (Fig. S3.4). Upon adoptive transfer in lymphopenic hosts, $Foxp3^-$ RTE purified from LN as Qa-2^{lo} gave rise to $Foxp3^+$ cells in similar frequency and number as thymocytes (Fig. 3.4A). In the same assay, peripheral mature cells purified as Qa-2^{hi}, therefore purged of RTE, showed a very poor capacity to differentiate into Treg, as indicated by less than 1% $Foxp3^+$ cells in the recovered CD4 T lymphocytes.

To confirm that most Treg progenitors in the periphery are encompassed in the RTE cell subset, we tested LN cells prepared from mice naturally purged of RTE following thymectomy. Donor mice were thymectomized (TxT) or, as a control, submitted to the surgery procedure except for thymus removal (Sham) and left to rest for at least 2 months. At this time point preexisting RTE in TxT mice had incorporated the mature T cell pool as indicated by the absence of $Qa2^{lo}$ cells in the peripheral organs (Fig. 3.4B). $CD4^+Foxp3^-$ LN

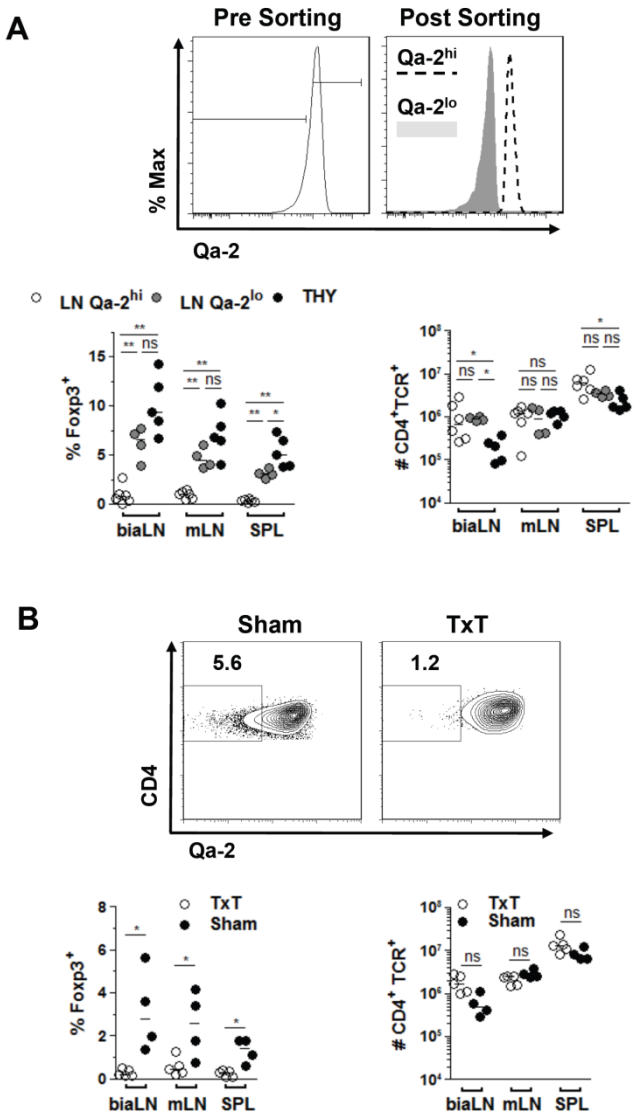

Figure 3.4. Recent thymic emigrants are the main precursors of peripherally differentiated Treg. Donor cell preparations, adoptive transfers and analysis were performed as in Fig. 3.1 with the following alterations: **A)** Donor LN cells were either purged or enriched for Qa-2^{lo} RTE (upper panels) before adoptive transfer (lower panels). **B)** Donor mice were thymectomized (TxT) or sham operated (Sham) 2-3 months before total LN CD4^{+}Foxp3^{-} cells were purified (upper panels) and transferred to TCRβ$^{-/-}$ recipient mice (lower panels).

cells isolated from either TxT of Sham mice proliferated equally well upon adoptive transfer in TCRβ$^{-/-}$ mice (Fig. S3.4). In contrast, *de novo* differentiated Foxp3^{+} cells were not or barely detectable in mice recipient of TxT donor cells, while as expected, cells from sham donors converted into Treg at low but readily detectable levels. Taken together, these results confirm that maturation stage defines T cell susceptibility to acquire Foxp3 expression and in turn, indicate that RTE are the main precursors of Treg differentiated in the periphery upon lymphopenia.

Peripheral T cell maturation associates with altered sensitivity to signals modulating Treg differentiation in vitro

Our results above revealing that mature peripheral cells poorly differentiate into Treg *in vivo* contrasted with the efficient production of iTreg from peripheral naïve T cells *in vitro*. However, several natural compounds likely to be produced in recipient mice inhibit iTreg induction *in vitro*. In addition, iTreg conversion *in vitro* requires TGF-β and TCR signaling, likely of comparatively reduced strength *in vivo*. In turn, this reasoning raised the possibility that immature and mature T cells display differential sensitivity to these inducing and inhibiting signals. Strikingly, LN cell, but not thymocyte, conversion was reduced when antigen presenting cells (APC), either BM-derived DC, T cell depleted splenocytes or CD11c^{+} purified splenic DCs were activated (Fig. 3.5A-D and not shown). This inhibitory effect was mediated by IL-6, as it was lost in cultures seeded with IL-6$^{-/-}$ splenic APCs (Fig. 3.5D). We further tested and confirmed that Qa-2^{lo} RTE, similarly to newly formed thymocytes (5), are resistant to IL-6 in an APC free system where IL-6 could be titrated (Fig. 3.5E and S3.5). However, preferential inhibition of mature vs. immature cells by IL-6 did not explain fully the lower capacity of peripheral T cell to convert into Treg upon lymphopenia. Thus, thymocytes performed better than LN cells at acquiring Foxp3 expression

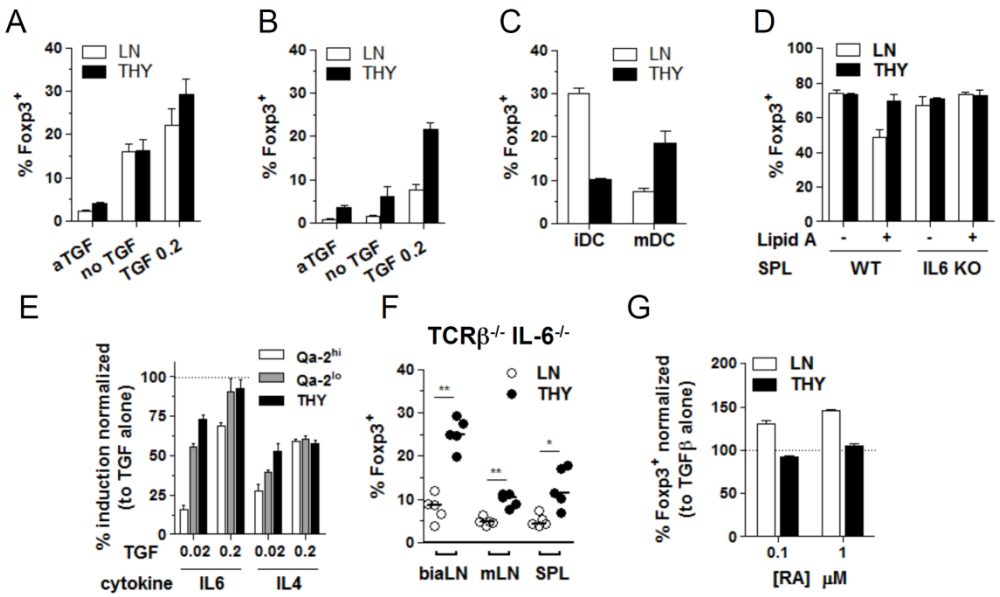

Figure 3.5. Enhanced sensitivity of mature T cells to modulators of Foxp3 induction. $CD8^{-}CD4^{+}Foxp3^{-}$ thymocytes and LN cells were purified as in Fig. 3.1 and set in cultures containing anti-CD3 Ab at 0.1 μg/ml for 3 days supplemented with varying APCs and compounds. **A, B**) APCs were BM derived DC either immature (A) or activated (B), media was supplemented with IL-2 and either neutralizing anti-TGF-β Ab or TGF-β. **C**) As previous, except that media did not contain IL-2 and TGF-β was fixed at 0.2 ng/ml. **D**) APCs were T cell depleted splenocytes (spAPC) from either WT of $IL6^{-/-}$ mice, activated or not overnight with Lipid A, media contained IL-2. **E**) LN cells were fractionated as mature resident cells ($Qa\text{-}2^{hi}$) and RTE ($Qa2^{lo}$), APCs were naive spAPC and media contained IL-2 as well as the indicated cytokines. Shown is normalized frequency of $Foxp3^{+}$ cells obtained in cultures supplemented with TGF-β and IL-6 or IL-4 relative to control cultures supplemented with TGF-β alone that were given a value of 100. **F**) Adoptive transfers were performed and analyzed as in Fig. 3.1 except that recipient mice were $TCR\beta^{-/-}$ $IL\text{-}6^{-/-}$ double mutants. **G**) APCs were naïve spAPC, media contained IL-2, 0.2 ng/ml TGF-β and the indicated concentration of retinoic acid (RA).

upon adoptive transfer into TCRβ$^{-/-}$IL-6$^{-/-}$ double mutant mice, despite an overall increased frequency of conversion, for both cell types, when compared to IL-6 sufficient recipients (Fig. 3.5F and S3.5). Among other cytokines that could affect Foxp3 induction, only IL-4 and to a lower extent TNF-α showed a noticeable inhibitory effect that affected mature more than immature T cells, possibly explained by lower level of surface receptor expression (Fig. 3.5E and S3.5), as shown previously for IL-6 (5). Retinoic acid is another factor that has been shown to modulate Treg differentiation, both *in vitro* and *in vivo* (21, 23, 24). However, while its addition to the cultures readily enhanced iTreg differentiation from peripheral T cells it had no effect on thymocytes (Fig. 3.5G). We conclude that several natural factors known to inhibit or enhance Treg differentiation specifically affect mature T cells.

We next assessed the sensitivity of each T cell subset to *bona fide* inducers of Treg, namely TCR triggering and TGF-β. We first monitored the response of anti-HY TCR transgenic cells to a large range titration of their nominal peptide presented by naïve APC (Fig. 3.6A). As suggested earlier (25, 26) the lower the TCR triggering, the larger the frequency of cells acquiring Foxp3 expression. More importantly, the slope of the dose response obtained with thymocyte cultures was much steeper than that of peripheral cells, indicating differential sensitivity to TCR triggering for Foxp3 induction. Next, fixing the peptide concentration and varying the concentration of TGF-β indicated a higher sensitivity of immature cells to this cytokine (Fig. 3.6B). Similar differential sensitivity to TGF-β was obtained when testing polyclonal cells stimulated with anti-CD3 (Fig. 3.6C). However, Ab-mediated neutralization of TGF-β abrogated Foxp3 induction in all subsets (Fig. 3.6A,B), confirming the dependency on TGF-β for iTreg induction. Yet, without exogenous TGF-β, conversion was frequently detectable, notably for thymocytes, likely the result of variable concentration of active TGF-β in the

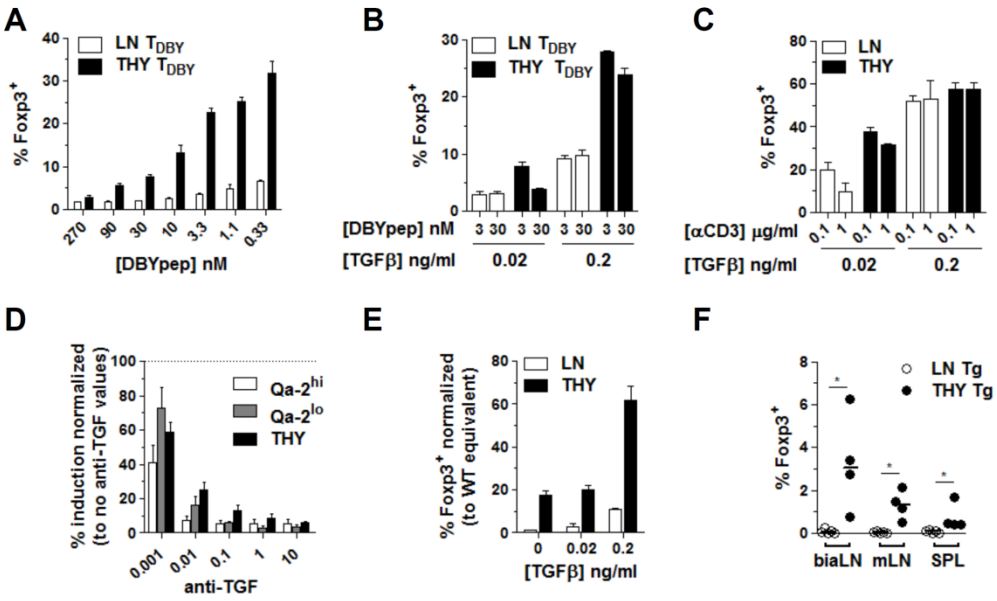

Figure 3.6. Enhanced sensitivity of immature T cells to TGF-β dependent induction of Foxp3 expression *in vitro*. A, B) $CD4^+$ thymocytes and LN cells isolated from female $Rag^{-/-}$ Foxp3-GFP anti-HY TCR tg mice were set in cultures containing spAPCs, IL2, 0.2 ng/ml TGF-β (in A) and the indicated concentration of nominal peptide. **C)** Polyclonal $CD8^-CD4^+Foxp3^-$ cells were stimulated as in (B) except anti-CD3 Ab was used instead of peptide. **D)** LN cells were fractionated as mature resident cells ($Qa\text{-}2^{hi}$) and RTE ($Qa2^{lo}$) and cultured in presence of spAPC, 0.1 µg/ml anti-CD3 Ab, IL-2 and the indicated concentration (µg/ml) of neutralizing anti-TGFβ Ab. Results are expressed as % of response in absence of neutralizing Ab. **E, F)** $CD8^-CD4^+Foxp3^-$ cells were purified from Foxp3-GFP, TGF-βRIIdn Tg double mutant mice and either cultured (E), as in C or adoptively transferred into $TCR\beta^{-/-}$ mice (F), as in Fig. 3.1.

serum supplement (not shown). Titration of neutralizing antibodies in such cultures revealed that thymocytes and RTE require less TGF-β than mature T cells to convert into a $Foxp3^+$ phenotype (Fig. 3.6D). As a confirmatory experiment we tested cells prepared from donor mice expressing a dominant

negative form of the TGF-β receptor (dnTGF-βRII) that are partially impaired in TGF-β signaling (27). Compared to the respective WT cells, mature mutant cells lost 90% of their capacity to convert into Treg as compared to only 40% for mutant thymocytes (Fig. 3.6E). Finally and in line with the results above, both LN and thymocytes isolated from mutant mice showed dramatically decreased capacity to convert into Treg when tested *in vivo*. This reduction resulted in rare to undetectable (max 0.3% of recovered cells) $Foxp3^+$ cells in recipient of LN cells and corresponded to a 7 to 10 fold decrease in recipients of thymocytes (Fig. 3.6F and S3.6). From this set of experiments, we conclude that T cell maturation associates with several features impairing their susceptibility to acquire Foxp3 expression, among which an increased sensitivity to inhibitory cytokines and a lower sensitivity to inducing signals such as TCR triggering and TGF-β.

Human RTE are more susceptible than mature cells to differentiate into Treg

We next tested whether our findings indicating that peripheral maturation limits T cells susceptibility to differentiate into Treg can be extended to humans. Human naïve $CD4^+CD25^-CD127^{hi}$ lymphocytes are devoid of Foxp3 expressing cells and can acquire Foxp3 expression *in vitro* upon stimulation with anti-CD3 in presence of TGF-β. Among these, $FOXP3^{bright}$ $CD25^{high}$ are *bona fide* Treg (28). Human RTE are enriched in the $CD31^+$ cell subset that represents 50 to 80% of the naïve $CD4^+CD25^-CD127^{hi}$ cells in peripheral blood of healthy donors (Fig. 3.7A), a variation previously associated with age and thymic involution (29). We tested $CD31^+$ and $CD31^-$ naïve cells isolated blood donor and $CD4SPCD25^-CD127^{hi}$ thymocytes isolated from patients undergoing heart surgery for their capacity to acquire a Treg phenotype *in vitro*. Upon culture in presence of plate-bound anti-CD3, IL-2 and TGF-β, cells in both subsets readily converted to a $FOXP3^{bright}$

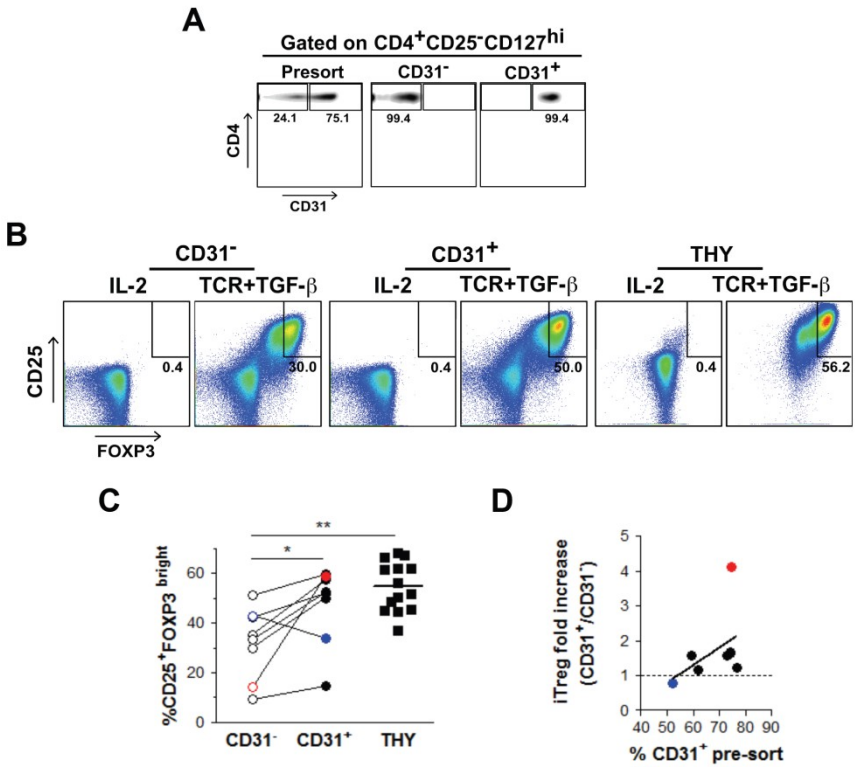

Figure 3.7. **Preferential differentiation of human thymocytes and RTE into Treg *ex vivo*.** Human thymocytes and peripheral blood mononuclear cells sorted as ($CD8^-$) $CD4^+CD25^+CD127^{hi}$ and for the latter, according to CD31 expression. Cells were cultured for 5 days in media containing IL-2 alone (IL-2) or supplemented with plate bound anti-CD3 mAb, soluble anti-CD28 mAb and TGF-β (TCR+TGF). **A)** Representative Flow cytometric analyses of CD31 expression within naïve $CD4^+CD25^+CD127^{hi}$ peripheral blood mononuclear cells before and after sorting. **B**) Representative dot plots show the frequency of $FOXP3^{bright}CD25^{high}$ cells within live lymphocytes after culture. **C**) Results of 8 independent blood donors, each pair $CD31^-/CD31^+$ represents one donor (paired t-test). Thymocytes were from another cohort (n=14). **D**) Correlation between the frequency of $CD31^+$ cells among naïve $CD4^+CD25^+CD127^{hi}$ cells in each blood sample before sorting and the respective fold increase in the frequency of $FOXP3^{bright}$ cells when comparing the induction efficiency of the $CD31^+$ versus $CD31^-$ cell subsets. Spearman coefficient test, *r* and *P* were 0.5238 and 0.0983, respectively.

$CD25^{high}$ Treg phenotype (Fig. 3.7B). A pair-wise comparison revealed that 7 out of 8 donors analyzed showed more efficient conversion when $CD31^{+}$ instead of $CD31^{-}$ cells were tested for either frequency (Fig. 3.7C) or number of $FOXP3^{bright}CD25^{high}$ cells, as well as level of FOXP3 expression, as defined by the mean fluorescence intensity (not shown). As the $CD31^{+}$ cell subset is heterogeneous, enriched in RTE but also encompassing more mature cells (30), it is expected that low frequency of $CD31^{+}$ cells would also associate with low number of RTE inside the $CD31^{+}$ cell subset. In agreement with this reasoning, the unique donor who showed lower iTreg induction in $CD31^{+}$ vs $CD31^{-}$ cell cultures presented with the lowest percentage of $CD31^{+}$ in total naïve cells, pre-sorting. Symmetrically, the donor who showed a 4 fold increased Treg induction in $CD31^{+}$ vs $CD31^{-}$ cell cultures presented with a high percentage of $CD31^{+}$ cells pre-sorting (Fig. 3.7D). Finally, analysis of thymocytes in comparison to peripheral blood cells further supported the notion that, in human as in mice, peripheral maturation progressively affects T cell susceptibility to differentiate into Treg.

Discussion

There has been increasing hope that inducing *de novo* differentiation of Treg would offer a therapeutic solution for autoimmunity and allergy. On the other hand, preventing *de novo* Treg development is needed to improve tumor therapies and vaccine efficacy. Our findings that extrathymic maturation of CD4 T cells limits their capacity to differentiate into Treg in mice and in humans bears several consequences on our understanding of immune regulation and on the development of novel therapeutic approaches.

Earlier works supported directly or indirectly the notion that RTE preferentially promote dominant tolerance to peripheral antigens (31, 32). In contrast, the more recent literature on Foxp3$^+$ pTreg assumes that any naïve cell can be a precursor of pTreg, provided they express the proper TCR specificity and are presented antigen in the right context. This notion likely derives from *in vitro* assays, in which most naïve peripheral cells can acquire Foxp3 expression. Intriguingly, the fraction of cells successfully induced to expressed Foxp3 *in vivo* in various assays, including in monoclonal TCR transgenic mice infused with the nominal peptide (6), is minute. To our knowledge this paradox has not been explored before and can be explained by our findings indicating that RTE, a limited cell subset in the periphery, are the preferential precursors of pTreg.

To test the hypothesis that limited number of Treg precursors relates to T cell maturation stage, we chose the lymphopenia induced Foxp3 expression assay. This experimental setting presents the advantage of testing polyclonal cells developed in a WT host for their spontaneous differentiation into Treg. It also mimics the severe peripheral lymphopenia that takes place in various patho-physiological situations, such as upon infection or in clinical settings, for instance upon high dose corticosteroid administration. Moreover, the physiological relevance of iTreg generated during LIP has been previously demonstrated in a model of colitis (8). In addition, induction

of Treg upon LIP is readily detectable which allows for reliable quantitative measurements. It is worth noticing that the frequency of Treg we obtained upon adoptive transfer of peripheral cells into TCR$\beta^{-/-}$ mice was above the average values reported in similar LIP assays for which RAG$^{-/-}$ recipient mice have been used (10). We confirmed that fully alymphoid mice poorly sustain pTreg differentiation (Lino in preparation) and nevertheless also favor conversion of immature rather than fully mature peripheral cells (not shown).

We further confirmed that T cell maturation limits their susceptibility to acquire Foxp3 expression *in vivo* by testing antigen-specific T cells triggered by their nominal peptide *in vivo*. Given that unmanipulated OTII Rag$^{-/-}$ mice are devoid of Treg, this set of experiments confirmed that we were monitoring true *de novo* Treg differentiation, as was already indicated by our voluntary contamination experiments. This approach also confirmed that our results did not owe to an enrichment of T cells pre-committed by TCR triggering in thymus, as also indicated by the experiments purging activated lymphocytes from polyclonal donor cells. Incidentally, these latter experiments provided the experimental design to exclude that our findings would owe to the fusion Foxp3-GFP allele, recently shown to be an occasional hypomorph and potentially unstable (33, 34). Exclusion of Foxp3^{+} cells from WT lymphocytes by selecting CD45RB^{high} CD25^{-} LN cells and CD25^{-} thymocytes before adoptive transfer fully reproduced the differential phenotype (Fig. S3.7).

Our findings indicating that cell maturation progressively restricts T lymphocytes susceptibility to acquire a Treg phenotype are consistent with other studies concerned with thymic Treg differentiation. Hence, thymocytes from newborns were more readily differentiated into Treg than from adults (35). Moreover, thymic APC were shown to be more potent at sustaining thymocyte rather than peripheral naïve cell conversion to iTreg (36). While these studies did not provide a molecular mechanism to these differences, our present work indicates that differential sensitivity to factors promoting

and inhibiting Treg differentiation explains the impaired ability of peripheral mature T cells to acquire a Treg phenotype. This finding completes our previous report indicating that recently differentiated thymocytes but not mature T cells, whether in the periphery or recirculating to the thymus, are refractory to IL-6 mediated inhibition of Foxp3 induction (5). Here, by testing RTE, we confirm that maturation stage defines CD4 cell sensitivity to IL-6. Yet, despite a clear inhibitory effect of IL-6 on LIP induced Treg, other factors beyond IL-6 determine the reduced susceptibility of mature cells to convert into Foxp3$^+$ cells. We further evidence a decreased sensitivity of immature cells to TGF-β. The role of this cytokine for the generation of thymic natural Treg has been debated and more recently, its requirement for *de novo* generated pTreg was also disputed (13-16). Our *in vivo* analysis of cells expressing a dominant negative form of the TGF-β receptor ascertained that engagement of this pathway promotes Treg differentiation during lymphopenia. Importantly, our finding that T cell maturation associates with increased sensitivity to both inhibitors (IL-6 or IL-4) and promoters/facilitators (TGF-β and RA) of Foxp3 induction, are also compatible with the notion that mucosa efficiently support the differentiation of Treg. In this microenvironment TGF-β and RA are abundant and inflammation maintained at check, such that Treg could also be recruited from resident mature peripheral cells. The novel concept brought about by our analysis is that systemic peripheral conversion, i.e. in situation of limited RA and TGF-β and/or in inflammatory context, is restricted to RTE.

Finally, our work reveals a third way by which the adult thymus contributes to peripheral tolerance. As first evidenced in the 1990s, selection of natural Treg takes place in the thymus upon TCR triggering by self-antigens, including those tissue specific genes expressed by TEC (2). Moreover, recirculation of peripheral antigens to the thymus also shapes Treg repertoire (5). Lastly, as we show here, recently differentiated T cells have a limited developmental time-window, before full maturation in the periphery,

to readily acquire Foxp3, placing RTE as the preferential precursors of pTreg. All together, because the thymus involutes with age, infections and upon various drugs administration, these properties call for specific attention to thymic activities when developing therapies to enhance or prevent immune tolerance.

Material and Methods

Mice

All mice were on a C57BL/6 background bred and raised in a strict SPF facility upon rederivation by embryo transfer when necessary. The Foxp3-GFP reporter mice (20) were bred to homozygocity with mice congenic for Thy1.1 (originally Jackson, USA), with animals transgenic for CD4-dnTGFβ-RII (27), with marilyn anti-HY TCR Tg $Rag^{-/-}$ mice (37) and with established OT-II $Rag2^{-/-}$ mice (originally OTII from Jackson and $Rag2^{-/-}$ from CDTA, France). $TCR\beta^{-/-}$ animals were crossed with $IL6^{-/-}$ mice (both originally from Jackson, USA) to generate $IL6^{-/-}TCR\beta^{-/-}$ mice. Nude-$Foxn1^{-/-}$ mice were originally from Taconic, UK. All animals were used between 6 and 12 weeks of age, unless otherwise indicated. When indicated mice receive i.v. injection of $3x10^5$ purified cells, and/or 100 μg/mouse of endotoxin free ovalbumin protein (endograde ovalbumin - hyglos). Thymectomies were performed by aspiration in 5-6 week old mice under ketamine/xillazine anesthesia. Sham operated mice underwent the full procedure but aspiration omitted. Mouse experiments were approved by the Institutional ethical committee and the Portuguese state entity DGV.

Human samples

Thymic specimens were obtained from routine thymectomy performed during pediatric corrective cardiac surgery at the Hospital de Santa Cruz, Carnaxide, Portugal, after parent's written informed consent. Buffy coats were provided by Instituto Português de Sangue, Lisboa, Portugal. Human studies were approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Antibodies

Mouse antibodies for FACS analysis and cell sorting were anti-HSA/CD24-Cy5 (M1/69), IL4-Rα/CD124-PE (mIL4R-M1), CD4-PE/PB/APC (RM4-5), CD45RB-PE (16A), CD44-biotin/PB (IM7), Thy1.2-biotin (53-2.1), Thy1.1-PE (Ox7), Qa-2-biotin (1.1.2), IL6-Rα/CD126-PE, all from Beckton Dikinson; IFNγ-RI/CD119-PE (2E2), Integrin alpha-IE/CD103-biotin (2-E7), KLRG-1-APC (2F1), GITR-biotin (DTA-1), Foxp3-FITC/PE/APC (FJK-16s) all from ebiosciences; anti-CD3ε-APC (500 A2-77), IL2-Rα/CD25-PE (7D4), CD8-PE/A647 (YTS169.4), produced in house and Helios-PE (22F6, Biolegend), Neuropilin-1-APC (polyclonal, R&D). Biotinylated Abs were revealed with streptavidin-A488/PE, A647, PB or APC-Cy7 (BD). For cell culture, anti-CD3ε (141.2C11), neutralizing TGFβ (1D11) and anti-Thy1.2 (30H12) were produced and purified on Protein G column in-house and anti-CD28 (37.51) was purchased from ebiosciences. Human antibodies were: CD3 (UCHT1 or OKT3), CD4 (RPA-T4), CD8α (RPA-T8), CD31 (WM59) and FOXP3 (PCH101) from ebiosciences, CD25 (2A3) from BD and CD127 (40131) from R&D.

Cell preparation

Mouse LN cells and thymocytes to be injected or maintained in culture were prepared from pooled thymi or pooled lymph nodes (inguinal, axillary, brachial, cervical, popliteal, mesenteric, para-aortic and pancreatic). Analyses were performed on single organs/single mice. Cell suspensions were maintained in 1xPBS/2% FCS at 4^{o}C for purification, or 1xPBS/2% FCS/0.05% NaN3 for analysis. Cell sorting was performed on a FACSaria or MoFlo FACS sorter. For analysis samples collected on BD FACScalibur or Cyan ADP analyzers. Staining was preceded by incubation with anti-Fc receptor blocking antibody and followed with propidium iodide staining.

Human thymocytes were separated on a Ficoll-Paque PLUS (GE Healthcare) density gradient while human PBMC were enriched in $CD4^+$ T cells using RosetteSep (StemCell Technologies), before cell sorting.

Cell culture

Mouse cells were cultured in complete RPMI supplemented with 10 mM Hepes, 1 mM Sodium Pyruvate, 50 μM β-mercaptoethanol (all from Gibco); 10 % FCS and 1% Penicillin/Streptomycin (Invitrogen) and 0.1% gentamicin (Sigma). For APC free cultures, flat bottom 96 well plates were coated with 2 μg/ml anti-CD3 antibody (2C11, in house) in PBS for at least 3 hours and media was supplemented with 1 μg/ml soluble anti-CD28 antibody (in house). APCs were either red blood cell lysed splenocytes depleted of Thy1 positive cells (by anti-Thy1 Ab and complement) and irradiated (3500 rad), or else BM derived DC from GMCSF supplemented cultures. When indicated, APCs were activated with 1 μg/ml Lipid A overnight. In APC containing cultures, media was supplemented with anti-CD3 (2 μg/ml), TGF-β (0.02 ng/ml peprotech), IL-2 (1/250 dilution of X63 cell line culture supernatant) and/or IL-4, IFN-γ, IL-12 or IL-6 (R&D), IL-17 (peprotech), TNFα (R&D), retinoic acid (Sigma) at 100 ng/ml. ^{3}H-thymidine added in the last 6 hours of culture to measure proliferation. Human cells were stimulated for 5 days with plate bound anti-CD3 mAb (2 and 1 μg/ml, respectively, clone OKT3 from Ebiosciences), soluble anti-CD28 mAb (1 μg/ml, clone CD28.2 from Ebiosciences) in medium supplemented with IL-2 (50 U/ml, (NIH)/AIDS Research and Reference Program, [IL-2] from Hoffman-La Roche) and TGF-β (5 ng/ml, peprotech).

Data analysis

FACS data were analyzed with FlowJo (Tree Star, Inc.). All numerical data were processed in Excell (Microsoft Corp.) and plotted in Graphpad Prism (Graphpad Software, Inc.). Statistical significance between sample pairs was determined using a non parametric Man-Whitney test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$), unless otherwise indicated.

Acknowledgements

The research leading to these results has received funding from the European Union's 7th Framework Programme (FP7/2007-2013) under grant agreement n°241447 (NAIMIT) to JD and from the Portuguese Research Council, FCT, under the form of grant, PTDC/SAU-IMU/113541/2009 to IC, fellowships to RSP, ACL, MLB and contract to IC. We acknowledge Drs. Miguel Abecasis and Rui Anjos from Hospital de Santa Cruz, Lisbon, Portugal for human thymus sample collection. We thank Rosa Santos, Ana Regalado and Catarina Martins for assistance with antibody production and mouse colonies maintenance; Rui Gardner and Telma Lopes for assistance in cell sorting and Manuel Rebelo for managing the animal house facility. We also thank Thiago Carvalho for critical reading of the manuscript and constructive input during the preparation of this project.

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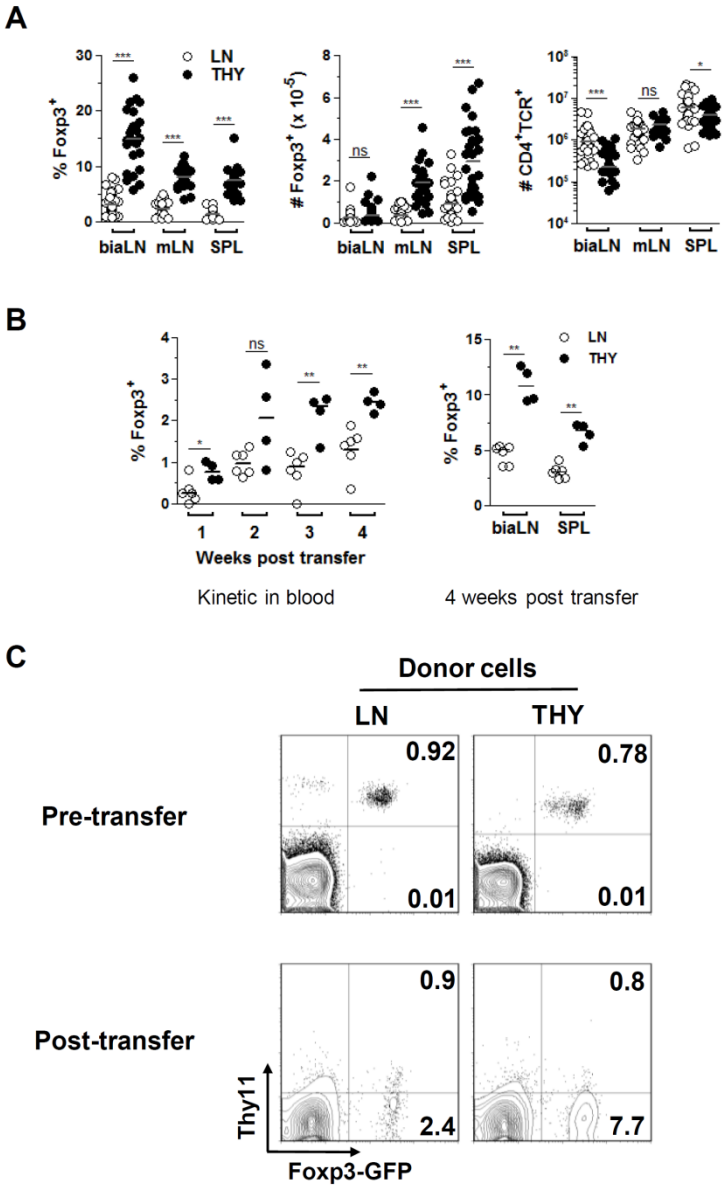
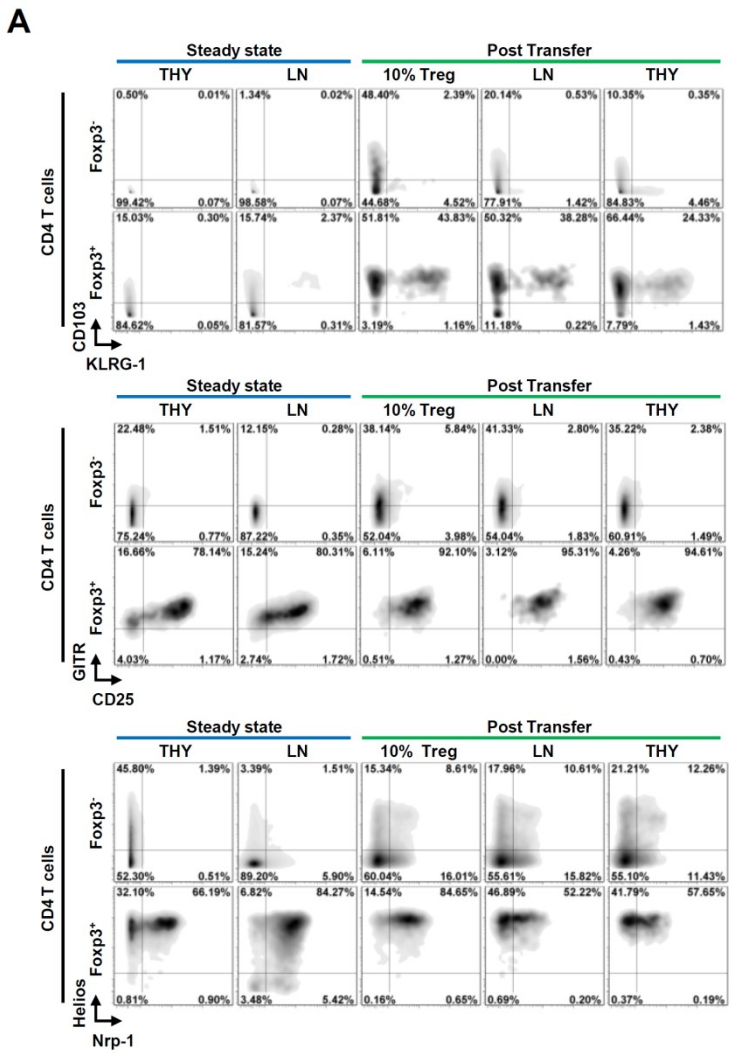
Appendix to Chapter 3 – Supplementary Figures

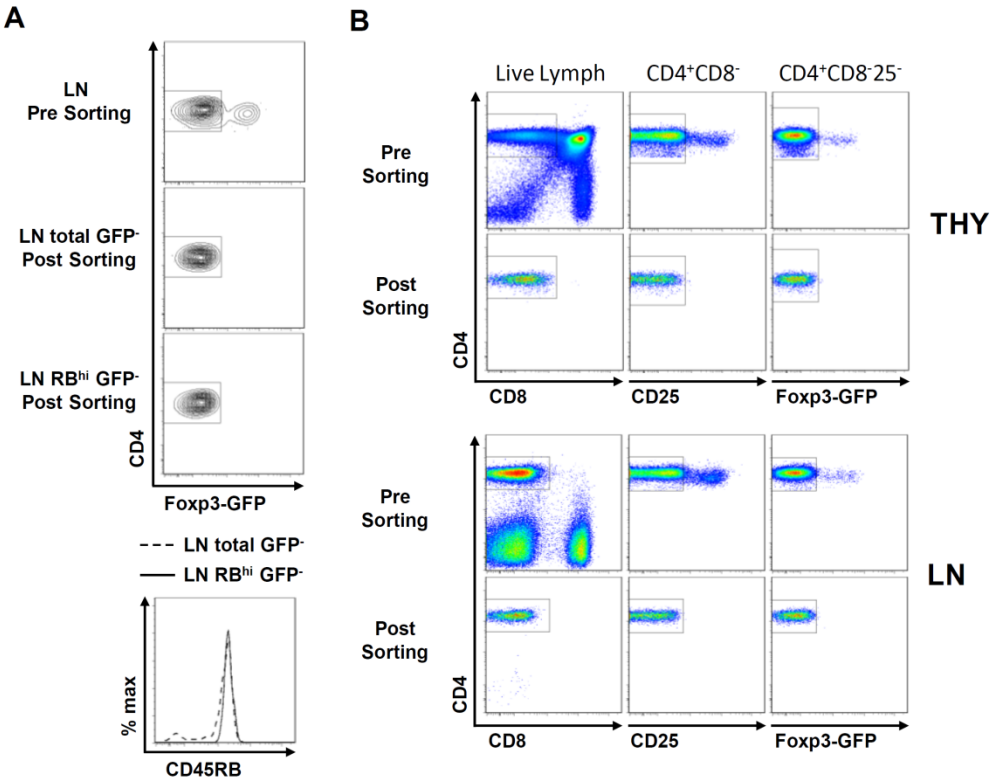


Supplemental Figure S3.1. **True Treg differentiation from peripheral Tconv cells. A)** Consistent preferential differentiation of thymocytes to a Foxp3$^+$ phenotype. Pool of 6 independent experiments conducted as in Figure 3.1. Frequency and numbers of Foxp3 and CD4 cells recovered. **B)** As in Figure 3.1, except that the recipients were Thy1.1 congenic C57BL/6 1 mice that had received 400 rad full body irradiation the day before. Shown is analysis 4 weeks later gated on donor Thy1.2$^+$ CD4$^+$ TCR$^+$ live cells. biaLN – pooled brachial, inguinal and

axillary LNs; SPL – spleen; mLN – mesenteric LNs; PP – Peyer's patches; LIL – large intestine lymphocytes). * p<0.05; **p<0.01; ***p<0.001, Mann-Whitney test. **C**) TCR$\beta^{-/-}$ recipients received a 90:1 mixture of $CD4^+CD8^-Foxp3^-$ (Thy1.2) and $Foxp3^+$ (Thy1.1) T cells, both prepared from either thymi or pooled lymph nodes of Foxp3-gfp reporter mice either Thy1.2 of Thy1.1 congenic, respectively (upper panel). Bottom panel shows analysis of recipient mice 4 weeks post adoptive transfer after gating on $CD4^+TCR^+$ splenocytes.

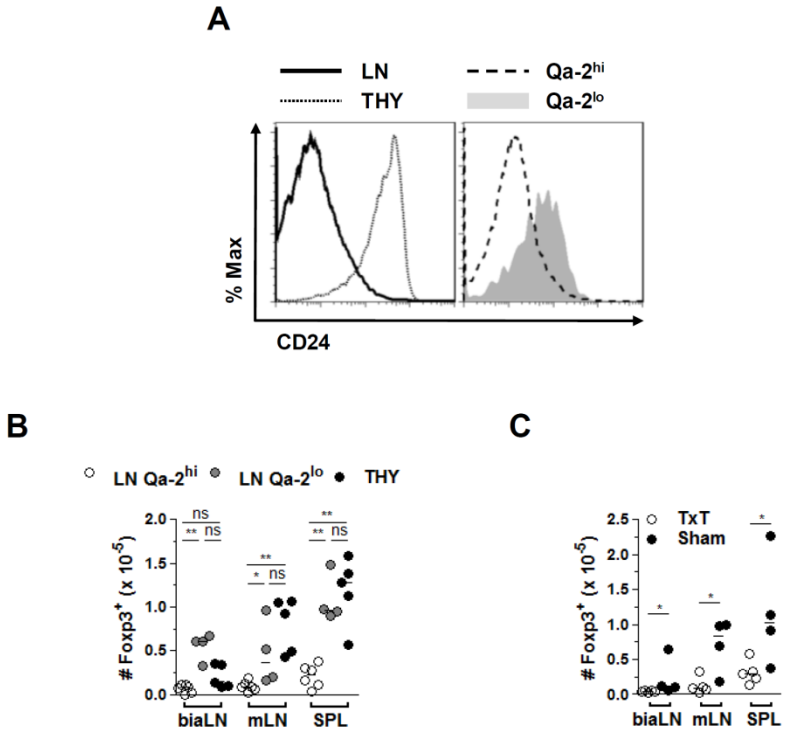


Supplemental Figure S3.2. Pair-wise phenotypic analysis of Foxp3$^-$ or Foxp3$^+$ cells from the spleens of animals treated as in Fig. 3.1A. Shown are representative FACS analysis of recovered Foxp3$^-$ and Foxp3$^+$ cells from thymocytes or LN transfers (two last right columns). Other Foxp3$^-$ and Foxp3$^+$ cell populations served as references: From animals kept at steady state, Thymic Foxp3$^+$ cells and Foxp3$^-$ conventional CD8$^-$CD4$^+$ thymocytes as well as peripheral Foxp3$^+$ and Foxp3$^-$ from LN (first two left columns); from TCR$\beta^{-/-}$ recipient mice that received 4 weeks earlier a mixture of Thy1.1 Foxp3$^-$ and Thy1.2 Foxp3$^+$ LN cells at a 9:1 ratio (central column).

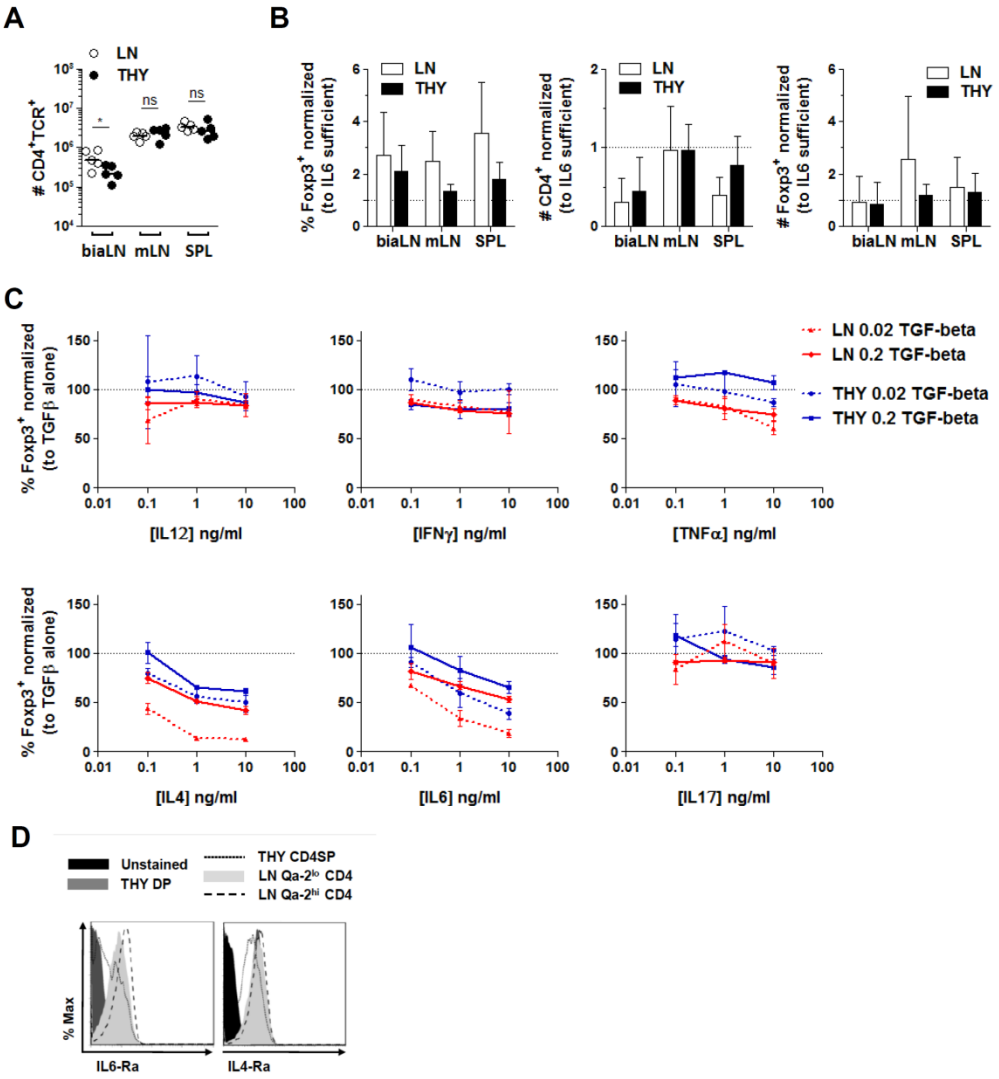


Supplemental Figure S3.3 Representative sorting samples for experiments presented in Fig. 3.3.

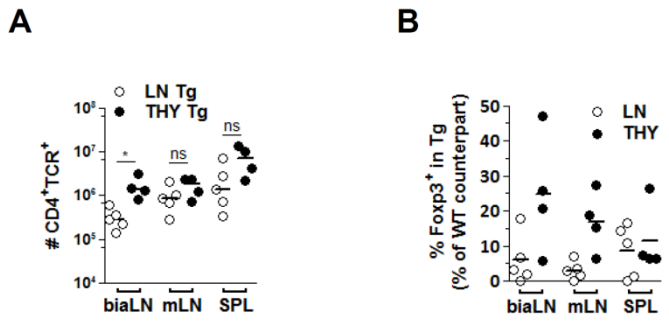
A) Purification of $CD4^{+}Foxp3^{-}CD45RB^{high}$ LN cells used in experiments presented in Fig. 3.3B. **B)** Purification of $CD8^{-}CD4^{+}Foxp3^{-}CD25^{-}$ LN cells and thymocytes used in experiments presented in Fig. 3.3D.



Supplemental Figure S3.4 RTE phenotypic analysis and Foxp3+ cell number of experiments shown in Fig. 3.4. A) Expression level of surface CD24/HSA on $CD4^+CD8^-Foxp3^-$ cells from LN cells and thymocytes (left) and on gated Qa-2^{hi} and Qa-2^{lo} LN cells from 2 months old mice. **B)** Numbers of $CD4^+TCR^+Foxp3^+$cells recovered upon adoptive transfer of peripheral resident mature cells (LN $Qa2^{hi}$), RTE (LN $Qa2^{lo}$) and thymocytes as in Fig. 3.4A. **C)** Idem for adoptive transfer of total $CD4^+Foxp3^-$ LN cells purified from thymectomized (TxT) or sham operated donor mice as in Fig 3.4B.

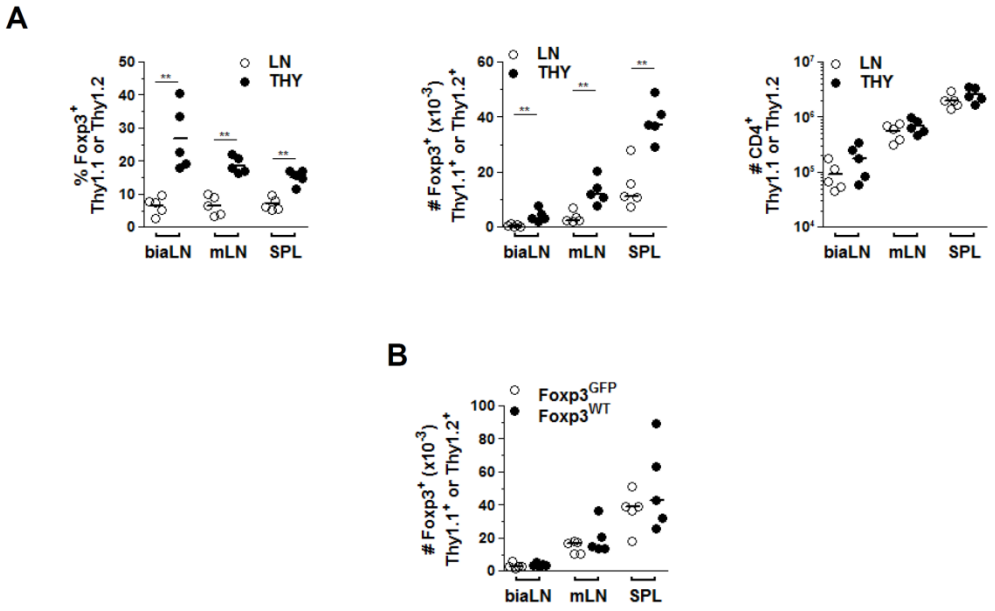


Supplemental Figure S3.5. Complementary data to experiments presented in Fig. 3.5. A, B) Number of $CD4^{+}TCR^{+}$ cells recovered in $TCR\beta^{-/-}$ $IL\text{-}6^{-/-}$ double mutants as in Figure 3.5F (A) and comparison with similar adoptive transfer into IL-6 competent recipient mice (B) where the later are given a value of 1. **C)** Detailed analysis of LN cells and thymocytes sensitivity to candidate inhibitors of Foxp3 induction **D)** surface expression of IL6-Rα and IL4-Rα by the three cell types analyzed in this work.



Supplemental Figure S3.6. Number of CD4 cells recovered and an alternative representation of data presented in Fig. 3.6.

Number of $CD4^{+}TCR^{+}$ cells recovered in $TCR\beta^{-/-}$ mice recipient of TGFβRIIdn transgenic cells as in Figure 3.6F (A) and comparison with similar adoptive transfer from WT Foxp3-GFP donors (B) where the later are given a value of 100.



Supplemental Figure S3.7. Preferential differentiation of Thymocytes vs. LN cells into Treg is not a singularity of the Foxp3-GFP allele. A) $CD8^-CD4^+CD25^-$ thymocytes and $CD8^-CD4^+CD25^-CD45RB^{high}$ LN cells were sorted from WT Thy1.1 and Thy1.2 mice, respectively and co-transferred into $TCR\beta^{-/-}$ mice. Recipient animals were analyzed as in Fig. 3.3C. **B**) $CD8^-CD4^+CD25^-$ thymocytes purified from either WT Thy1.1 or Foxp3-GFP Thy1.2 mice were co-transferred into $TCR\beta^{-/-}$ animals and analyzed as in A.

Chapter 4

in vivo Induced Regulatory T Cells Limit the Extent of Lymphopenia Induced Proliferation

Chapter 4 preliminary notes

The author of the thesis designed and planed the experiments together with the supervisor Dr. Jocelyne Demengeot. The author also participated in all the experiments presented with the participation of the remaining authors mentioned in the beginning of the chapter in special cases. The author also prepared the figures. The analyses and interpretation of the results were mainly performed by the author and the supervisor Dr. Jocelyne Demengeot.

Chapter 4

in vivo Induced Regulatory T Cells Limit the Extent of Lymphopenia Induced Proliferation

manuscript in preparation by

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Abstract

Foxp3 expressing regulatory T cells constitute a subset of CD4 T cells present in humans and mice that has potent regulatory activities. While initially thought to originate exclusively in the thymus it is nowadays accepted that Treg can develop from Foxp3$^-$ T cells isolated from the periphery. By performing adoptive transfer of Foxp3$^-$ T cells to lymphopenic recipients, in which some cells upregulate Foxp3 during the ensuing expansion, we explored the potential of Tregs differentiated from peripheral cells to regulate the global T cell population transferred. To this aim we designed a new protocol to obtain Foxp3 deficient T cells in a naïve state and applied a loss of function approach. Transfer of Foxp3 competent or deficient naïve T cells revealed that the ability of naïve T cells to upregulate Foxp3 allows the control of the expansion of the global T cell population transferred. However, it does not have an impact on T cell activation nor helper cell differentiation that takes place during lymphopenia induced proliferation. By performing secondary adoptive transfers of the Treg developed from peripheral cells together with fresh Foxp3$^-$ T cells we found that these Treg while displaying a low suppressive activity, likely due to low cell numbers present, are a stable lineage in respect to maintaining Foxp3 expression. We also document that iTreg differentiated during lymphopenia upregulate IFNγ receptor which indicates the differentiation of a parallel phenotype to the dominant Th1 polarization associated with the experimental system used. Understanding the regulatory potential and stability of Treg differentiated from peripheral cells is of utmost importance to support the investment in future therapeutic applications aiming at performing adoptive transfer of Treg differentiated from cells of peripheral origin.

Introduction

Foxp3 expressing regulatory T cells (Treg) are in control of pathological immune self reactivity and limit the immune response during infection (1-5). Treg may be generated in the thymus from developing thymocytes (nTreg) or in the periphery after T cells have been exported from the thymus (iTreg) (5-8). Differentiation at both stages follows similar rules with the less mature T cells being more prone to give rise to Treg due to lower sensitivity to pro-inflammatory cytokines, that inhibit Foxp3 expression in more mature T cells, and being able to differentiate to Treg in lower TGFβ availability that diminishes as T cells age (Chapter 2 – manuscript in preparation). While the role of Treg found in the periphery of a naïve mouse, which will likely include both thymic and peripheral derived Treg, has been extensively described and mechanistically characterized, the contribution of Treg generated from each T cell compartment has just started to be explored. However, the technological advances have not yet completely fulfilled the requirements for a definite determination of the relative contribution of thymic and peripheral origin to the peripheral Treg pool. Initial reports have made use of *in vitro* derived Treg to illustrate the functionality of iTreg (6), but recently more elaborated approaches have been applied and *in vivo* derived iTreg have been implicated mainly in the control of mucosal associated antigen specific CD4 reactions. For instance, iTreg seem to be able to control certain aspects of asthma, IBD and successful pregnancy (9-13).

On the other hand, the stability of Foxp3 expression and the phenotypic plasticity of Treg are other crucial features to be understood about Treg if full therapeutic and/or diagnostic value is to be extracted from this T cell subpopulation. Work based in *in vitro* derived iTreg showed that iTreg are more prone to lose Foxp3 expression than Treg isolated from the periphery of naïve mice. This phenomenon has been mainly ascribed to lower demethylation of the Foxp3 promoter region in iTreg when compared to Treg from the periphery of naive mice (14). However, when investigators

evaluated the methylation status of iTreg derived *in vivo* upon adoptive transfer, and therefore thymic independent, of TCR Tg T cells with the following administration of the cognate Ag, the Foxp3 promoter of the Treg that differentiated was fully demethylated as it happens in Treg isolated from naïve mice (15). This report indicates that *in vitro* differentiation of Treg does not fully mimic *in vivo* Treg differentiation, at least in the experimental conditions tested so far. Therefore, the stability of *in vivo* derived iTreg differentiated in different conditions remains an open question.

In a similar phenotypic concern about Treg recent reports have showed that Treg depend on genetic programs elicited in the responding population to control the respective response. Of example is the need for Tbet, IRF4 and Stat3 expression in $Foxp3^+$ cells to be able to control the respective Th1, Th2 and Th17 polarized responses (16-18). These studies have opened the concept of phenotypic plasticity beyond maintenance or loss of Foxp3 expression to a full genetic program that directs Treg activity to the environment which they are exposed to (19). Based on the observations of iTreg differentiation upon lymphopenia induced proliferation (LIP) of Tconv we decided to test if Treg differentiated after adoptive transfer to lymphodeficient recipients would bear the classical Treg function of controlling T cell proliferation. For this aim, we set up a loss of function strategy and developed a new protocol where T cells that cannot become Treg develop in a non inflammatory environment and can be harvested in a naïve state. This is an important detail since mice that are deficient for Foxp3 develop a multi organ auto immune lymphoproliferative disease and are therefore comprised almost exclusively of activated T cells (20). Using naïve peripheral T cells as donor population and assessing the T cell number 4 weeks after transfer to TCRβ deficient recipients we found that the population of T cells that were not able to express a functional Foxp3 expanded more than the Foxp3 sufficient T cell population. Co-transfer of naïve cells from Foxp3 sufficient and deficient background confirmed that

iTreg that differentiate during the transfer are able to control the expansion of the global population of T cells transferred. After confirming functionally and phenotypically that iTreg display a classical Treg phenotype, we decided to test iTreg control of proliferation after a secondary transfer to provide a final proof of its functionality *in vivo*. To our surprise, iTreg transferred with a fresh naive T cell population were not maintained for 4 weeks in the secondary transfer, while if mice recipients of a single transfer were observed 8 weeks after, iTreg were still detectable, indicating that the cells had the potential to be maintained for the entire 8 week period. An evaluation of iTreg surface levels of receptors for cytokines linked with different Th polarization indicates that iTreg cells developed in this condition are likely to depend in IFNγ signaling and their disappearance in co-transfer with fresh conventional T cells suggests iTreg generated through the protocol presented here are only maintained in this inflammatory condition.

This work provides further support to strategies based on the adoptive transfer of iTreg cells but at the same time raises questions to the design of its differentiation according to the type of response that is to be regulated.

Results

Generating Foxp3 deficient T cells in a naïve state

The identification and study of regulatory T cell physiology and function has profited from the discovery and later use of the mutation affecting the scurfy mouse (Sf), found to be a Foxp3 null mutant. These mice suffer from an autoimmune lymphoproliferative syndrome that develops early in life and leads to death of the majority of mice by 4-6 weeks of life (20). The phenotype observed in mice that have a full Foxp3 deficiency (hemizygous males or homozygous females, since the Foxp3 gene is located in the X chromosome) was one of the original pieces of evidence that led to the identification of the Foxp3 transcription factor as a master regulator for the Treg genetic program and perfectly illustrates the essential nature of Treg's regulatory capacity (21). However, to be used in a loss of function approach in the study of iTreg, the direct use of Sf cells is a potential source of confounder effects, given that the vast majority of CD4 cells found in Sf mice are activated and embedded in a complex cytokine milieu. It has been reported that WT recipients of Sf BM donors are healthy, even if the recipients are subjected to lethal irradiation regimens before transfer. The lack of disease transfer to WT recipients has been ascribed to radio resistant Treg that are not eliminated through radiation (22). This report is a good example of Treg dominant mode of tolerance which allows the use of cells carrying a WT Foxp3 allele to keep Foxp3 deficient T cells under control.

In this work we made use of this knowledge and designed a bone marrow (BM) transfer system to obtain naïve Foxp3 deficient T cells. The protocol used is depicted in Figure 4.1A and consists of transferring C57BL/6J (Thy1.2) Sf BM to congenic C57BL/6J Thy1.1 recipients (hereafter referred to as Thy1.1) that had received a 900 rad total body irradiation regimen the day before. We also transferred 10^7 total splenocytes from Thy1.1 donors a

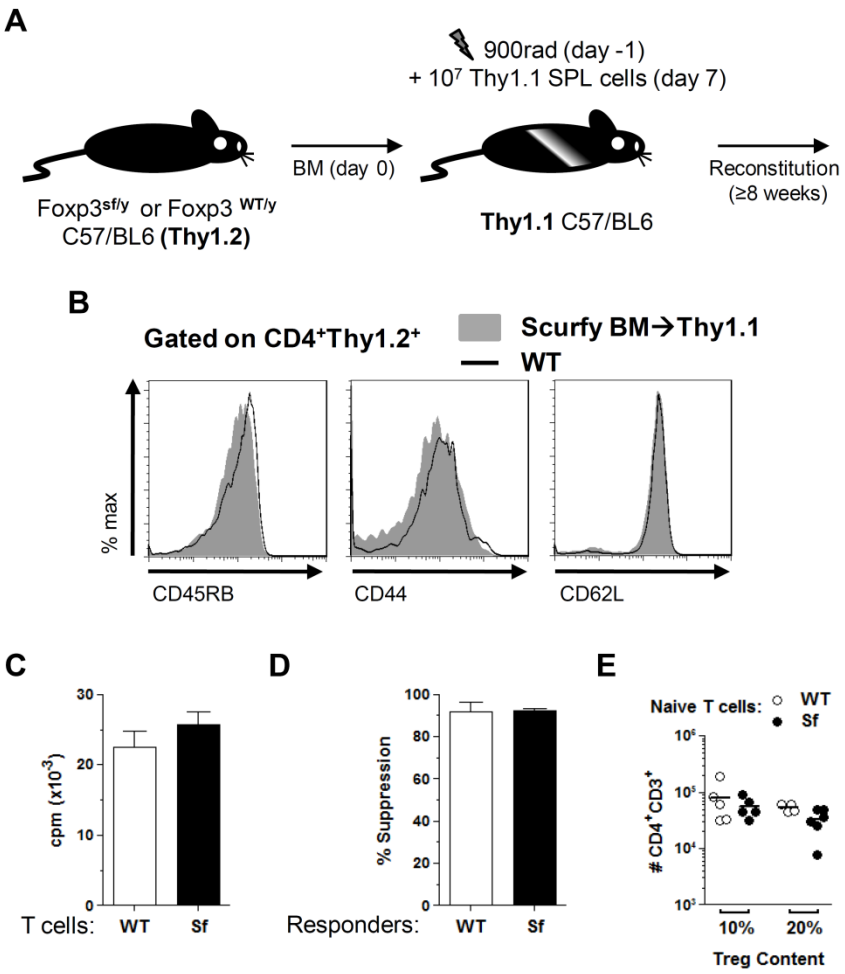

Figure 4.1. Foxp3 deficient Naive T cells can be prepared by transferring Scurfy bone marrow to WT recipients. (A) Bone marrow transplantation protocol to obtain Foxp3 deficient T cells and the respective WT controls. Briefly, bone marrow was extracted from femurs and tibias of Scurfy or WT (Thy1.2) donors and $5x10^6$ cells, after T cell depletion and red blood cell lysis, were transferred i.v. to WT (Thy1.1 congenic) recipients that had received 900 rad total body irradiation the day before. Recipients of bone marrow received a transfer of 10^7 WT (Thy1.1) total splenocytes 7 days after to minimize the activation and expansion of the first cells to differentiate from the transplanted bone marrow. The peripheral T cell pool of recipient mice was allowed to reconstitute for at least 8 weeks before mice being used as donors of Scurfy or WT naïve T cells. (B) Mice receiving Scurfy bone

marrow following the protocol described in A were analyzed by flow cytometry for the expression of the activation markers, CD45RB, CD44 and CD62L and were found to display a naïve phenotype. Shown are representative histograms of $CD4^+Thy1.2^+$ cells from at least 5 mice recipients of Scurfy bone marrow (grey shaded) and fresh WT cells (solid line). (C-E) $CD4^+CD25^-CD45RB^{hi}Thy1.2^+Thy1.1^-$ cells from LNs of mice prepared as in A were FACS sorted and: cultured *in vitro* for 3 days in the presence of plate bound αCD3, soluble αCD28, IL-2 and anti-TGFβ (C); co-cultured in the presence of Treg at a ratio of 2:1 and T cell depleted irradiated splenocytes and αCD3 (D); or co-transferred in the presence of $CD4^+Foxp3\text{-}GFP^+$ Treg. Shown are the c. p. m. derived from tritiated thymidine incorporation during the last 6 hours of culture (C and D) and the number of cells recovered 4 weeks after transfer (E).

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week after the BM transfer, to minimize lymphopenia induced proliferation of the first nascent T cells and therefore guarantee a naïve and undisturbed peripheral cell pool of Foxp3 deficient T cells. The peripheral pool of lymphocytes in BM recipients was then allowed to reconstitute for at least 8 weeks before mice being used as T cell donors or analyzed. Haribhai *et al* have recently used a similar approach to achieve the same goal. Their protocol was based on transferring splenocytes to newborn Sf mice (10, 11). However, we prefer, and recommend, the use of a BM transplant protocol since it allows for the maximization of the usually limiting Foxp3 deficient mice, given that a single donor can contribute with bone marrow to at least 5 recipients. In parallel, WT (Thy1.2) BM was also transplanted to similar Thy1.1 recipients to be a source of control, Foxp3 sufficient, T cell donors in subsequent experiments.

BM chimeras prepared according to our protocol survived for several months without showing signs of disease (data not shown). We first analyzed the peripheral T cells of Thy1.1 mice reconstituted with Sf BM and found that

$CD4^+$ $Th1.2^+$ T cells presented similar levels of CD45RB, CD44 and CD62L as fresh naïve T cells isolated from WT non manipulated donors (Fig. 4.1B). We also found Sf naïve T cells to be functionally equivalent to WT naïve T cells in *in vitro* proliferation assays (Fig. 4.1C). Likewise, naïve Sf T cells were equally suppressed by fresh peripheral Treg from naïve WT mice, both *in vitro* and *in vivo* (Fig. 4.1D and E). These data confirm that Sf cells maintain a naïve state in our system, are as functional as WT naïve cells generated through the same protocol and, as such, are a valid sample for Foxp3 loss of function approaches in naïve T cells when used in comparison to WT naïve T cells generated through the same protocol, for which the main differences to be observed are due to the expression, or the lack of, a functional Foxp3 protein.

Loss of function of Treg differentiated from peripheral cells in vivo during Lymphopenia Induced Proliferation – control of T cell activation

Treg can be induced to differentiate *in vivo* from peripheral cells by different protocols, such as slow low dose antigen delivery, antigen delivery together with T cell activation modulating antibodies, like αCD4, oral delivery of antigen or direct Ag delivery to Dendritic cells (23-26). However, these are approaches which best fit the use of TCR Tg systems and pose difficulties when applied to polyclonal T cell populations, mainly due to the low frequency of clones existing at steady state to any given antigen. On the other hand, one can transfer WT polyclonal $Foxp3^-$ CD4 T cells (Tconv) to lymphopenic recipients and a proportion of these cells upregulates Foxp3 expression while expanding (27). While this system is more demanding to test for Treg function, it allows the use of polyclonal WT cells avoiding potential TCR Tg derived artifacts. This system allowed us to assess the differentiation and role of *in vivo* derived iTreg.

To test iTreg function we performed transfers of Scurfy or WT naïve T cells to TCRβ KO mice analyzing lymphoid organs 4 weeks after transfer as depicted in Figure 4.2A. Naïve T cells were isolated by FACS sorting $CD4^+$ $Thy1.2^+$ $Thy1.1^-$ $CD25^-$ $CD45RB^{hi}$ cells. This sorting strategy displayed a typical contamination of $Foxp3^+$ cells below 1% when sorting WT cells (Fig. 4.2B). While offering an efficient Treg depletion when sorting WT cells, this protocol also allowed us to sort the equivalent naïve T cells from Sf origin and, according to the report by Gavin *et al* (28), exclude cells that were supposed to be $Foxp3^+$ in the Foxp3 deficient background.

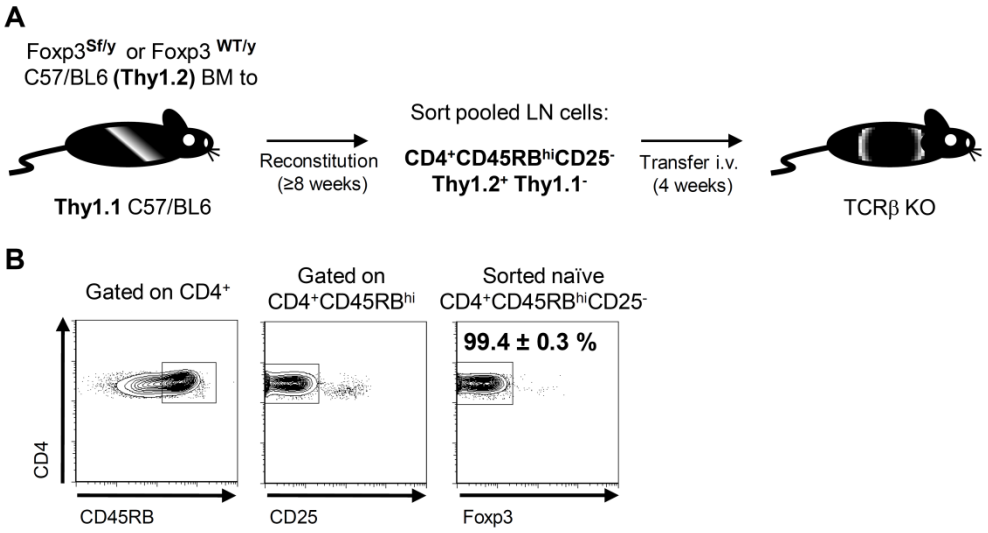

Figure 4.2. Protocol to differentiate iTreg *in vivo* during lymphopenia induced proliferation. (A) Mice prepared as in Fig. 4.1A were used as donors of $CD4^+CD25^-$ $CD45RB^{hi}CD25^-$ T cells which were transferred i.v. to TCRβ deficient recipients. Mice were then analyzed 4 weeks after transfer. (B) Gating strategy used to sort naïve T cells. Number inside the last plot represent the frequency of $Foxp3^-$ cells inside $CD4^+CD45RB^{hi}CD25^-$ lymphocytes (n=5) in an analysis of WT mice.

Adoptive transfer of naïve T cells to lymphopenic recipients has been widely used as an experimental model of colitis and the protective effect of Treg co-transfer in this system a landmark in setting the role of Treg in regulating T cell activity (29). While it was expected that the majority of naïve T cells gets activated in this protocol we decided to assess if the ability of the naïve T cell population transferred to express Foxp3 would have an impact in the extent of such activation. We followed T cell activation by analyzing CD44 upregulation and CD62L downregulation in cells recovered from recipients of Sf or WT naïve T cells' transfer. Analysis of these markers showed a similar activation of naïve T cells irrespective of their Foxp3 genotype (Fig. 4.3A, B and C).

As another read-out of T cell activation, and to test if the ability of naïve T cells to upregulate Foxp3 had an impact in regulating helper T cell differentiation during LIP, we performed single cell analysis of cytokine secretion in T cells harvested after the same transfers as above by flow cytometry, after in vitro stimulation of the harvested T cells with PMA/Ionomycin followed by standard intracellular stainings. This analysis revealed a similarly high frequency of Th1 cells, little Th17 and undetectable Th2 cells, as measured by the frequency of cells secreting IFNγ, IL-17 and IL-4, respectively, in cells recovered from recipients of both WT and Sf origin. In the same line of the above findings, the frequency of IL2 producing cells was also similarly high in both expanded WT and Sf T cells (Fig. 4.3D-H). These results indicate that Treg developed during LIP are not able to control the activation and helper cell differentiation of the expanding naïve T cell population.

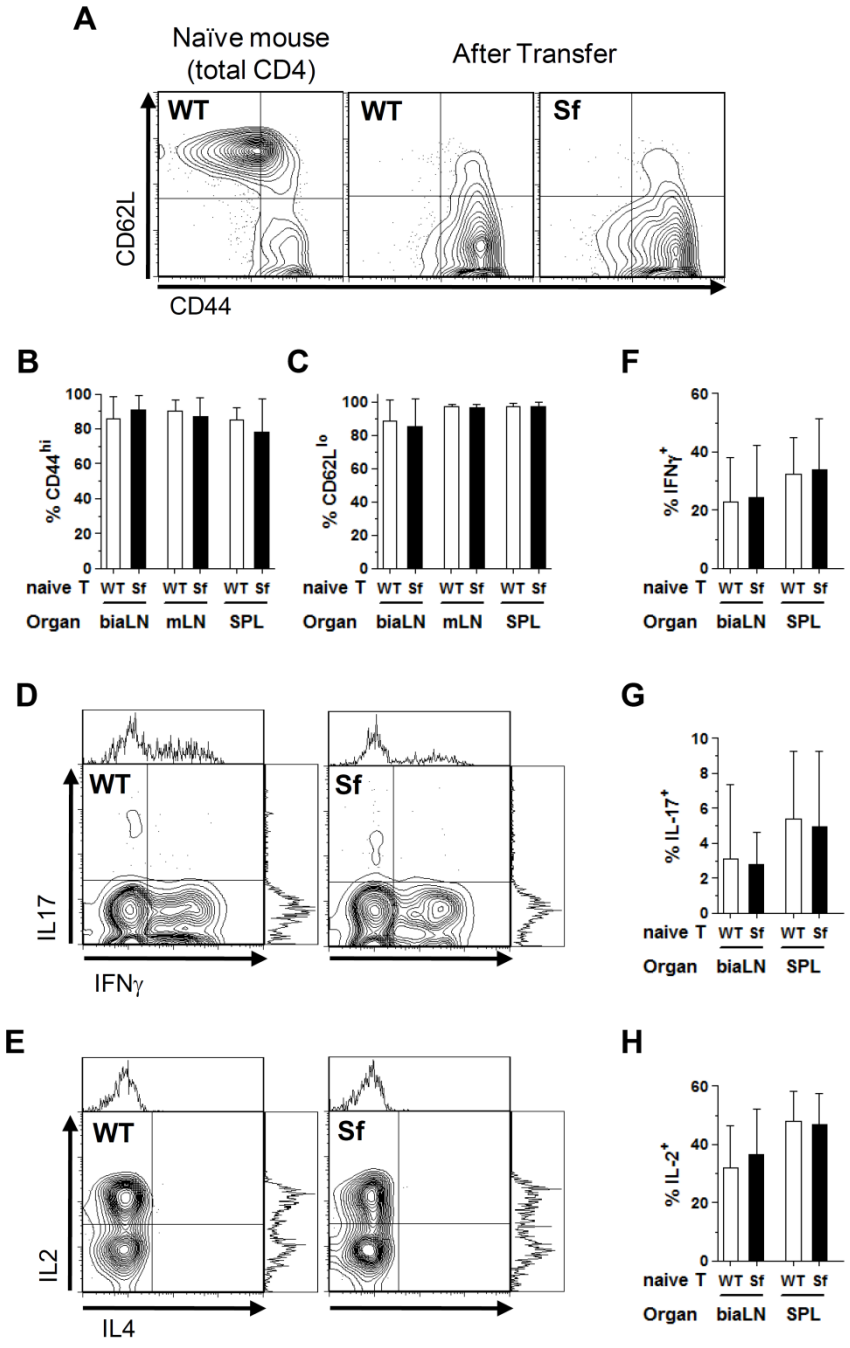

Figure 4.3. *in vivo* derived iTreg do not control lymphopenia induced T cell activation. (A-G) Analysis of recipients of transfers performed according to the protocol depicted in Fig. 4.2A. $CD4^+CD3^+$ cells were analyzed for activation by CD44 and CD62L expression (A-C). (A) Representative FACS plots for CD62L *vs*. CD44

profiles of $CD4^+CD3^+$ cells from naïve WT mice (left) or from TCRβ KO mice recipients of WT (middle) or Sf (right) naïve T cells 4 weeks after transfer. Percentage of activated T cells represented as upregulation of CD44 (B) and downregulation of CD62L (C) inside $CD4^+CD3^+$ cells from all mice analyzed is shown. (D-H) $CD4^+CD3^+$ cells from the same recipients were also analyzed for IL-17, IFNγ, IL-4 and IL-2 secretion after in vitro stimulation with PMA and ionomycin. Shown are the representative FACS plots of IL-17 *vs.* IFNγ profiles (D) and IL-2 *vs*. IL-4 profiles (E) with the corresponding percentage of $IFN\gamma^+$ (F), $IL\text{-}17^+$ (G) and $IL\text{-}2^+$ (H) cells within $CD4^+CD3^+$ cells recovered from the mice analyzed. IL-4 secreting T cells were not detected.

The differentiation of iTreg during lymphopenia induced proliferation upon adoptive transfer to T cell deficient recipients limits the expansion of the T cells transferred

As introduced above, it is known that $Foxp3^-$ T cells upregulate Foxp3 expression upon transfer to lymphopenic recipients (27). With our protocol we found that 1 to 40% of the recovered T cells (15.8±10.5; 6.1±3.3; 4.9±2.9; Mean±SD for $\%Foxp3^+$ inside $CD4^+CD3^+$ cells recovered from biaLN, mLN and SPL, respectively) expressed Foxp3 (Fig. 4.4A). At the same time we confirmed that the majority of recipients of Sf cells were devoid of $Foxp3^+$ cells, with a small number of exceptions where a minor contamination of Thy1.1 cells from the sorting were probably co-transferred, without any measurable effect in the parameters measured. Together with the sorting strategy used this result allowed us to ascribe the observed differences to the presence of iTreg. The seminal paper by Sakaguchi *et al* that identified $CD25^+$ cells as the CD4 T cell subset largely enriched in the long sought after Treg, showed that the co-transfer of $CD25^+$ cells could control the

expansion of naïve T cells upon transfer to T cell deficient recipients (1). The capacity of Treg to control the proliferation of Foxp3$^-$ T cells later became a prototypical property of Treg. Therefore, we followed the number of T cells recovered from recipients of WT and Sf naïve T cells to test if the differentiated iTreg were actively controlling the proliferation of the transferred T cell population. Despite WT naïve T cells displaying a robust expansion, as expected, Sf cells originated approximately 4 to 6 fold, between minimums and maximums, respectively, and about 6 fold, between averages, higher cell numbers (Fig. 4.4B - WT and Sf samples). This result is an indication that Foxp3 expression by naïve T cells during LIP contributes to control the extent of expansion of the transferred T cell population. However, and despite our careful design of the naïve T cell preparation protocol, we could not fully exclude that some unforeseen difference between WT and Sf naïve T cell populations was the source of the increased cell number obtained from Sf cells. To test if the presence of iTreg was indeed sufficient to limit the expansion of naïve T cells under LIP we made use of the dominant mode of regulation known to be a property of Treg, and designed co-transfer strategies where naïve Sf cells would expand in the presence of iTreg originating from a WT naïve T cell subset. First we transferred the same number of T cells again to TCRβ deficient recipients, with WT and Sf naïve T cells each composing half of the population. Analysis 4 weeks after transfer revealed that the number of T cells recovered was similar to the transfer of the WT cells alone and decreased when compared to the transfer of Sf cells (Fig. 4.4B - mix). This result strongly suggests that iTreg indeed control the expansion of the global naïve T cell pool transferred, irrespective of half not being able to upregulate Foxp3. On the other hand, this system was not designed to retrospectively identify WT and Sf donors which made it impossible to determine if both cells expanded similarly. However, another experiment was performed using WT Thy1.1 cells harvested from a Thy1.2 recipient of Thy1.1 BM, using the same strategy illustrated in Figure 4.1A but with the allotypic markers

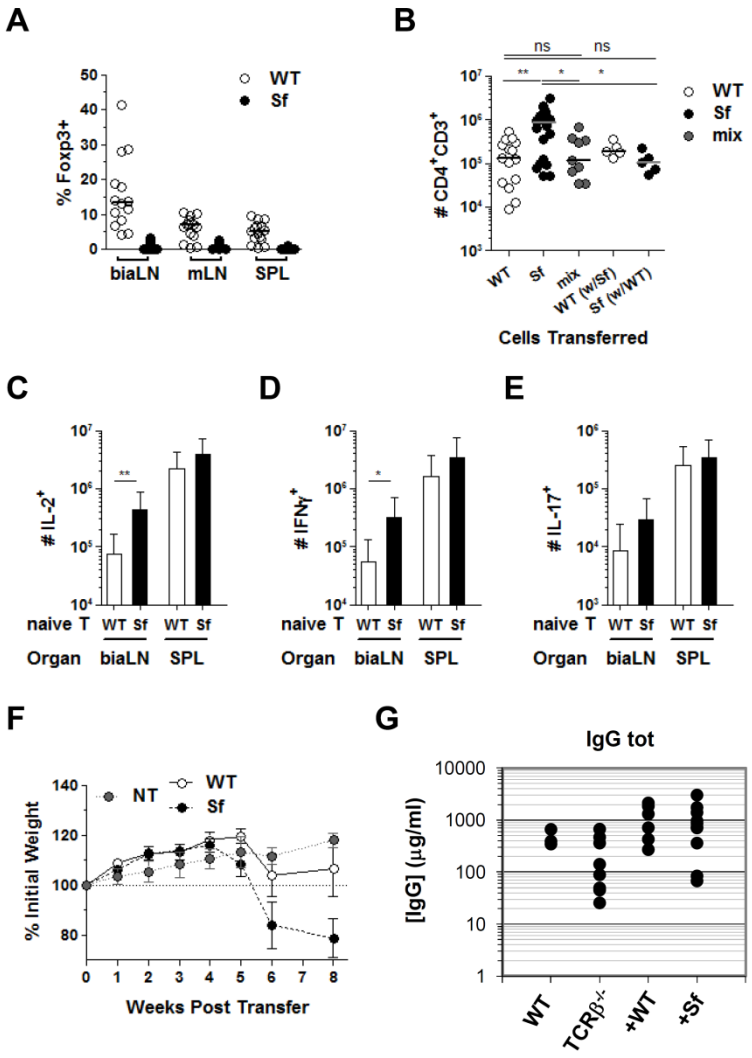

Figure 4.4. Lymphopenia derived iTreg limit the number of T cells recovered after naïve T cell transfer to lymphopenic recipients. (A,G) 10^6 Naïve T cells were transferred to TCRβ KO recipients according to the protocol depicted in Fig. 4.2A, with adaptations for some cases (B,E). (A) Frequency of $Foxp3^+$ in $CD4^+CD3^+$ cells. (B) $CD4^+CD3^+$ Cell numbers for the transfers performed as illustrated in Fig. 4.2A (WT and Sf samples), for experiments with a mix of 50% WT and 50% Sf naïve T cells (mix sample) and for co-transfers of WT and Sf cells at 1:1 ratio but analyzed in separate (WT (w/Sf) and Sf (w/WT), for the calculation of WT cells recovered from transfers in the presence of Sf cells, and for the calculation of Sf cells recovered in the presence of WT cells respectively). (C-E) Number of $IL\text{-}2^+$ (C), $IFN\gamma^+$ (D) and IL-

17^+ (E) $CD4^+CD3^+$ cells. (F) Weight change of mice subjected to the protocol depicted in Fig. 4.2A was followed up to 8 weeks after transfer. (G) Seric total IgG detected 4 weeks post transfer. * P<0.05; **P<0.01; ***P<0.001; Mann-Whitney U test.

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switched for the case of WT BM donor and recipient. In this case each T cell donor contributed with the same number has in the transfer of single populations, making the number of cells transferred double in total. In this setup, the number of cells harvested from WT donors was similar to the single transfer, while the donors of Sf cells gave rise to reduced number of cells (Fig. 4.4B - WT (w/Sf) and Sf (w/WT)) possibly indicating an unexpected impact of Sf cells in the function of iTreg, and/or vice-versa. This collection of data shows that the differentiation of iTreg during LIP contributes to limit the extent of naïve T cell expansion.

In spite of the undetectable effect in T cell activation reported in Figure 4.3, it is plausible that the higher number of T cells due to the absence of iTreg leads to some effect at a functional level, such as increased pathology. First, the higher number of T cells recovered from recipients of naïve T cells of Sf origin resulted in the total number of IL-2, IFNγ and IL-17 producing T cells developing from Sf naïve T cells to be increased in comparison with the number of cells recovered from recipients WT cells (Fig. 4.4C, D and E). On the other hand, while most experiments were analyzed 4 weeks after transfer, before any disease manifestation was observable, recipients of scurfy cells did show an accelerated weight loss 5-6 weeks after T cell transfer (Fig. 4.4F) without showing an increase in the seric total IgG levels 4 weeks post transfer (Fig. 4.4G), both in comparison to recipients of WT cells. This effect indicates that the control of T cell number but not of activation

leads to a different kinetic in disease manifestation without changing other properties.

To further test the capacity of iTreg to control the lymphopenia induced expansion of naïve T cells we performed a sequential transfer of naïve T cells in which a group of TCRβ KO mice first received cells of Sf or WT origin and a second population of WT Foxp3-GFP$^-$ Tconv cells being transferred to all recipients 14 days after (Fig. 4.5A). In this setup it was predicted that iTreg differentiated in the recipients of WT cells would contribute to limit the number of cells recovered from the second transfer. However, while there was a small trend to recover less cells from the second transfer from the recipients that had received WT cells in the first transfer in comparison to recipients of Sf cells, the effect was small and not statistically significant (Fig. 4.5B).

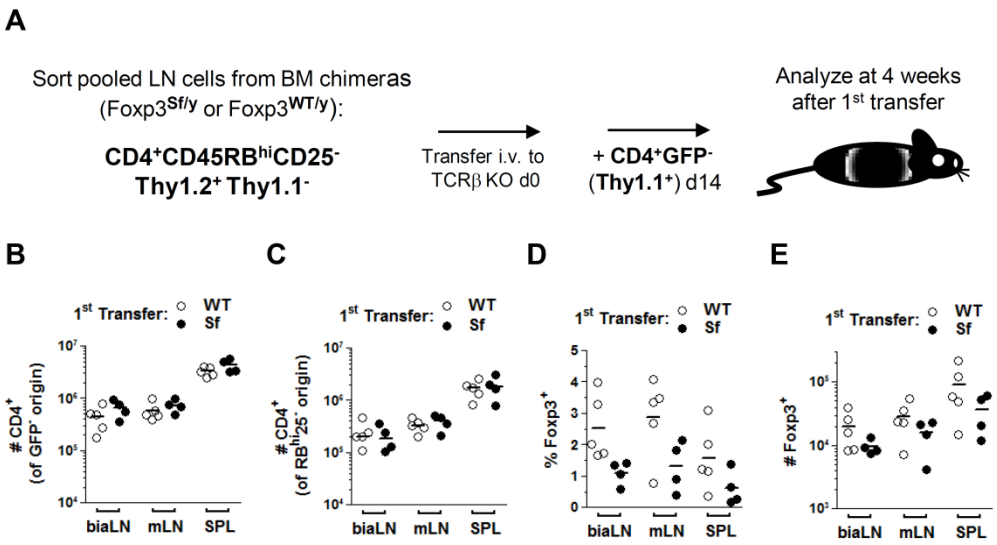

Figure 4.5. iTreg differentiated while the expansion of naïve T cells is ongoing are able to control the final number of T cells. (A) Illustration of the protocol of sequential naïve T cells transfer. Briefly naïve T cells of WT or Sf origin prepared as indicated in Fig. 4.1A were transferred to TCRβ KO recipients. 14 days later all recipients received a second transfer of Foxp3-GFP$^-$ CD4 cells and were analyzed 4

weeks after the first transfer. (B) Number of $CD4^+$ cells of Foxp3-GFP^- origin (second transfer) recovered in recipients of WT or Sf cells in the first transfer. (C) Number of $CD4^+$ cells of naïve origin (first transfer) recovered in recipients of WT or Sf cells in the first transfer. (D) Percentage of $Foxp3^+$ in total $CD4^+$ cells and total number of $CD4^+Foxp3^+$ cells (E).

In the same direction, the number of T cells recovered from the first transfer was also similar irrespective of their WT or Sf origin (Fig. 4.5C). However, when the frequency and number of Foxp3+ cells in both groups of recipients was analyzed there was not a significant reduction of Treg in recipients of Sf cells (Fig. 4.5D). Surprisingly, this experiment revealed that even if iTreg are only allowed to differentiate past 2 weeks post transfer they can have a strong effect in the proliferation of the expanding T cell population. On the other hand, from the data in Figure 4.4B there were already indications that having only half of the population of naïve T cells able to differentiate to Treg was sufficient to limit the lymphopenia induced T cell expansion. Alternatively, if the second transfer would be performed with Sf cells, which was not possible due to the lack of a Thy1.1 congenic Foxp3 deficient strain, one would expect the recipients of WT naïve T cells in the first transfer to limit the expansion of T cells from both first and second transfer, in contrast to the recipients of Sf cells in both transfers. Nevertheless, this collection of data reinforces that iTreg developed during LIP have a regulatory role in the expansion of naïve T cells.

iTreg differentiated during lymphopenia induced proliferation display stable Foxp3 expression but fail to be maintained by naïve T cells

One of the most active discussions in the Treg field is to what extent iTreg are different to nTreg. The recent literature has however focused the

attention in rare differences identified rather than in the more common similarities. In fact some recent papers (10, 11, 30) have found that the majority of the transcriptional signature found in Treg isolated from the periphery of naïve mice, or subjected to the same protocols as iTreg, is shared. The work by Feurer *et al* where iTreg generated by different methods were compared to both thymic nTreg and peripheral Treg is rather striking in showing that the transcriptional profiles of Treg vary according to the conditions the cells are exposed to (30). While not biologically surprising, it illustrate how shallow most approaches to identify iTreg specific markers may have been so far. At the same time this work clearly illustrates the plasticity of Treg to adopt a transcriptional program that matches its microenvironment and opens the possibility that iTreg and nTreg are equivalent but have the potential to present diverse phenotypes, what may be expected is that Treg differentiated in the periphery display a set of phenotypes more frequently than Treg generated in the thymus that differentiate in the presence of a rather constant set of cues. To further address the properties of iTreg differentiated during lymphopenia we first assessed the expression of classical Treg associated markers to ascertain that iTreg presented a classical Treg phenotype beyond Foxp3 expression. Flow cytometry analysis revealed the expression of high levels of Foxp3 and the Treg classical markers CD25, GITR and CD103 (Fig. 4.6A and B), which were accompanied by an increase in the representation of cells positive for the same markers (Fig. 4.6C), all in comparison to peripheral Treg isolated from naïve mice. In vitro proliferation assay revealed that iTreg were equally able to suppress the proliferation of $Foxp3^-$ T cells and were equally anergic as Treg isolated from the periphery of naïve mice (Fig. 4.6D). Having confirmed that iTreg differentiated in our protocol resembled peripheral Treg, probably in an activated state, we decided to test if the iTreg cells we obtained displayed stable Foxp3 expression. The ability of Treg to maintain Foxp3 expression is another property that has been explored and met significant advances in recent works. On one hand, it has been shown that

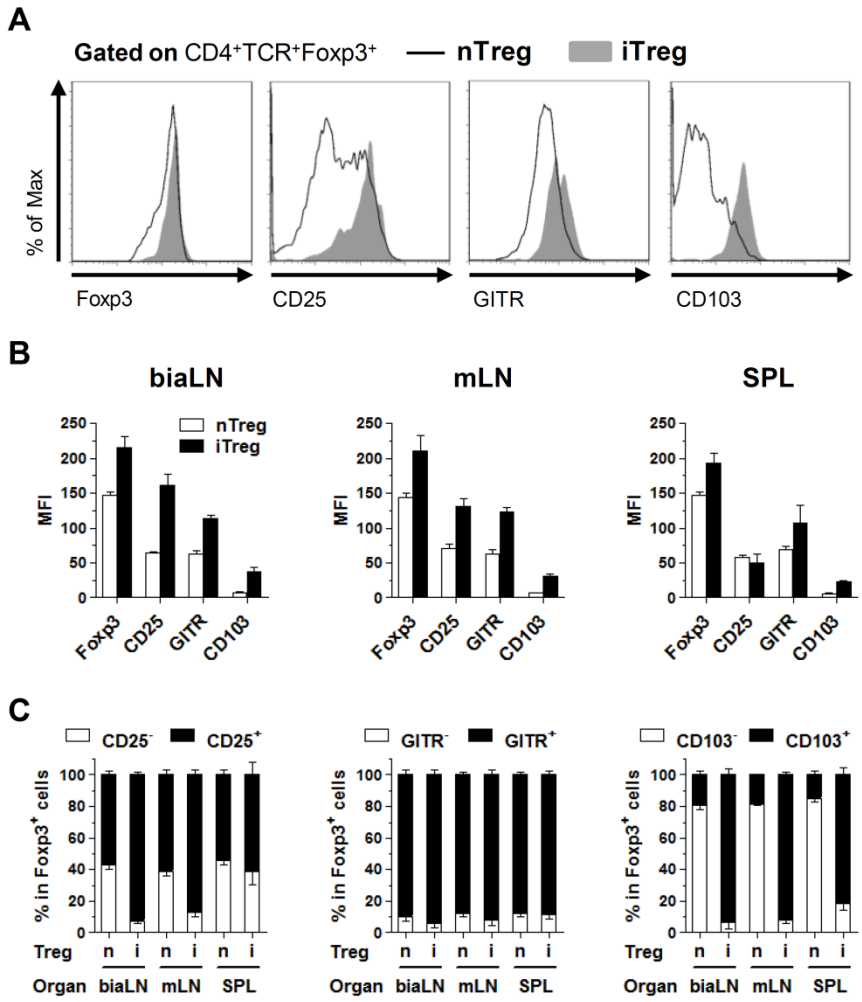

Figure 4.6. iTreg display characteristic Treg phenotype. (A) Representative histograms of Foxp3 (far left), CD25 (middle left), GITR (middle right) and CD103 (far right) expression in steady state fresh Treg or iTreg developed during naïve T cell expansion in TCRβ KO recipients according to the protocol illustrated in Fig. 4.2A. (B) Mean fluorescence intensity (MFI) detected in cells isolated from several lymphoid organs, in the analysis shown in A. (C) Relative composition of positive cells for each marker inside $CD4^+Foxp3^+$. (D) Proliferation and suppression assays. Foxp3-

GFP$^-$ T cells were incubated *in vitro* in the presence of T cell depleted and irradiated splenocytes and stimulated with soluble αCD3 and αCD28 for 3 days, either alone (Tresp) or in the presence of half the amount Treg isolated from the periphery of naïve mice (+nTreg) or from the LNs of TCRβ KO recipients of Foxp3-GFP$^-$ T cells 4 weeks earlier (+iTreg). Both Treg populations were also incubated in the same conditions in the absence of responder T cells (nTreg and iTreg). Shown are the c. p. m. resulting from the incorporation of tritiated thymidine during the last 6 hours of culture

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peripheral Treg have the potential to lose Foxp3 expression and gain a helper cell phenotype, a process that may undermine Treg based therapeutic approaches (31, 32). On the other hand, one of the initially identified differences between Treg induced from peripheral cells and Treg isolated from the periphery of naïve mice, was the capacity of the later to sustain Foxp3 expression in contrast to the rapid loss presented by the former (14). However, the later interpretation may be misleading given that the iTreg tested in that work was prepared using the in vitro protocol alone, which may not represent in vivo derived iTreg. To test the stability of iTreg differentiated during LIP we sorted Thy1.1 or Thy1.2 cells, either iTreg from transfers of Foxp3-GFP$^-$ Tconv cells to TCRβ KO recipients that had lasted 4 weeks or Foxp3-GFP$^+$ Treg from naïve mice. We also sorted Thy1.2 or Thy1.1 Foxp3-GFP$^-$ Tconv cells from the Foxp3-GFP reporter mouse to be the respective Thy1.1 or Th1.2 Treg pairs. Thy1.1 + Thy1.2 pairs of iTreg + Tconv and nTreg + Tconv were then co-transferred to TCRβ KO recipients and analyzed 4 weeks after. Since this experiment also had the potential to test the regulatory capacity of iTreg in re-transfer system we monitored the number of Tconv cells originated in recipients of each Tconv pairing with the different Treg. Surprisingly, iTreg displayed no control of Tconv expansion illustrated by similar numbers of Tconv recovered from Tconv transferred

alone or with iTreg, while co-transfers with nTreg resulted in the expected reduction of Tconv cells recovered (Fig. 4.7A). Given the data presented in Fig. 4.3B it was expected that iTreg would be able to control the expansion of Tconv cells, at least to some extent. However, when we assessed the number of cells from Treg origin recovered we found that very little cells from iTreg origin were present (Fig. 4.7B). The absence of iTreg cells at the end of the experiment provides a simple explanation to the absence of suppression of T cell proliferation. Still, regarding the main aim of the experiment, the few cells recovered still expressed Foxp3 (Fig. 4.7C) indicating that iTreg differentiated during LIP are able to maintain Foxp3 expression. Another pertinent question that this experiment allowed us to address is if the presence of iTreg or nTreg has an impact in the differentiation of iTreg from the Tconv population. This was achieved by simply measuring the Foxp3 frequency inside the cells originally $Foxp3^-$, which was found to be similar irrespective of Tconv cells being transferred alone (Fig. 4.7D), in the presence of iTreg or nTreg, showing that Treg presence has little impact in the differentiation of iTreg during LIP.

The fact that little cells of iTreg origin were recovered after a secondary transfer raised the question of iTreg persistence. One hypothesis could be that iTreg are generated and are not kept for long, however, using the same protocol illustrated in Fig. 4.2A, but performing the analysis 8 weeks post transfer, we still found $Foxp3^+$ cells (Fig. 4.7E) with a slight increase in frequency as what was found in the analysis at 4 weeks (Fig. 4.7E vs Fig 4.4A), showing that iTreg cells can be maintained for the entire 8 week period that the experiment with the secondary transfer adds up to for the iTreg cells. An alternative explanation is that iTreg cells once differentiated and maintained in the inflammatory milieu that takes place during LIP are, or become, dependent on some of those inflammatory cues. This is not a completely

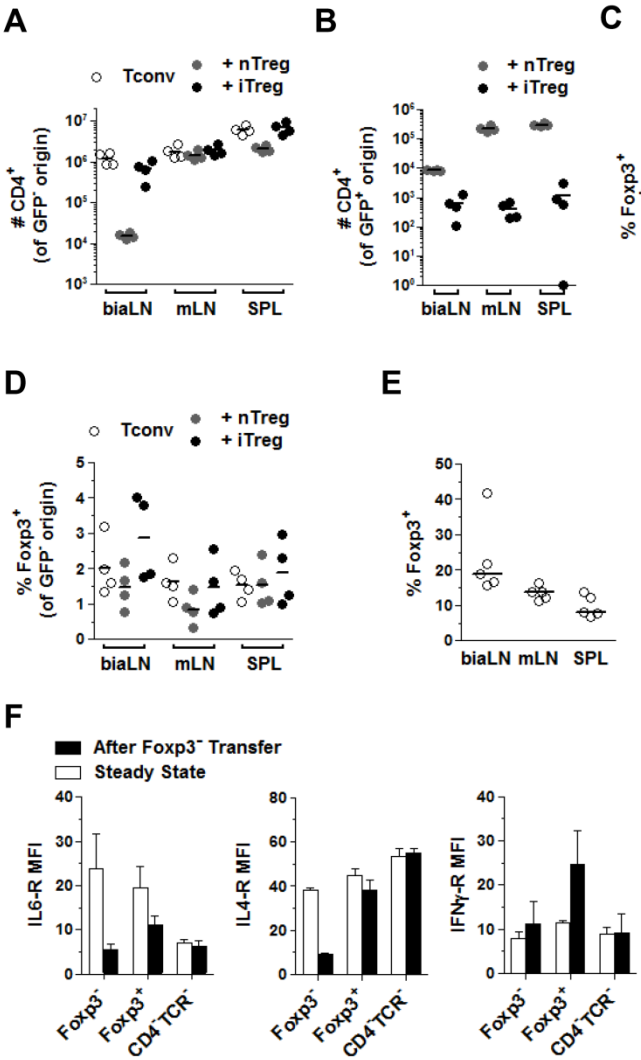

Figure 4.7. iTreg developed from naïve T cells upon transfer to lymphopenic recipients display stable Foxp3 expression but fail to control the expansion of fresh Foxp3$^-$ cells. Foxp3-GFP$^+$ cells were isolated from the LNs of TCRβ KO mice that had received Foxp3-GFP$^-$ CD4 T cells 4 weeks before (iTreg) and were compared to fresh Foxp3-GFP$^+$ cells isolated from naïve WT mice (nTreg) in several assays. (A-E) Foxp3-GFP$^-$ CD4 T cells (Tconv) were transferred to TCRβ KO recipients either alone or in the presence of iTreg or nTreg at a ratio of 1:3. (A) Shown is the number of CD4$^+$ cells originating from the Tconv subset recovered from the indicated lymphoid organs of the several recipients. (B) Number of CD4$^+$ originating from the Foxp3-GFP$^+$ subset. (C) Percentage of Foxp3-GFP$^+$ cells within cells originally Foxp3-GFP$^+$. (D) Percentage of Foxp3 in cells originally Foxp3-GFP$^-$. (E) Percentage of Foxp3-GFP$^+$ cells in CD4$^+$CD3$^+$ cells isolated from recipients of naïve T cells following the protocol illustrated in Fig. 4.2A but with the analysis being performed 8 weeks post transfer instead of 4 weeks as for the data in Fig. 4.3B. (F) IL-6 (left), IL-4 (middle) and IFNγ

(right) receptor surface levels represented by mean fluorescence intensity (MFI) in fresh Foxp3-GFP$^+$ and Foxp3-GFP$^-$ CD4 cells (steady state) or isolated from TCRβ KO recipients of Foxp3-GFP$^-$ CD4 T cells 4 weeks before. MFI of CD4$^-$TCRβ$^-$ cells are shown for comparison.

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new mechanistic interpretation of Treg stability and function, since the maintenance of Foxp3 expression by nTreg as been ascribed to IL-2 produced by Tconv cells, the number of nTreg has been shown to be dependent on the IL-2 produced by Tconv cells and Treg seem to adopt a genetic program that matches the T cell response that takes place (19). A small flow cytometry assessment of the surface levels of cytokine receptors know to be involved in different T cell polarizing conditions, IFNγ, IL-4 and IL-6, revealed that iTreg express higher levels of IFNγ receptor than peripheral steady state Treg, while they maintain IL-4 and down modulate IL-6 receptors (Fig. 4.7F). The upregulation of IFNγ matches the Th1 polarization reported in Fig. 4.2 (Fig. 4.3D-H). This last piece of data shows that iTreg, while being a stable lineage, seem to be fragile and possibly depend on the inflammatory environment to be kept.

Discussion

The differentiation and function of regulatory T cells developed outside the thymus has recently been a focus of attention in the Treg field. After the initial demonstration that peripheral cells could be induced to express Foxp3, and gain regulatory properties, there have been many reports of methods to differentiate iTreg and to characterize the differences between peripheral steady state Treg and iTreg. However, the lack of markers that irrefutably discriminate Treg differentiated in the thymus from the ones differentiated in the periphery has been a major hurdle in the study of iTreg developed in WT mice. As a consequence TCR Tg T cells and in vitro protocols have been the major source of iTreg to be studied.

In the first part of this work we made use of the classical experimental system of adoptive T cell transfer to T cell deficient recipients and applied a loss of function approach to track *in vivo* derived iTreg function. The use of a loss of function approach for iTreg requires the use of Foxp3 deficient naïve T cells. This, however, poses the challenge of obtaining such T cells in a naïve state since Foxp3 deficient mice develop a multi organ systemic disease where T cells present unrestricted activation. To circumvent this effect we developed a protocol involving the transfer of bone marrow from Foxp3-deficient donors to WT recipients, where WT Treg, some radioresistant from the recipients and an extra source provided by the transfer of total splenocytes, are enough to keep Foxp3 deficient T cells under control and similarly functional to their WT counterpart (Fig. 4.1).

Naïve T cells from BM chimeras were sorted according to high expression of CD45RB and no expression of CD25 (Fig. 4.2A and B), which allowed us to isolate equivalent T cells of WT and Sf origin from independent BM chimeras. By transferring these cells to TCRβ KO recipients and analyzing the T cell number and phenotype, serum IgG and weight change we found that the ability of naïve T cells to upregulate Foxp3 contributed to limit the

cell number recovered 4 weeks after transfer. This ability did not, however, have an impact in T cell activation, helper cell polarization and any functional output (Fig. 4.3 and Fig. 4.4). The fact that the differentiation of iTreg had no impact on the activation of transferred T cells was not particularly surprising, since the transfer of naïve T cells to lymphopenic recipients has for long been a protocol to induce colitis, and an original model to show the regulatory activity of $CD25^{+}$ T cells (29). In summary this experimental setting represents a context in which naïve T cells are known to expand and get activated even when able to upregulate Foxp3 expression (1, 27). What was more surprising was the ability to limit the number of T cells recovered by iTreg differentiated during the T cell expansion. While this was one of the predicted outcomes for an iTreg functional readout the fact that the system ultimately leads to multi-organ disease with unrestricted T cell activation poses a challenge for iTreg differentiation and function due to the inflammatory milieu that installs. While the most straight-forward explanation is the induction of Foxp3 expression by WT naïve T cells and the lack of by Sf T cells, several alternative hypotheses could be put forward and confounder effects be in place. First the report by Gavin *et al* (28), in which a GFP mediated truncation of the Foxp3 allele was used as a reporter system to study Foxp3 deficient T cells, assures that the sorting strategy used excludes T cells of Sf origin that had received signals to express Foxp3. This guarantees that the differences observed are not due to differences in the initial T cell pool. On the other hand, the results from the co-transfer of WT with Sf naïve T cells, where we found again a reduction of the number of T cells recovered when compared to the transfers of Sf cells alone, proves that there is no cell autonomous property in the Sf cells that make them expand more, but it is rather the differentiation of iTreg in the WT cells pool that exerts a dominant regulation on the expanding T cell population. Despite this data irrefutably showing that iTreg limit the T cell expansion in lymphopenia it does not allow to pin point the effect of would be iTreg in the transfer of Sf cells. This is because the strategy of transferring Foxp3 deficient naïve T

cells while resulting in the loss of iTreg function it does not eliminate the cells that receive the signals to upregulate Foxp3 but do not become iTreg due to the lack of a functional Foxp3 protein. In this regard the results observed are linked to naïve T cells not being able to develop a full Treg transcriptional program and not to the elimination of iTreg cells. As a result cells that would turn on Foxp3 expression in the WT scenario but cannot in the Sf background can still contribute to any output observed by adopting a different phenotype in the later case.

Given the results discussed above, the outcome of the experiment reported in Figure 4.5 was unexpected. In this case, we found that the same number of cells was recovered in recipients of WT and Sf cells that received a second transfer of Foxp3-GFP^{-} conventional T cells 2 weeks after, and were analyzed at 4 weeks after the beginning of the experiment. What was expected was that the recipients of WT cells in the first experiment due to the presence of iTreg from both transfers would present a lower cell number of cells. First the number of cells from the second transfer was supposed to suffer some regulation from the iTreg that had already developed from the first transfer in the recipients of WT but not in recipients of Sf cells. Secondly, the number of cells recovered from the first transfer could also have been higher for cells of Sf than of WT origin. Both predictions were not verified. While it is possible that these results indicate lack of iTreg capacity to regulate the expansion of a second wave of conventional T cells it is also possible that the iTreg developed from the secondary transfer were able to provide enough regulation to control both its own expansion as to limit the final output of the Sf cells resulting in a similar number of Sf and WT naïve T cells. In fact the total number of Foxp3^{+} cells in both recipients of WT and Sf T cells in the first experiment, while reduced in the recipients of Sf cells, was not statistically different. It is also possible that the microenvironment at 2 weeks after transfer of Sf and WT cells is somehow different in the recipients of Sf cells. This could lead to a higher activity of iTreg differentiated from the

second transfer in the recipients of Sf cells than in the recipients of WT cells resulting in a similar number of cells from the first transfer in both groups. This is however a speculative interpretation and further work has to undertaken to clarify the full potential of iTreg differentiated during lymphopenia as well as to possibly achieve some quantitative information. Meanwhile, others have published several works starting the elucidation of iTreg roles and its relevance. Curotto de Lafaille *et al* first shown that Foxp3 induction in TCR Tg T cells is important to control the chronic phase of Asthma in an experimental setting (9). In a more recent study, Josefowicz *et al* showed that iTreg, for which Foxp3 expression is dependent on a given enhancer in the Foxp3 locus, have a determinant role in preventing mucosal associated T cell pathology (12). And Haribhai *et al* have shown that iTreg enrichment synergize with nTreg contributing to a more efficient recovery from an IBD experimental model, caused itself by a conventional T cell transfer, and multiorgan autoimmune pathology caused by Foxp3 deficiency (10, 11). While the later two reports the first was performed with *in vitro* derived Treg, the second study demonstrated the functionality of *in vivo* differentiated iTreg, which was ultimately ascribed to a complementation of the steady state regulatory T cell repertoire by iTreg. Albeit, this study fails to acknowledge that steady state Treg may, and most likely do, contain iTreg. This leads to the proposition that in regulation demanding conditions the differentiation of iTreg complements the steady state regulatory T cell repertoire. This notion does not exclude a baseline contribution in the absence of external challenge but states that upon challenge the differentiation of iTreg can increase the efficiency of T cell based regulation, irrespective of any iTreg that may develop naturally in a naïve mouse. The steady state differentiation of iTreg is, in theory, expected to take place as a product of intrathymic Ag level/density and presence probabilities together with the fact that T cells have a grace period to become Treg just after leaving the thymus, as our data in the previous work shows (Chapter 2).

In the last part of this work and using the same experimental model of T cell adoptive transfer to lymphopenic recipients but with Foxp3-GFP reporter mice as donors we sought to track iTreg stability. After finding that iTreg differentiated in our system resemble Treg isolated from the periphery of naïve mice (Fig. 4.6) we performed secondary transfers of iTreg, which had developed during LIP for 4 weeks in T cell deficient recipients, to similar recipients together with Foxp3-GFP$^-$ conventional T cells. This experimental setup allowed us to determine that iTreg differentiated during LIP give rise to low number of cells after the re-transfer, while maintaining Foxp3 expression and showing little impact on the expansion of the Tconv cells (Fig. 4.7A, B and C). The lack of control of Tconv expansion was initially surprising since we report in the previous sections that the differentiation of iTreg is important to control the expansion of Foxp3$^-$ T cells. However, the low number of iTreg cells recovered is probably the explanation for the absence of regulatory activity. The clarification of why so little iTreg cells are recovered after re-transfer needs further investigation. The simpler hypotheses being that the cells display low survivability or low proliferative potential. On the other hand, as for the sequential transfer results (Fig. 4.5), it is also possible that the presence of iTreg differentiating later during the expansion provide enough regulation to mask the impact of the initial Treg. While it is clear that the regulation exerted by iTreg on the secondary transfer was much lower, since it was undetectable, than the one provided by fresh peripheral Treg, only a co-transfer with naïve Scurfy cells will undoubtedly eliminate the possibility of iTreg to be exerting some regulation in this system. In any case, the later proposition depends on the iTerg now being maintained in such a setup. In this regard, we did find an upregulation of the IFNγ receptor (Fig. 4.7F) which indicates that iTreg isolated after 4 weeks of Tconv expansion in lymphopenic recipients seem to have adapted a phenotype sensitive to the Th1 polarization displayed by the expanding population, as shown in Figure 4.3. It seems therefore plausible to propose that iTreg may require the milieu

from which they were isolated to survive, proliferate or exert regulatory activity.

In fact the phenotypic adaptation of Treg to helper T cell polarization goes towards a concept that has recently been gaining shape within the T cell physiology field, which is the notion of phenotypic plasticity. This has been fueled by both Th17 and Treg studies. For Th17 several studies indicate that there are states of differentiation that, while fitting in a terminal differentiation phenotype by normal parameters, when analyzed more carefully cells are found that may still be re-diverted to another lineage previously thought to be mutually exclusive with the first, mainly Th1 (33). The recently described Th9 seems to be another of such cases, with Th2 driving signals not being a final commitment to Th2 but leaving the possibility for the cells to be pushed to an IL-9 producing fate if other factors are added (34, 35). For the Treg field there are several studies describing the loss of Foxp3 expression by peripheral Treg cells as there are studies describing Treg genetic programs that mirror the program of Th response to be regulated. Mainly, Treg regulation has been shown to be dependent on several master transcription factors ascribed to a given Th polarization phenotype (19). In summary, the fact that we find a regulatory activity while iTreg differentiate together with the polarization of the helper T cells, fail to find one when the differentiation is temporally decoupled by having the iTreg from the beginning of the T cell activation, and not simultaneously, and the fact that we detect the upregulation of the receptor for the hallmark cytokine of the helper cell polarization that takes place, opens the possibility that it is not only the maintenance of Foxp3 expression that maybe dependent on helper T cell cues, but also Treg functional competence. If this proves to be correct the design of therapeutic strategies relying on the adoptive transfer of Treg differentiated from peripheral cell clones may require that the cells to be transferred to be differentiated in conditions that are contingent on the immune system activity to be regulated.

By showing a potential regulatory capacity in iTreg but also some possible frailties in the cells themselves, we hope this study is stimulates the further understanding of Treg physiology regarding their plasticity and adaptability to the immune system activity and raises questions that may one day guide the rational design of adoptive Treg based therapies.

Material and Methods

Mice

C57BL/6J (WT – Thy1.2), C57BL/6J Thy1.1 congenic (Thy1.1) C57BL6/J$^{Sf/y}$ (Scurfy – Sf), C57BL6/J TCR$\beta^{-/-}$ (TCRβ KO), C57BL6/J Foxp3$^{GFP/Y}$ (Foxp3-GFP), were all bred and kept in our animal facility in SPOF conditions. Mice were used between 8-12 weeks of age at the beginning of each experiment.

Preparing Foxp3 deficient naive T cells

BM cells were extracted from femurs and tibias of C57BL/6J WT or Scurfy mice in HBSS by flushing with a 20G syringe. Cells were then spun down at 300G for 10 min and incubated for 30 min at 37ºC with αCD4 and αCD8 antibodies at 1 μg/ml and complement that was pre-treated by a 30 min incubation with splenocytes on ice and passed through a .5 μm filter. T cell depletion was stopped by adding FCS to achieve a 20% final concentration. Cells were then washed twice, re-suspended in PBS, counted and a minimum of 5x10^6 transferred *i.v.* by retro-orbital injection into C57BL/6J Thy1.1 congenic recipients which had been subjected to 900 rad of total body irradiation the day before. 7 days later 10^7 total splenocytes isolated from C57BL/6J Thy1.1 congenic mice were transferred to each recipient. Mice were kept under standard conditions for 8 weeks before being used as donors of WT or Sf naïve T cells. For the experiment where WT cells and Sf cells were co-transferred, and analyzed separately at the end of the experiment, WT (Thy1.2) mice were used as recipients of WT Thy1.1 bone marrow, and the Thy1.2 and Thy1.1 markers used to discriminate Sf and WT cell origin.

Sorting and Cell Transfer

iTreg differentiation was performed by transferring 10^6 naïve ($CD45RB^{hi}CD25^-$) or conventional (Foxp3-GFP^-) $CD4^+$ T cells *i.v.* to TCRβ KO mice. For the case of Thy1.1 donors that had been reconstituted with WT bone marrow, Thy1.2 inclusion and Thy1.1 exclusion gating was applied. iTreg loss of function assays were performed by using naïve T cells from Thy1.1 mice reconstituted with Sf BM as described above. Sf naïve cells were sorted using the same strategy as for WT cells and were both transferred in parallel to $TCR\beta^{-/-}$ recipients. For sorting, cells were harvested from mesenteric, inguinal, brachial, axillary, popliteal, cervical, and para-aortic lymph nodes of donor mice and turned into single cells suspension in sorting buffer (2% FCS in PBS). After washing, cells were re-suspended in sorting buffer and stained with the according antibodies for 30 min on ice. Last, cells were again washed, passed through a 100 μm mesh and sorted in a MoFlo or FACSaria cytometers. After sorting, cells were washed twice and ressuspended in PBS at a concentration of 10^7 per ml and 100 μl were injected per recipient. Recipient mice brachial, inguinal and axillary pooled LNs (biaLN), mesenteric LN (mLN) and spleen (SPL) were analyzed at 4 weeks after transfer except if otherwise stated. For some experiments recipient mice were weighed at the day of transfer and once a week thereafter until the day of cellular analysis. Each mouse weight change was calculated as a percentage of day 0 weight. Total IgG was determined by standard sandwich ELISA technique.

Cellular analysis

Cellular phenotypes were analyzed by flow cytometry using a FACScalibur or Cyan ADP flow cytometers. For *ex-vivo* analysis brachial, inguinal and axillary pooled LNs (biaLN), mesenteric LN (mLN) and spleen (SPL) were

analyzed. Cellular preparation for FACS analysis was performed as for sorting. For cytokine expression analysis cells were plated for 4 hours with PMA/ionomicyn stimulation, with brefeldin A being added in the last two hours of culture. Cells were then stained for surface CD4 and CD3, fixed for 30 min with 1% paraformaldehyde, permeabilized for 30 min with Saponin and intracellular cytokine staining was then performed. Recipients of transfers were analyzed 4 weeks after transfer, except if otherwise stated. *Ex-vivo* analysis of Thy1.1 recipients of bone marrow was performed between 8 and 12 weeks post transfer. iTreg samples were obtained from LNs harvested from TCRβ KO recipients that had received an adoptive transfer of naïve T cells (for ex-vivo analysis) or conventional T cells (for sorting and re-transfer) 4 weeks before.

In vitro proliferation and suppression assays

For *in vitro* proliferation of naïve T cells flat bottom 96 well plates were coated with αCD3 by incubating plates for 3 hours at 37ºC with a solution of 2 μg/ml antibody in PBS. Plates were then washed with PBS and $2.5x10^4$ naïve T cells cultured in the presence of IL-2 (20 U/ml) αTGFβ (5 μg/ml) and soluble αCD28 (1 μg/ml) in complete RPMI. For iTreg proliferation and suppression assays cells were plated in round bottom plates in the presence of 10^5 splenocytes (after red blood cell lysis and T cell depletion, in a similar protocol as for the bone marrow depletion omitting complement pre-treatment) and 1μg/ml αCD3 for 3 days at 37ºC. For Treg proliferation $5x10^3$ iTreg or nTreg were plated alone. For suppression assays $2.5x10^4$ Tconv responder cells were plated either alone or together with $5x10^3$ iTreg or nTreg. For detection of proliferation 1 μCi tritiated thymidine was added to each well in the last 6 hours of culture. Plates were frozen and thawed at the end of the culture and cell extracts harvested into a paper filter, covered with meltilex and scintillations read in a microbeta counter.

Acknowledgements

The research leading to these results has received funding from the European Union's 7th Framework Programme (FP7/2007-2013) under grant agreement n°241447 (NAIMIT) to JD and from the Portuguese Research Council, FCT, under the form of grant, PTDC/SAU-IMU/113541/2009 to IC, fellowships to RSP, ACL, MLB and contract to IC. We thank Rosa Santos, Ana Regalado and Catarina Martins for assistance with antibody production and mouse colonies maintenance; Rui Gardner and Telma Lopes for assistance in cell sorting and Manuel Rebelo for managing the animal house facility.

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Chapter 5

General Discussion

This Chapter aims at discussing the general concepts involved in the findings presented in this Thesis offering a personal evaluation of the ideas they bring about. At the same time it intends to leave a final note on the state of the art of the Treg field both in academic terms and in respect to potential clinical applications. The comparative analysis of the results in respect with the currently available literature is not the main aim of this chapter given that the general introduction together with each chapter's introduction and discussion should already fit that purpose.

5.1 Brief Historical Contextualization

Since the original propositions on factors determining the establishment of immune tolerance, Immunology has advanced greatly in the characterization and understanding of the immune system. Astonishingly, the initial theories captured essential features of immunological tolerance, namely a developmental dependent factor for its establishment in physiological conditions. From Medawar and Burnet (1, 2) to Lederberg (3) and later Modigliani (4), with respective co-workers and contemporary scientists, and now to the work presented here in Chapter 3 as well as work by others (5), it is now clear that the organism age as well as the cell age are essential factors in the susceptibility of T cells to give rise to tolerance. By showing that RTE are more susceptible to differentiate to Treg in the experimental setting tested, our work is the latest confirmation of the cell age model for T cells and confirms that Treg differentiation is to be included in it.

Other notion that is now known to be essential for robust immune tolerance and healthy homeostatic conditions is the system nature of immunoregulatory networks. The immune system being kept under control so that responses harmful to the host do not take place, or are minimized, with invading pathogens still being efficiently eliminated, is dependent in the correct functionality of a wide variety of cell types, different pathways in each

cell type and a proper interaction between all these factors. Two components that have gained increased attention recently are B cells with regulatory properties (6) and the necessity for an intact dendritic cell compartment (7). Treg have nevertheless maintained prime attention due to their capacity to regulate a wide variety of responses, affect the function of many cell types and being essential for the survival of mice and Humans. Naturally, all these factors turned Treg one of the main hopes for future therapies aiming at manipulating the immune system activity.

Since Treg act by downmodulating or preventing immune responses, two possible angles are usually envisaged. In one hand, the aim can be to enhance Treg function and abolish an unwanted immune reaction, such as in autoimmune conditions, and on the other it is possible to prevent their action or cause their removal to allow the immune system to act, as it would be desired to attain more efficient anti tumor immune surveillance. The general lack of efficient treatment for several cases fitting in both types of situations makes Treg manipulation a very attractive candidate for clinical interventions. However, it is this same potential of Treg to either allowing immune responses to take place, if absent, or inhibit them, if present, that make a comprehensive knowledge about Treg physiology a requirement to guide the development of Treg based therapeutic applications. Mainly, due to the fact that erroneously generating tolerance to a pathogen may compromise the host by allowing infection to establish and inhibiting tolerance has the inherent risk of allowing autoimmune reactions to be initiated. It is therefore of paramount relevance that we understand both the requirements and constraints of Treg differentiation, maintenance and function. This knowledge shall help in evaluating safety and efficiency of potential protocols for therapies aiming at controlling particular immune system activities through Treg manipulation. Moreover, this knowledge should also make clear which conditions are required for Treg to provide long lasting tolerance when generated by external manipulation. The fulfilling

of this task has the potential of leading to the cure of several immunopathologies untreatable to date.

5.2 Antigens in the Thymus and T cell selection

Tolerance is commonly divided in central and peripheral mechanisms (8). Central tolerance is one that is established in primary lymphoid organs, the thymus and bone marrow, while the cells are developing, while peripheral tolerance is established in the periphery, that is, outside primary lymphoid organs, like the spleen or lymph nodes when the cells are already mature. Since it is during the initial cellular development that the repertoire is built and an initial assortment of clones to a regulatory or effector devoted phenotype takes place, central tolerance has to be taken into account when addressing T and B cell based tolerance. During their maturation in the thymus, T cells undergo several quality control steps, among which, proper TCR selection. Both positive and negative selection are essential to generate a T cell compartment that is efficient in mounting responses against invading microorganisms while sparing the host from its damaging potential.

While peptides involved as antigens in positive selection are essential to guarantee the generation of a repertoire that interacts correctly with the host's MHC and may even be have an impact in the peripheral maintenance of T cells (9), deletion and Treg induction are considered the essential steps in assuring a self tolerant repertoire (8). These two outcomes are intrinsically constrained by the antigens that are presented by the cells that mediate the selection steps. In fact, the T cell repertoire that arises in the thymus is a product of the interaction between the TCRs generated and the antigens that are present in the time window for which the cells are susceptible to receive deletion and Treg induction signals, not excluding other contributing factors. Regarding the contribution of the antigen, it is mainly determined by its

diversity, quantity and distribution. All this factors are in their turn dependent on the process that makes antigens available in the thymus. The three main mechanisms contributing for intrathymic antigen diversity are simple diffusion of small molecules transported in the blood stream (10), ectopic expression of antigens that are specific of peripheral tissues mediated by the transcription factor Autoimmune Regulator (AIRE) (11), and transport by antigen presenting cells migrating from the periphery to the thymus (12).

AIRE is expressed in medullary thymic epithelial cells (mTECs) which are found in the same thymic niche as thymocytes that are in the differentiation stage where deletion and Treg induction occur (11, 13). AIRE promotes random expression of antigens in a way that each cell expresses its own set. Its function is essential for robust tolerance since mice that cannot express a functional version of this transcription factor display autoimmune pathologies, albeit of mild severity and late in adulthood. A recent report has identified the main contribution of AIRE driven intrathymic antigen diversity to very early age (14). These data and the nature of mechanism by which AIRE induces peripheral antigen expression indicate it is important to set a baseline tolerance to self-antigens that may be present in the periphery. On the other end, blood borne and dendritic cell based mechanisms provide a source of antigen diversity and/or quantity adjustment that are directly contingent on the actual diversity of antigens present in the periphery at the respective moment. The work presented in Chapter 2 adds some new information about this process, particularly regarding Treg differentiation against peripheral antigens made available in the presence of inflammatory signals. In contrast, with the main research focus taken by the Treg field, which has been on Treg induced to novel antigens from peripheral naïve T cells, we have revealed an intrathymic source of Treg recognizing peripheral antigen. The main difference lays in the potential of thymocytes to differentiate to Treg under inflammatory settings while peripheral cells are inhibited by such immunogenic signals. As a consequence, even if an

antigen is provided in an together with inflammatory signals, it can access the thymus and shape the phenotype of exported T cells. As expected, providing antigen that thymocytes recognize can lead to both deletion and Treg differentiation depending on the antigen dose and TCR affinity/avidity, which shows that this process also has the potential to shape the repertoire of T cells being exported from the thymus. Importantly, in the lower end of Ag dose, Treg differentiate and deletion is minimal, while at higher Ag doses deletion may be close to complete and no cells that recognize the antigen with high affinity/avidity are exported.

5.3 Intrathymic Treg differentiation – Tolerance vs. Immunity

However, the potential to generate Treg in the thymus against antigens that are being targeted in an immune response in the periphery creates an apparent conundrum, as it creates the possibility of generating tolerance to antigens arising from a source that has also triggered receptors that lead to a ridding immune response. Moreover, Treg activity being so powerful, leads to the question of how is the response efficient if Treg are generated against the same antigens that are being targeted. Our work also showed that the differentiated Treg are functional, since they were essential to prevent the development of spontaneous autoimmune disease that occurs in the mouse model that was used. The solution for this potential conflict is not known, but it may involve a timing issue and/or an antigen property effect. Timing wise, it is possible that Treg only meet the antigen in the periphery once the response is ongoing, not preventing it from taking place but possibly contributing to limit its intensity. If true, this mechanism may represent a tradeoff between ensuring the control of immune responses to limit harm to the host by the immune response itself, at the cost of potentially allowing the persistence of infectious agents when they are not readily eliminated. The later scenario should only take place, however, when the initial response did

not produce efficient cellular or humoral effector mechanisms in the time window before Treg come into play. On the other hand, the persistence of the antigen may also play an important role. Once Treg against peripheral antigens are exported from the thymus the maintenance of the regulatory phenotype likely depends on interacting with the antigen in the periphery. In that way, if an invading pathogen is being eliminated the antigen will become unavailable and the cells selected against its antigens will lose Foxp3 expression incorporate the conventional T cell pool and possibly participating in a re-challenge, or may even die if the antigen is providing essential survival signals. In any scenario they will no longer contribute with regulation that would inhibit a secondary response. In contrast, if the antigen reaching the thymus is self derived it will remain present in the periphery and the adaptive Treg will maintain their regulatory properties incorporating the Treg pool. The later is closer to the case represented by the main mouse model system used in our work, where the monoclonal T cells recognize a self antigen and generated Treg could prevent auto-immune disease that develops in untreated mice. As such, the protocol employed and its outcome can be taken as an anti-autoimmune vaccine which efficiency depends on the thymus function.

Irrespective of the apparent contradiction of generating regulatory T cells against antigens being targeted in an ongoing immune response, the fact that the thymic output of Treg is able to adapt to the peripheral antigenic landscape is an important concept to understand the development of the Treg repertoire. This notion however needs to be incorporated in the constraints that exist upon Treg differentiation. For instance, the confirmation of the existence of a limited niche for intrathymic Treg differentiation, that most likely includes antigen availability as a constraint, has recently received experimental support. By generating new genetically modified mice carrying a TCR Tg recovered from Treg and investigating the intrathymic Treg differentiation in these mouse lines, it was found that the efficiency of Treg

differentiation of a given clone is dependent on its clonal size (15, 16). In short, high frequency of Treg in such clones is only observed when the clonal size is small, while, when the clonal size increases the frequency of cells maturing as non regulatory also increases. This new finding has introduced a new angle in the constraints ruling Treg induction, which is the fact that the clonal size has an impact in the chance cells have to be diverted to this phenotype. A possible interpretation of these results is that it is rather a confirmation of the antigen as a limiting factor. In this line of thought, the lower frequency of Treg, when clones are represented in a big clonal size, is a result of the physical limit placed by how many antigen presenting cells a thymocyte can interact with before being exported. In cases of self reactive clones of big clonal sizes, only the fraction of thymocytes that interact with the cells presenting the cognate antigen undergo deletion, or are induced to become Treg, with the remaining being exported as naïve. Noticeably, when the authors of such studies diluted the TCR Tg cells the frequencies of Treg observed tended to a 100% figure. This result indicates that, at lower clonal sizes, all cells of a given clone have the chance to interact with the cognate antigen. It is true that this did not take place for all TCR Tg lines, which could be a result of how much of the antigen is available. Alternatively, the lines in which a high frequency was reached could be the result of a compounded effect from having higher multireactivity, which should translate in a more diverse set of antigens with which the clones can interact with, directly increasing the antigen niche. Still, for the cases where the frequencies went up to close to 100%, it could also be a result of diluting the clone to the extent of the antigen being in excess in relation to the clones being generated, in a way that all thymocytes could meet the antigen. Nevertheless, this scenario implies that there would be more antigen available that was not being presented to a thymocyte bearing the Tg TCR, indicating that full Treg differentiation of a clone recognizing an antigen present in the thymus may require the antigen to be in excess. If the antigen availability limit is proven to be the main determinant in allowing self reactive

clones to escape Treg induction, this finding could be extrapolated to deletion and indirectly provide an experimental proof of a limited capacity for all clones to be correctly deleted, except for the antigens that are in excess in relation to the clones that recognize it with high affinity/avidity being generated.

Nevertheless, the Treg niche is likely determined not only by the availability of antigen, but also by Treg inducing factors, such as gamma chain cytokines and TGFβ. The fact that these components are limiting, allows cells that recognize self to escape the central mechanisms of tolerance and be exported to the periphery. One of the main questions that arise from these limitations on repertoire assortment regards the way the Treg repertoire is built so that it is able to control the self reactive cells escaping deletion. Do Treg need to recognize all peripheral antigens or is there an effect such as immune dominant regulatory epitopes or antigens. This may allow Treg to regulate reactivities against other antigens of the same organ by some sort of cross regulation. On the other hand, it is possible that for a given reactivity to be regulated it is sufficient that a fraction of the cells of a clone recognizing self with high affinity/avidity are diverted to the regulatory pool. Our understanding of how the thymic repertoire is selected in respect to the peripheral image of self and environment derived innocuous antigens is still not complete and this is an important piece of information that may help in tracing defects in autoimmune or allergic reactions as well as provide insights into therapeutic opportunities to manipulate the activity of the immune system. Another possible route to complement incomplete thymic deletion and Treg differentiation of self reactive T cells is the differentiation of Treg in the periphery.

5.4 Thymic Contribution to Peripheral Treg Induction

The observations laid above and the fact that transferring Treg depleted T cell preparations from healthy donors to lymphopenic recipients lead to autoimmune manifestations clearly indicate that self reactive T cells are exported from the thymus as non regulatory and the adjustment of the T cell compartment in the periphery to achieve full regulation may be a requirement. Following the concept prompted by the results presented in Chapter 2, showing the differential sensitivity of thymocytes and peripheral cells to inflammatory cues in respect to the upregulation of Foxp3, and early theories indicating that immature cells are more prone to be diverted to regulatory phenotypes (5), we decided to test if recently made T cells already found in the periphery shared those properties.

The results from this part are presented in Chapter 3 where we did find that immature T cells are more prone to differentiate to Treg, at least in adoptive transfer of Foxp3 negative T cells to lymphopenic recipients. Recent thymic emigrants (RTE) display an intermediate sensitivity to IL-6 and IL-4 which translates in a higher susceptibility to upregulate Foxp3 in the presence of these inflammatory factors when compared to the same process in mature peripheral cells, in which Foxp3 expression is strongly inhibited by such factors. At the same time, we showed that immature cells, both thymocytes and RTE, require less TGFβ available to upregulate Foxp3 again when compared to mature peripheral cells. The fact that immature cells are more prone to differentiate to Treg seems to rely in a combination of differential sensitivity to signals inhibiting and inducing Treg differentiation. However, the fact that immature T cells require less exogenous TGFβ than more mature cells, indicates that the contribution of immature T cells to Treg differentiation is likely to be relevant in settings where TGFβ is a limiting factor, and not only in lymphopenia.

Having a more specific characterization of the RTE maturation stage will be important to reveal potential new opportunities for interventions aiming at inducing tolerance. In summary, the main contribution of this part of the work was to show another contribution of the thymus for tolerance, this time by providing cells to the periphery that are more amenable to be polarized to a tolerizing phenotype. On the other hand understanding the maturation process of T cells after thymic export is also required to fully understand how the T cell repertoire is built and the T cell pool formed. Going back to the initial proposition by Burnet and Medawar, that tolerance is a property of neonatal life, our finding with RTE during Lymphopenia fits it perfectly. The newborn peripheral immune compartment is a lymphopenic system which is being filled in by thymic output, that is, it is mainly composed of RTE. Without excluding the effect of other factors, such as the innate immune system activation, our finding of RTE being particularly efficient in differentiation to Treg during Lymphopenia induced proliferation, guarantees an initial buffering to thymic selection regarding the establishment of tolerance early in life to self and environment derived innocuous antigens. At the same time, the enrichment in RTE puts Treg differentiation from peripheral cells as a likely participant in the original observations of neonatal tolerance.

5.5 Treg precursors and the Two Step Model

The differentiation of Treg has long been marked by a discussion of which cells can indeed upregulate Foxp3. When intrathymic differentiation of Treg was the only origin proven, the discussion focused on the models already mentioned in the introduction of elective or instructive mechanisms. After the experimental proof of Treg differentiation from peripheral cells some groups have addressed the existence of preferential precursors of Treg. The most successful data reported so far showed that CD25 expressing Foxp3

negative CD4SP thymocytes, as well as peripheral CD4 cells, do display an increased efficiency in giving rise to Treg when compared to the CD25 negative counterpart (17-19). The upregulation of Foxp3 by this subpopulation of T cells is dependent only on cytokine signaling, namely IL-2, and the CD25 expression is, in its turn, dependent on TCR stimulation. This interpretation led to a model of a two step mechanism for the upregulation of Foxp3 expression, in which cells first receive TCR stimulation in a favorable microenvironment, which leads to the upregulation of CD25, and later receive cytokine signal that then leads in the expression of Foxp3 and the establishment of a regulatory T cell phenotype. While we did not detect an impact on excluding such CD25 expressing Foxp3 negative cells of our preparations, the authors had already put forward that in the lymphopenia system CD25 negative cells can upregulate CD25 and then receive the cytokine stimulation to become Treg. At the same time the possibility that both signals are received simultaneously was not addressed. As such our study is not incompatible with this notion, but if this intermediate step is mandatory for Foxp3 expression immature cells should more frequently upregulate CD25 than mature cells when transferred to lymphopenic recipients. This proposition is so far untested but certainly deserves some further investigation. What is not clear is what the mechanism behind a potential increase in the frequency of cells that upregulate CD25 would be. What we have excluded in our work is that it would arise from a different repertoire between mature and immature cells, given that monoclonal TCR Tg cells displayed the same maturation dependent Treg susceptibility variation. Alternatively, it could lay in a differential integration of TCR signaling by T cells at different maturation stage. Regarding this possibility we did find a differential response to antigen titration with monoclonal T cells stimulated with the cognate antigen but not with α-CD3. This piece of data confirms the potential differences in TCR signal integration and highlights that different qualities of TCR signaling have the potential to alter the efficiency of Treg differentiation. In summary, while

we did identify several factors that are known to influence Treg differentiation having a different impact in immature and mature cells, we did not find a fundamental molecular basis for the different sensitivity, that is what is behind a thymocyte lower expression of IL6 and IL4 receptors. Nevertheless, one of the most active researchers studying RTE has herself proposed that the rules behind the phenotype of RTE and their transition to mature cells are likely to depend in a collection of factors and modifications, and this multiparameter nature of immature vs mature T cells transition has made it difficult to understand RTE phenotype and mechanisms (20).

5.6 Peripherally Differentiated Treg – Phenotype and Function

Induced Treg studies have been mainly performed using TCR transgenic T cells. While being an invaluable tool to clarify antigen specific responses this tool is not always complemented with experiments with polyclonal WT T cell samples, leaving questions about the physiological relevance of iTreg unanswered for a long time. This scenario suffered a positive step forward with some recent reports using genetically modified mice where the genetic region controlling Foxp3 expression in peripheral cells is deleted (21). While the original report of this mouse line did not show any pathology the backcrossing of the mutation to a C57BL/6 background revealed a T cell based immunopathology. A further evaluation of the condition showed that the absence of iTreg differentiated through the activity of this genetic element led to a Th2 based uncontrolled immune response at mucosal interfaces. While the regulatory potential of Treg differentiated from peripheral cells had been demonstrated this was the first system showing a role for iTreg in an immune system that develops from the beginning without the capacity to generate iTreg.

Both Chapter 2 and Chapter 3 give a strong indication that preventive therapies against auto-immune disorders may profit from being administered

at the youngest age possible. The fact that thymic activity decreases with age (22) leads to a lower chance of harnessing intrathymic Treg differentiation and the lower amount of RTE produced reduces the pool of cells in the periphery that are more prone to differentiate to Treg. An alternative to deal with this physiological constraint is to couple strategies that boost thymic activity with interventions relying on young peripheral cells and thymocytes as the source of cells differentiating to Treg. While such strategies are not yet available in the clinic there is active research ongoing with that goal in mind (23). Once both are established, the potential of the feature of young T cells being efficient in generating tolerance may help design applications to be performed in adults.

It is nowadays widely accepted that Treg differentiate *in vivo* in different anatomical locations and at different maturation stages, namely from recently formed thymocytes in the thymus and from T cells already exported to the periphery. Moreover, Treg can also be generated through a quite diverse set of experimental manipulations (24).

What has also been shown is that the phenotype obtained in the different conditions is not always the same. It seems important to have a full understanding of the factors influencing the different phenotypes Treg may adopt and address how each phenotype translates into functional properties. While studies that explore potential differences between Treg found at steady state of naïve mice and Treg differentiated from peripheral non regulatory T cells tend to interpret any discrepancies as arising from the differences in the Treg origin, precautions against confounder factors have not always been taken into consideration. In most of the studies published so far Treg from different sources are not subjected to the same treatment. As such, there is the possibility that the conditions to which Treg are subjected are an important determinant to obtain the observed phenotypes and not the cells that gave rise to them. However, it is possible that Treg originating from conventional T cells in the periphery will have a particular

phenotype simply due to being exposed to a particular environment that Treg made in the thymus are not frequently in contact with. This would be a scenario of an extrinsic influence on Treg characteristics by which iTreg would differ from nTreg simply due to being exposed to particular cues and not related to differences in the phenotype related to the maturation stage at which the cells were first induced to express Foxp3. Such influences could explain why many studies report higher or lower level of expression of potential markers instead of a positive negative discrimination. The proposition of Helios as a marker of nTreg is an example of how comparing a narrow sample of iTreg may generate a wrong interpretation. While the original report compared only *in vitro* derived iTreg with steady state Treg, albeit both subjected to the same treatments. Nevertheless, works performed later, clarified that Treg induced from peripheral cells *in vivo* rather than *in vitro* can express Helios. Our own analysis which is presented in Chapter 3 shows that iTreg differentiated in the lymphopenia induced proliferation system also express Helios (25-27). On the other hand, two reports published back to back while this thesis was being finished, showed that Neuropilin-1 is preferentially expressed in thymic derived Treg. However, iTreg in a given environment also upregulate Nrp-1 expression which provides an example supporting that the main determinant for the different Treg phenotypes that can be observed is the environment in which Treg are in contact with (28, 29).

This is a similar concept to what has been observed in the plasticity of Treg, particularly in regards to the loss of Foxp3 expression. The main studies published so far have shown that Treg depend on IL-2 and TGFβ to keep Foxp3 and the regulatory phenotype. More recently, a notion of Treg adaptability in the form of sub-phenotypes with particular functions has also started to take form (30). In these studies it was established that Treg require a functional transcriptional program that is orchestrated by transcription factors that are also determining the phenotype of helper cell

polarization of Tconv cells. Once Treg are made deficient in these pathways, by Foxp3 driven genetic manipulation, T cell mediated responses of the corresponding polarization are found deregulated. The work we present in Chapter 4 hints at a behavior of adaptability and dependence on the microenvironment the Treg are exposed to, to adopt a particular phenotype. In the lymphopenia system we used, where naïve or conventional T cells are transferred to lymphopenic recipients, a Th1 response takes place concomitantly with the differentiation of iTreg. While this iTreg seem to have little impact in controlling the activation of the global population of transferred T cells they do have an impact in the number of T cells recovered. However, the fact that little cells are recovered in a secondary transfer indicates they are either dependent in the microenvironment they differentiated in or are short lived. While we do not have data to provide a final answer to explain this phenomenon, we did find indications that the first possibility may be involved. iTreg differentiated in this system upregulate the IFNγ-R with no relevant changes in other cytokine receptors tested. Since IFNγ is the main cytokine secreted by Th1 polarized cells, it is likely that iTreg adopted a response to the IFNγ rich environment originating from the Tconv cells. It seems worth to explore this finding further and understand what the effect of INFγ in iTreg differentiated in this setting may be.

Irrespective of the potential to generate Treg from peripheral cells, one must not forget that immune responses do take place. When exogenous antigens are presented together within an inflammatory context T cells get activated and a response is mounted despite the presence of Treg recognizing self antigens that are likely presented simultaneously. This implies that Treg activity can to some extent be circumvented by Tconv provided there are the right signals leading to an immune response and there are no Treg recognizing such antigens. The fact that Treg administration in experimental models usually works preemptively, as a prophylactic measure, rather than as a treatment against an ongoing response is likely to be a result of such

mechanism. However, immune responses can indeed be terminated and there maybe ways of making Treg efficient in dealing with ongoing immune system activity. One possible proposition is that the activity of Treg having a particular sub-phenotype may be more efficient at terminating a particular ongoing response than a blind mix of Treg isolated from steady state or differentiated in “neutral” conditions. Again, the studies with mouse models where Treg are deficient for transcription factors known to orchestrate a particular phenotype in helper T cells fit this hypothesis. While not many Treg are found expressing these transcription factors at steady state, they do develop together with the helper T cell polarization and keep the helper T cell activation under non pathological limits. In fact, this new concept on the potential of Treg to adapt to the immune system activity is another model that fits well with the adaptive nature of T cells. Together with the potential to adapt to the antigens present in the correct microenvironment at the correct time, Treg may also be able to adapt to the ongoing T cell responses to guarantee the maintenance of self-tolerance and minimize harmful side effects from exacerbated immune responses. This can be looked at as the Treg mirror image of the adaptability of the effector immune responses to any conceivable antigen and the adaptability of the type of response to be mounted according to the different challenges posed by different threats.

A clarification of which immune system activities do Treg differentiated in different conditions influence and to what extent can it be influenced will provide invaluable information to set up efficient Treg boosting protocols. For instance, such differences may translate in how many cells need to be present to achieve the desired regulation. If a small population of Treg generated in one condition is more efficient than Treg generated in another in controlling a given activity, the implementation of a therapeutic intervention may be facilitated. At the same time, if a given Treg phenotype is particularly adapted to regulate a given response, it may be desirable to

only promote this sub-type of Treg so that unwanted over regulation of activities unrelated to the one to be targeted are prevented.

5.7 A Potential System to Allow the Discrimination of Treg Differentiated in the Thymus and in the Periphery

Irrespective of the lack of markers that distinguish Treg differentiated in the thymus and in the periphery, characterizing the contribution of both origins is important to understand how the Treg repertoire is shaped. The inability to define a distinctive phenotype of induced and natural Treg make this task more difficult but there should be ways to achieve it. We have indeed designed a system based on genetic manipulation to map the origin of Treg that should label Treg made in the thymus differently from Treg differentiated in the periphery. While the technical state of the art turn the project a high risk one, the concept should be valid for when the tools become available.

The system basically requires a method to label cells under the exclusive condition of being expressing two genes simultaneously. Such a system would be integrated to label cells expressing Foxp3 at the same time as they are expressing a thymocyte stage specific gene, for which we found the proximal Lck promoter a valid candidate. While genetic labeling systems dependent on the activity of two genes are not fully developed we did find a few reports targeting this issue by expressing the Cre recombinase in two different halves, inactive when alone and only active when co-expressed through heterodimerization (31-33). In theory, If one half of Cre is driven by Foxp3 and the other by the proximal Lck promoter only cells upregulating Foxp3 in the thymocyte stage would have the potential to recombine a genetic label, permanently and exclusively tagging Treg made in the thymus. Then by coupling a reporter construct encoding GFP and RFP with a floxed stop codon separating them, also driven by Foxp3 together with one of the halves of the Cre, should reveal recombination events by Foxp3 driven

expression of both GFP and RFP. As such, Treg cells that first upregulated Foxp3 at the thymocyte stage would be GFP and RFP positive while cells upregulating Foxp3 after the proximal Lck promoter activity had been shut down would be GFP alone. However, the fidelity and efficiency of the system do not seem up to par for the task of efficiently labeling such a small population of cells to the extent of attributing a particular origin to a small percentage within them.

The capacity to efficiently track Treg depending exclusively on the maturation stage at which Treg upregulate Foxp3 will provide invaluable information about the potential of generating Treg in a WT scenario. An advance in understanding iTreg differentiation will be essential to evaluate the potential of generating tolerance outside the thymus and help clarify the best manipulations to harness its full power.

5.8 Concluding Remarks

While the work presented in this thesis was designed from an academic interest with a particular focus in elucidating rules governing Treg differentiation, it is impossible to disregard the potential interest of future clinical applications when studying Treg. This was pursued by analyzing the findings here reported not only as clarifications of biological phenomena but also in the light of their potential in enhancing or maintaining tolerance, which has by itself a direct correlation to clinical settings. In Chapter 2 the potential access to the thymus by peripheral antigens offers information about how the Treg repertoire may be selected against antigens that are exclusively peripheral but at the same time gives an example of a proof of concept for an anti autoimmune disease vaccine. Chapter 3 is focused on the impact of cell age in the differentiation of Treg in the periphery. While extending the findings presented in the previous chapter, Chapter 3 is fully focused in Treg differentiation outside the thymus and provides an example

of an age dependent property that may be taken advantage of in the application of therapies aiming at generating tolerance. Then, Chapter 4 presents a brief study of Treg differentiated in the periphery, their function and stability in lymphopenia, where we obtained indications that Treg differentiated in the periphery can act on the naïve T cell population from which they arise, but that they may also be fragile and/or dependent on particular conditions to survive and exert their function. *In toto,* the work presented here provides new findings regarding the origin and physiology of Treg in respect to their adaptation to the peripheral immune compartment.

In conclusion, the body of work elucidating Treg physiology indicates Treg are a heterogeneous population, however this heterogeneity may not translate in a constitutive phenotype identifiable by surface markers particularly reflecting the maturation stage of the cells that were the precursors. In turn, Treg seem to display a great degree of plasticity that represents the milieu in which they are inserted in a transitory manner. As such, Treg may lose Foxp3 and gain effector functions, adopt transcriptional profiles that match the immune responses to be regulated and can be differentiated from T cells at different stages. These features fit perfectly the notion of adaptive immunity which is based on the ability of the immune system to recognize and respond concordantly to previously uncharted challenges. This emerging picture of Treg dynamics represents anticipatory capacity to tolerance as the generation of a random repertoire of antigen receptors does for immunity.

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