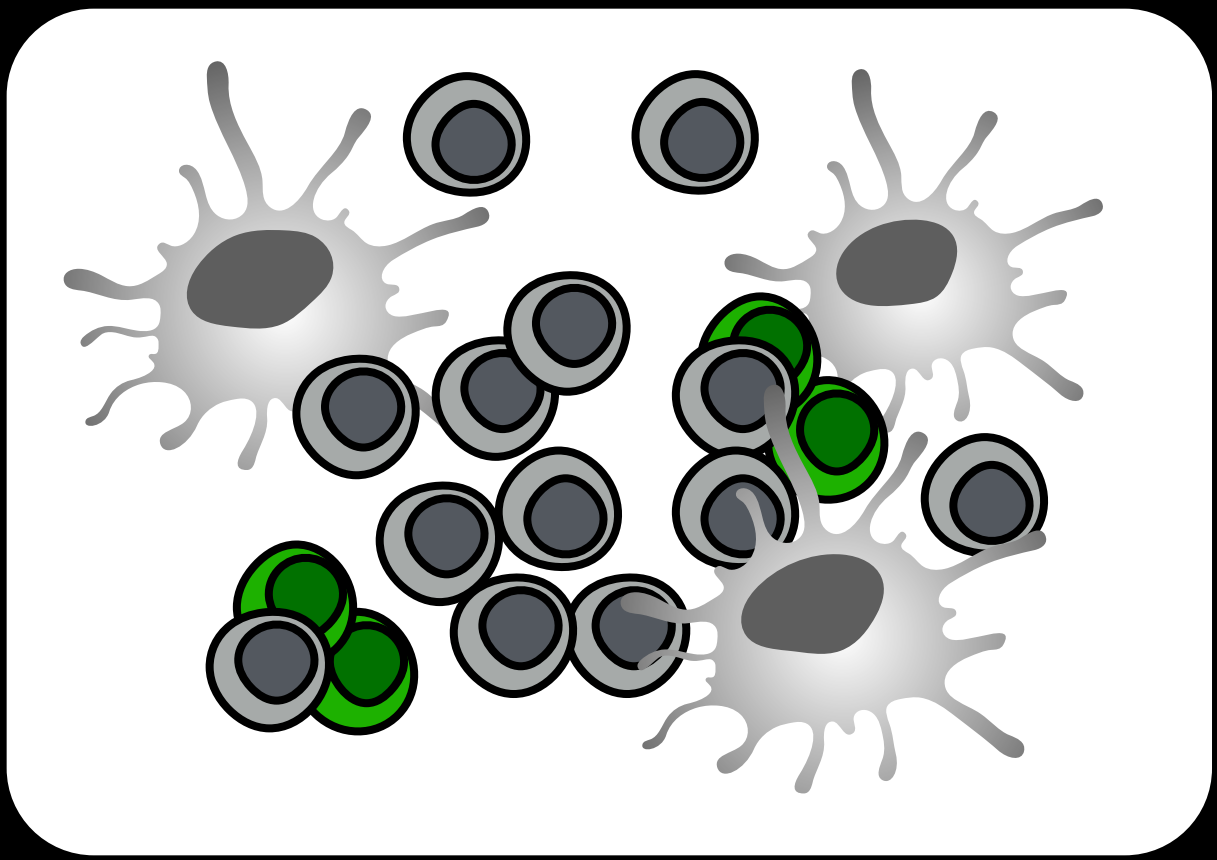
Robust tolerance out of volatile Regulatory T cells

Lessons from mathematical modelling

Eleonora Tulumello



Dissertation presented to obtain the Ph.D degree in Integrative Biology and Biomedicine

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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UNIVERSIDADE NOVA DE LISBOA

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Summary

Natural tolerance is the ability of the immune system to prevent any immune response against body components and tissues. This capacity coexists with and cannot be disentangled by the ability of the immune system to recognize and fight against any kind of fast evolving pathogen that enters the body. The process of natural tolerance is so robust and inconspicuous in healthy individuals that it shows its actual immunological nature when it fails, in pathologic conditions referred as autoimmune diseases. Autoimmunity consists in the disruption of body tissues orchestrated by the immune system cells. Namely during autoimmunity, the immune system uses against its own tissues and organs the very same destructive mechanisms otherwise used for fighting pathogens. It occurs together with an uncontrolled clonal expansion of auto-reactive lymphocytes.

Natural tolerance is the result of developmental processes that take place, first, in the thymus and in the bone marrow, and continue in the periphery. As a result, activation and proliferation of the auto-reactive lymphocytes, that have escaped the negative selection, is controlled by specific Regulatory T cells (Tregs).

It is nowadays clear in the field that tolerance relies on Tregs, a subset of CD4 T cells enriched in auto-reactivity which selectively express the transcription factor Foxp3. It is also widely established that, in particular, tolerance emerges as the result of the interactions and the dynamics that maintain Tregs population in balance with the population of auto-reactive CD4 T effector cells (Teffs) that they suppress. The interactions among Tregs and Teffs are mediated by the antigen presenting cells (APCs) that present auto-antigens as peptides complexed with MHC class II on their surface. These interactions, through which Tregs feed on the very same population they suppress, are strongly non-linear and density dependent.

It is also known that Foxp3 expression is necessary for Tregs development

and regulatory function, as much as individuals whose CD4 T cell cannot express functional Foxp3 protein suffer from multi-organ and lethal autoimmune diseases. In the past years several observations *in vivo* of Foxp3 dynamics have been gathered. In healthy individuals Foxp3 expression in Tregs is stable over several rounds of cells divisions, persisting for several months. In some "pathologic" experimental conditions, such as lymphodeficiency or under inflammation, loss of Foxp3 expression in Tregs and the accumulation of cells that used to express Foxp3 (exFoxp3) is observed over time. Moreover, cells that lose Foxp3 expression can become pathogenic, having the ability to infiltrate tissues and expressing inflammatory cytokines.

In the past, robustness of tolerance, has been partially attributed to the robustness of Tregs. The experimental observations about Foxp3 dynamics *in vivo*, in which Tregs lose their identity, push to probe other possible scenarios.

The present work aims at investigating theoretically and quantitatively the mechanisms that make tolerance robust. Robustness of tolerance in physiologic condition is achieved at two complementary levels: on the one hand, at a cellular level, Foxp3 expression in Tregs is stably maintained, and on the other hand, at a multicellular level, Tregs population is maintained in balance with the Teff population they suppress. For this reason our approaches also followed two complementary levels.

At a population level, we asked whether Tregs could be maintained as a stable population, even in case Foxp3 expression is determined by the inputs the cells receive from the other immune cells population, in a density-dependent way. We built upon the previous developed cross regulation model, which describes the temporal evolution and interaction between Teffs and Tregs mediated by APCs. We introduced the loss of Foxp3 expression and their consequent conversion into Teffs, in Tregs that lack the pro-Foxp3 stimuli. We found parameter regimes in which, as long as the Foxp3 loss rate is slow enough, not only Tregs population can be stably maintained, but also their robustness in response to perturbation is increased as compared to a scenario of purely committed Tregs. Differently from the original model, in which, in response to a perturbation, homeostasis is restored through oscillation, when Foxp3 loss is allowed, homeostatic restoration happens much more smoothly. Avoiding oscillations could be a key point for tolerance robustness, being that both over-shootings and strong declines in ei-

ther Teff and Treg population densities may cause the system to collapse toward autoimmunity.

At a cellular level, we asked whether the stability of Foxp3 expression, observed in physiologic condition, can be due to the context, rather than to a programmed developmental process. To this aim, we investigated how the assumption of context-dependent Foxp3 loss, introduced in the population dynamics model, could be explained at a cellular level, in CD4 T cells. We developed a stochastic model for Foxp3 expression in CD4 T cells which allowed us to investigate the characteristic time scale of Foxp3 loss *in vivo*, and the commitment and plasticity of Tregs. The model accounted for heterogeneity in Tregs: it considers committed Tregs, that in no case can lose Foxp3 expression, and plastic Tregs, whose Foxp3 expression depends on the context. We used this model to fit *in vivo* data on temporal evolution of $Foxp3^+$ cell frequency in cohorts of cells that were either $Foxp3^+$ or $Foxp3^-$ at a given time, in different experimental conditions. We found that experimental data are compatible with Tregs being a homogeneous pool of CD4 T cells, in which Foxp3 expression is either stable or labile depending on the context, in particular depending on Teffs number and proportion. This result means that the data are compatible with Foxp3 expression dynamics in CD4 T cells being dependent on immune populations densities. We were also able to quantify the average time a Treg is able to maintain Foxp3 expression, in the periphery and in absence of pro-Foxp3 stimuli, to be of four weeks.

We went further, asking which cellular mechanisms could explain the found slow Foxp3 loss rate. To do that, we extended the previous model for Foxp3 expression in CD4 T cells, introducing the epigenetic dynamics along with the transcriptional/translation dynamics, already present. We tried to put forward the idea that slow epigenetic dynamics, dependent on the transcriptional state of the cell, together with a fast and context-dependent transcriptional/translational dynamics could explain both stability and lability of Foxp3 expression. Data fitting suggests that this could be the case. The model predicts that in physiologic condition, where Tregs are stable, the vast majority of them has an active state of chromatin in the Foxp3 locus. It also predicts that the slow dynamics of Foxp3 loss, observed in lymphopenia, would be mainly explained by the epigenetic remodeling dynamics of the Foxp3 locus.

In conclusion, our work indicates that robustness of tolerance can be explained as a result of non-linear and density-dependent dynamics among Teffs and Tregs, mediated by APCs. These interactions happen in a context in which Tregs identity, exemplified by stable Foxp3 expression, is not committed, at least for a conspicuous part of Tregs. On the contrary, Tregs identity largely depends on the interactions Tregs make with the other two populations, therefore ultimately depends on the population densities themselves. Furthermore, the slow dynamics observed in the loss of Foxp3 expression in Tregs that do not receive enough pro-Foxp3 stimulation from the context can be attributable to epigenetic mechanisms. Epigenetic mechanisms here involved are consequences of the transcriptional state of the cell, without being necessarily and directly determined by inputs the cell receives. Finally, these epigenetic mechanisms take also place in physiologic conditions. However, they are counterbalanced by fast and context-dependent Foxp3 up-regulation which results in the apparent stability of Foxp3 expression which is rather a dynamical process.

Resumo

A tolerância natural refere-se à capacidade do sistema imunológico de evitar a resposta destrutiva contra os tecidos e orgãos corporais. A tolerância é indissociável da capacidade do sistema de reconhecer e responder contra todo e qualquer tipo de patogénio. Em indivíduos saudáveis, o processo de tolerância natural é tão robusto e inconspícuo que só se revela quando falha em situações patológicas chamadas doenças autoimunes. A autoimunidade patológica corresponde à ruptura da fisiologia e destruição dos tecidos corporais orquestrada pelas células do sistema imunológico, através dos mecanismos destrutivos usados para combater patógenios. A autoimunidade ocorre concomitantemente com a expansão clonal descontrolada de linfócitos autoreativos.

A tolerância natural é o resultado de processos de desenvolvimento que ocorrem, em primeiro lugar, no timo e na medula óssea e que prosseguem na periferia. Estes processos asseguram que a ativação e proliferação dos linfócitos autoreativos, que escaparam à seleção negativa, sejam controlados especificamente por células T reguladoras (Tregs). As Tregs são um subconjunto de células T CD4 predominantemente autoreactivas que expressam seletivamente o fator de transcrição Foxp3. A tolerância surge como resultado das interações e da dinâmica que mantém a população de Tregs em equilíbrio com a população de células efetoras T CD4 autoreativas (Teffs), que as primeiras inibem. As intera cões entre Tregs e Teffs dependem de células apresentadoras de antigénios (APCs), que apresentam à sua superfície antígenos sob a forma de péptidos complexados com as moléculas de MHC de classe II. Essas interações, nas quais as Tregs são estimuladas pelas células Teffs que suprimem dão origem a dinâmicas fortemente não lineares dependentes das densidades das várias popula cões celulares intervenientes.

A expressão de Foxp3 é necessária para o desenvolvimento e a função reg-

uladora das Tregs, sendo que os indivíduos cuja células T CD4 não podem expressar a protéina Foxp3 funcional sofrem de doenças autoimunes letais que afetam múltiplos orgãos . Em indivíduos saudáveis, a expressão de Foxp3 em Tregs é estável ao longo de várias divisões celulares, persistindo durante vários meses. Em algumas condições experimentais ?patológicas?, como a linfodeficiência ou a inflamação, observa-se a perda de expressão do Foxp3 em Tregs e a acumulação, ao longo do tempo, de células que anteriormente expressavam este factor de transcrição (exFoxp3) . Além disso, as células que perdem a expressão de Foxp3 podem tornar-se patogénicas, tendo a capacidade de se infiltrar nos tecidos e expressar citocinas inflamatórias.

No passado, a robustez da tolerância natural foi parcialmente atribuída à robustez da identidade das Tregs. Contudo, as observações experimentais da dinâmica de perda de expressão de Foxp3 *in vivo* sugerem a necessidade de investigar de cenários alternativos. O presente trabalho tem como objetivo investigar, teórica e quantitativamente, os mecanismos que tornam a tolerância robusta. A robustez da tolerância em condições fisiológicas é alcanccada em dois níveis complementares: por um lado, a nível celular, a expressão de Foxp3 em Tregs é mantida de forma estável; e, pelo outro, ao nível multicelular, as populações de Tregs e Teffs mantêm-se em equilíbrio dinâmico. Por essa razão, abordamos estes dois níveis.

Ao nível da dinâmica populacional, perguntamos se as Tregs podem ser mantidas como uma população estável, mesmo quando a expressão de Foxp3 seja determinada pelos sinais que as células recebem das outras células imunes, sinais esses que são dependentes da densidade celular. Desenvolvemos o modelo de regulaição cruzada proposto anteriormente, introduzindo a perda da expressão de Foxp3 em Tregs que não recebendo estímulos extracelulares promotores da expressão de Foxp3 se convertem em Teffs. Encontramos regimes de parâmetros nos quais, desde que a taxa de perda do Foxp3 seja suficientemente lenta, se observa a manutenção estável da população de Tregs. Mais ainda, o estado em que as Tregs predominam torna-se mais robusto em resposta á perturbação quando as Tregs podem transformar-se em Teffs em comparação com um cenário em que as Tregs mantêm a sua identidade. No modelo original, em resposta a uma perturbação, a homeostasia é restaurada após oscilações da densidade das c'elulas. No entanto, quando a perda de Foxp3 é permitida no

modelo, a restauraccão do equilíbrio ocorre suavemente sem oscilações. Evitar oscilações pode ser um ponto chave para a robustez da tolerância, sendo que tanto o crescimento excessivo quanto o forte declínio das densidades populacionais de Teff e Treg podem causar o colapso do sistema ou a sua evolução para o estado de autoimunidade.

Ao nível celular, perguntamo-nos se a estabilidade da expressão de Foxp3 observada em condições fisiológicas poderia ser causada pelo contexto em vez de o resultado de processo de desenvolvimento programado. Com esse objetivo, investigamos como a hipótese de perda de Foxp3 dependente do contexto, introduzida no modelo de dinâmica populacional, poderia ser explicada ao nível celular. Desenvolvemos portanto um modelo estocástico da expressão de Foxp3 nas células T CD4, o que nos permitiu investigar a escala de tempo característica da perda de Foxp3 *in vivo*, bem como a determinação e a plasticidade das Tregs. Tendo em conta a heterogeneidade das Tregs, o modelo considera Tregs irreversivelmente determinadas, que em nenhum caso podem perder a expressão de Foxp3, e Tregs plásticas, cuja expressão de Foxp3 é dependente do contexto. Esse modelo permitiu ajustar dos dados *in vivo* relativos à evolução temporal da frequência das células $Foxp3^+$ nas coortes de células $Foxp3^+$ ou $Foxp3^-$ num determinado momento, em diferentes condições experimentais. Descobrimos que os dados experimentais são compaíveis com a hipótese de que as Tregs seriam um conjunto homogêneo de células T CD4 nas quais a expressão do Foxp3 é estável ou l ábil, dependendo do contexto, em particular dependendo do número e da proporção de Teffs. Este resultado implica que os dados são compaíveis com uma dinâmica de expressão Foxp3 nas células T CD4 dependente das densidades das populações de células imunes. Foi também possível quantificar em quatro semanas o tempo médio em que uma Treg consegue manter a expressão de Foxp3 na periferia e na ausência de esímulos pró-Foxp3.

Interrogamo-nos ainda sobre os mecanismos celulares que poderiam justificar a lenta taxa de perda de Foxp3 pela Tregs. Assim, estendemos o modelo anterior para a expressão de Foxp3 introduzindo a dinâmica epigenética. Tentamos avan car o conceito de que uma dinâmica epigenética lenta (dependente do estado de transcrição da célula) juntamente com a dinâmica rápida da transcrição e tradução dependente do contexto poderia explicar a estabilidade e a volatilidade da expressão de Foxp3. O ajustamento dos dados confirmou essa

possibilidade. O modelo prevê que, em condições fisiológicas onde os Tregs são estáveis, a grande maioria destes possui um estado de cromatina activo no locus Foxp3. O modelo também indica que a dinâmica lenta da perda de Foxp3, observada na linfopenia, poderá ser explicada principalmente pela dinâmica de remodelação epigenética do locus Foxp3.

Em conclusão, o nosso trabalho indica que a robustez da tolerância pode ser explicada como o resultado da dinâmica não linear das densidades de populações de Teffs, Tregs e APCs. As interações entre estas células ocorrem num contexto em que a identidade das Tregs, exemplificada pela expressão estável de Foxp3, não está determinada, pelo menos para uma parte conspícua de Tregs. Pelo contrário, a identidade das Tregs depende em grande parte das interações que estas fazem com as células das outras duas populações, dependendo portanto, em última análise, da densidade da sua própria população. Além disso, a lenta dinâmica observada na perda da expressão Foxp3 em Tregs que não recebem suficientes estímulos pró-Foxp3 pode ser atribuída a mecanismos epigenéticos. Os mecanismos epigenéticos envolvidos são determinados pelo estado de transcrição da célula, sem serem necessaria e diretamente determinados pelos estímulos que a célula recebe. Finalmente, esses mecanismos epigenéticos também ocorrem em condições fisiológicas. No entanto, eles são contrabalançados pela regulação rápida da transcrição do Foxp3, dependente do contexto, o que resulta numa aparente estabilidade da expressão de Foxp3, que é no entanto o resultado de um processo dinâmico.

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1

General Introduction

1.1 Tolerance is robust

The adaptive immune system has evolved in a way that makes it able to mount immune responses against any kind of fast evolving pathogens, such as viruses and bacteria. At the same time, evolution has ensured the adaptive immune system to be tolerant to body components, body tissues and non-harmful third parties, such as commensals and food.

Although along the history of immunology it has not always been clear, these two abilities of the immune system, not only are intertwined, but they constitute two sides of the very same coin and one cannot be understood without the other.

Individuals are naturally tolerant, this status generally persisting during their entire life. Moreover, the developmental processes that lead to natural tolerance are so robust that it took long until natural tolerance started to be perceived as an immunological process and therefore was integrated into an immunological perspective. In 1900 the immunologist Erlich used the term *horror autotoxicus* to describe what he thought was the body's innate aversion to immunological self-destruction [Ehrlich and Morgenroth, 1899, Ehrlich, 1902]. At that time antibodies were the accepted hallmark of immunity, despite it was not yet known which cells produced them. It was clear that several vertebrates are able to produce a large variety of antibodies which could recognize, in principle, any protein. Yet, there was no clear idea on how the immune system could then distinguish between ”non-self” and ”self”, targeting the former while sparing the latter.

Natural tolerance can be clearly recognized as an immunological process when it fails, during autoimmune disease.

Autoimmunity occurs as a consequence of massive clonal expansion of auto-reactive lymphocytes, namely when the immune system redeploys against its own tissues and organs the very same destructive mechanisms, otherwise used for fighting pathogens. As a result, entire organs are infiltrated with auto-reactive immune cells. They can be literally liquified by auto-reactive cytotoxic T cells and with the help of autoantibody-producers B cells. Although CD8 T cells and B cells are the one responsible for immune pathology, we know that their clonal expansion cannot occur without the necessary trigger of auto-reactive CD4 T cells, which actually orchestrate the immune response.

The reason why immunity and tolerance are deeply connected resides in the peculiar characteristics of the adaptive immune system, which makes it able to cope with evolving pathogens. In particular, it resides in the vast repertoire of antigen receptors that are generated and that lymphocytes express in their surface. Through this large variety of antigen receptors, lymphocytes have the potential to recognize any possible antigen that enters the body and is presented as peptides complexed with MHC class II on the surface of antigen presenting cells (APCs). The generation of this vast repertoire is achieved in lymphocyte precursors, by random recombination of the gene segments that code for the receptor chains, such as the variable (V), the joining (J) and, in some cases, the diversity (D) gene segment. This process is called V(D)J recombination. Most lymphocytes have a unique antigen receptor, able to recognize and react to specific antigen presented. Also, lymphocytes can undergo clonal expansion, if activated through receptor engagement.

The drawback of the potentiality to recognize any kind of antigen, is that lymphocytes that recognize body antigens are inevitably generated. These auto-reactive lymphocytes can cause autoimmunity, unless their clonal expansion is prevented in the periphery.

For this reason the key mechanism to build and maintain natural tolerance, preventing pathologic autoimmunity, boils down to prevent the clonal expansion of auto-reactive lymphocytes in the periphery. The mechanisms through which this goal is achieved by the immune system have been a hotly debated topic during the history of immunology.

1.2 Clonal selection and natural tolerance by deletion of immature lymphocytes

The original clonal selection theory [Burnet et al., 1957], introduced in 1957 by Burnet, first claimed that each lymphocyte bears a single type of receptor, with a unique specificity, that would undergo clonal expansion upon receptor engagement and subsequent activation. In his theory, Burnet justified the prevention of auto-rective lymphocyte clones with their deletion, that would take place during embryonic development. However, we know that generation of lymphocytes is a lifelong process in mammals.

Two years later, Lendeberg proposed that the deletion of potentially auto-reactive lymphocytes would take place during lymphopoiesis, in the bone marrow and in the thymus [Lederberg, 1959]. Indeed immature lymphocytes that express auto-reactive receptor are deleted either in the bone marrow or in the thymus [Renno and Acha-Orbea, 1996, Kisielow et al., 1988]. This process is often referred as negative selection and central tolerance. In particular, in T cells negative selection a key role is played by the transcription factor autoimmune regulator (Aire), thanks to which tissue specific antigens are expressed in the thymus [Taniguchi and Anderson, 2011].

1.3 Natural tolerance by deletion of circulating lymphocytes

Negative selection that takes place in thymus and in the bone marrow cannot account for tolerance to peripheral antigens that are not expressed in either of the two lymphopoietic organs. In 1987 Langman and Cohn proposed a solution to this puzzle, introducing a second version of the so called ”two signal model”. This model predicts deletion of auto-reactive lymphocytes circulating in the periphery.

1.4 Regulation among lymphocytes: meet the Regulatory T cells

Neither central nor peripheral deletion of immature auto-reactive lymphocytes can, by themselves, explain natural tolerance. Indeed, the presence of significant number of mature auto-reactive lymphocytes circulating in the periphery in healthy animals is experimentally well documented [Ramsdell et al., 1989, Heath et al., 1992]. This suggests that their presence, per se, is not sufficient to break tolerance. Nevertheless, several experimental evidences have shown that auto-reactive circulating lymphocytes can undergo massive clonal expansion and cause autoimmune pathology, unless they are controlled by specific Regulatory T cells (Tregs). Tregs are a subset of $CD4^+$ T cells, nowadays recognised as the one selectively expressing the transcription factor forkhead box protein 3 (Foxp3) and high level of the interleukine 2 (IL-2) receptor α-chain (CD25).

Let us recapitulate the experiments that led to the notion that natural tolerance emerges as a result of the interactions among lymphocyte populations.

Mice that have been thymectomized in perinatal period, and within a small time window, develop multi-organ autoimmune disease in adulthood. However, autoimmunity does not occur in case the thymectomy is performed later [Nishizuka and Sakakura, 1969, Sakaguchi et al., 1982]. This suggested the existence of a special wave of production of Tregs in the thymus early after birth, as proposed by Modigliani [Modigliani et al., 1996].

Beside this special period, Tregs are known to be produced in the thymus throughout the entire life [Seddon and Mason, 1999, Itoh et al., 1999, Jordan et al., 2001, Fontenot et al., 2005], although to a lesser extend, mainly due to thymic involution [Boehm and Swann, 2013]. One possible interpretation is that thymectomy performed during that perinatal window, results in a strong imbalance in the bulk of the Tregs that colonize the periphery [Dujardin et al., 2004]. The imbalance is later amplified by the cells population dynamics that takes place in the periphery, as suggested by Carneiro [Carneiro et al., 1995, Carneiro et al., 2007].

Autoimmune pathologies that develop in adulthood mice upon thymectomy in perinatal period, can be prevented by adoptive transfer of $CD4^+$ Tregs, sorted from healthy adult mice [Suri-Payer et al., 1998]. However, transfer of $CD4^+$ T

cells, that lack a proper amount of Tregs, is not able to prevent the development of autoimmunity in the recipient.

Many interesting experimental observations have been performed in lymphodeficient mice, which lack adaptive immunity. Adoptive transfers into empty mice of small numbers of $CD4^+$ T cells that are poor in Tregs, result in large lymphoproliferative pathology, whether the pool of cells are sorted as $CD25^-$ [Sakaguchi et al., 1995], $CD45Rb^{high}$ [Morrissey et al., 1993, Powrie et al., 1993] or $Foxp3^-$ [Wan and Flavell, 2005]. However, massive clonal expansion and autoimmunity are prevented in case mice are reconstituted with $CD4^+$ T cells population, either enriched in Tregs or in proper mixture of Tregs and non-Tregs [Annacker et al., 2000, Annacker et al., 2001, Almeida et al., 2002].

Tregs sorted from donor that lacks specific tissue, fail to prevent autoimmune response against that specific tissue in the host [Seddon and Mason, 1999]. This finding highlights the antigen specificity, or at least tissue specificity, of Tregs. Also it indicates that persistence of Tregs as population requires sustained stimulation by peripheral antigens.

Furthermore, it was observed that break of tolerance can be triggered by perturbations that cause disequilibrium between Tregs and their target T cells population. These perturbations encompass either direct perturbations of T cells proportions, perturbations of other leukocytes, or perturbations of the innate immune system, such as the one occurring upon massive local inflammation that follow immunization with adjuvants [Panoutsakopoulou and Cantor, 2001]. Interestingly enough, when tolerance is broken, reverting the situation seems to be a very difficult task, even more difficult than breaking tolerance.

Concerning the possibility of inducing tolerance to specific antigens, decades of experiments in grafts have shown that, although successful grafts can be performed during embryonic development, in most of the cases tissues are rejected as soon as immunocompetence develops. Billingham, Medawar and Brent showed that hematopoietic tissue constitutes an exception. In their seminal experiment, performed in 1953, they managed to induce chimerism and homograft acceptance in mice, by transferring spleen cells into intrauterine fetuses [Billingham et al., 1953]. Later, it was shown that also thymic epithelial cells are able to induce tolerance in the host to themselves and to other tissues from the same donor [Modigliani et al., 1995, Ohki et al., 1987].

1.5 The cross regulation model of cell populations dynamics: an integrated framework for tolerance and immunity

All the observations recalled in the previous section indicate that the robustness of tolerance in adults is the result of density-dependent interactions that Tregs make with other T cells and APCs populations, as put forward by the cross regulation model (CRM) developed by León and Carneiro [León et al., 2001, León et al., 2003, León et al., 2004b, León et al., 2004a, Carneiro et al., 2005, Carneiro et al., 2007].

The CRM describes the temporal evolution of Treg and Teff population densities whose interactions are mediated by the APC populations. APC-mediated interactions happen through multicellular conjugates that Tregs, Teffs and APCs of the same specificity form and brake over time, in a density-dependent way. Through these interactions, Tregs control the proliferation of the Teffs that react to the same set of antigens. According to the model, tolerance emerges as a result of the population dynamics occurring in the periphery, mediated by APCs.

Antigen presentation is fundamental in the sense that it determines, in a robust way, the configuration of equilibrium reached by the populations of Tregs and Teffs recognizing that specific antigen. In particular the population dynamics in the periphery shapes the $CD4^+$ T cell peripheral repertoire into two subsets [Carneiro et al., 2007]. The first subset is constituted by a more diverse set of clones, which are barely auto-reactive and whose clonal expansion is limited by the APCs availability. These clones react to antigens that are rarely presented in the periphery and, most likely, are the ones responsible for mounting immune responses against pathogens. The second subset of clones is constituted by a less diverse set of clones of auto-reactive Teffs and Tregs that react to the same antigen(s) and regulate each other's growth. These clones are supported by a larger density of APCs, if compared with the previous case. Bi-stability characterizes the latter set of clones in the sense that there are two possible configurations of equilibrium for them. In healthy individuals Tregs and Teffs of the same clone coexist, with Tregs regulating the Teffs. Nevertheless, in case a strong perturbation leads the system far from the healthy equilibrium, the population dynamics can amplify the perturbation and the system can eventually collapse into the other

configuration of equilibrium, characterized by no Tregs.

Perturbations of the system can occur early in development or later in life. Nevertheless, considering that a perturbation early in development would affect the seed of Tregs that colonize the periphery, it appears much clear the fundamental role of thymus in early stages of development. In fact the thymus ensures an appropriate seeding of clones in the periphery.

The bi-stability of the second subset of clones, which guarantee natural tolerance and can potential cause autoimmunity, can be explained as a result of the competition of Teffs and Tregs for APCs. In fact the CRM assumes that APCs are limited in density and the interactions among T cells and APCs determine T cells activation. Activated T cells can proliferate, provided growth factors (mainly IL-2) are available. Given that activated Teffs produce IL-2 [Almeida et al., 2006, Malek, 2008], it is enough a critical APCs density for Teffs to sustain their own proliferation. On the other hand, because Tregs do not produce IL-2, their maintenance as a population requires a higher density of APCs. In fact, higher density of APCs can sustain enough activated Teffs, which are source of IL-2, while preventing Teffs to outcompete Tregs in the competion for APCs.

Therefore the APCs-mediated population dynamics defined by the cross regulation model explains the self *versus* non-self discrimination operated by the adaptive immune system and the partitioning of T cells repertoire, based on their degree of auto-reactivity. It provides an explanation for the observation that autoreactive T cells leaving the thymus, are accompanied by Tregs that control them. Finally it explains the high auto-reactivity of Tregs [Kim et al., 2007]. In summary, the CRM clarifies in which sense immunity and tolerance are two faces of the very same coin.

1.6 Tolerance robustness as a result of non-linear density-dependent populations dynamics

As already mentioned, the CRM interprets natural tolerance as a result of the non-linear and density-dependent interactions among Tregs and Teffs, mediated by APCs. Along with the importance of the APCs discussed in the previous section, the relative density of Tregs and Teffs plays a fundamental role in the popula-

tion dynamics. The interactions among T cells and APCs need proximity and the CRM approximates proximity by multicellular conjugates formed among APCs and T cells. In particular, the model assumes that Tregs can inhibit Teffs when they form multicellular conjugates together with them and APCs. As a result the relative densities of the three populations determines which kind of conjugates are more likely to be formed. In turn, the type of conjugates formed influences the kind of interactions that are more likely to take place and which can either induce or inhibit cell proliferation. Interactions determine both the population dynamics and the populations densities of equilibrium.

The density dependent-interactions among the three cell populations, at the end of the day, shape robustly the repertoire ensuring auto-reactive T cells to be controlled by Tregs of similar specificity. Density dependency is therefore responsible for a robust self *versus* non-self discrimination.

If we consider a given couple of Treg and Teff populations that react to the same set of antigens, presented by a given population of APCs, we can refer to these populations as a niche. Within each niche, the density-dependent interactions are responsible for the robustness of the configuration of equilibrium. In this context, robustness of the healthy equilibrium refers to the perturbations the niche can cope with without Tregs being outnumbered, which would result in autoimmunity.

Moreover, density dependent interactions explain tolerance robustness in terms of the ability the system has in coping with increasing auto-antigens presentation, without breaking tolerance. This ability persists as as long as the increase happens slowly. An increase in the density of APCs presenting auto-antigens can occur when individuals are under immunological challenge and tissues are destroyed as a consequence. In this case it is fundamental that tolerance is maintained, preventing the immune system to redirect toward body components the immune response mounted against pathogens.

The cross regulation model, that provides an integrated framework for explaining immunity, tolerance and its robustness. However assumes that Tregs and Teffs identity is defined, during their interaction. Along the years, several experimental observations has been gathered suggesting that this assumption might be relaxed. Let us dig into some fundamental knowledge about Tregs and their lineage-specifying transcription factors Foxp3.

1.7 Origin of Tregs and Foxp3 expression

Tregs are a subset of CD4 T cells which selectively express the transcription factor Foxp3 (forkhead domain DNA-binding transcription factor box protein 3). For long time, before Foxp3 gene was discovered to be essential for Tregs development and function, in 2003, regulatory T cells used to be identified based on high level of expression of the high affinity IL-2 receptor α-chain (CD25) in their surface. In 1995, Sakaguchi et al. first described thymically derived cell type, characterized by the expression of CD4 and CD25, able to prevent and rescue multi-organ autoimmunity and lethal systemic inflammation [Sakaguchi et al., 1995, Asano et al., 1996]. Because CD4 and CD25 are not cell-specific surface markers, the discovery of the transcription factor Foxp3 as unique marker for Tregs, at least in mice, constituted a turning point in the study of Tregs. In both humans and mice, Foxp3 is essential for Tregs development and suppressive function [Hori, 2003, Fontenot et al., 2003, Khattri et al., 2003, Tran et al., 2007]. However, in humans also recently activated Teffs express Foxp3 [Tran et al., 2007]. The fact that Foxp3 expression is necessary for regulatory function is proven by the evidence that individuals whose CD4 T cells cannot express Foxp3 die due to multi-organ autoimmunity early in development. Mutations or lack of Foxp3 lead to the scurfy phenotype in mice and to immuno-dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans, characterized by break of tolerance and by the development of a spectrum of autoimmune diseases [Bennett et al., 2001, Brunkow et al., 2001]. This is actually the way in which Foxp3 gene was first discover, when it was realized that a mutation in the gene, resulting in a truncated Foxp3 protein, was the cause of the scurfy phenotype [Brunkow et al., 2001].

Tregs are enriched in self-reactivity [Kim et al., 2007] and constitute around 10 to 15% of the total CD4 T cells in the peripheral lymphoid compartment, in mice in physiologic condition. Most of Foxp3 transcription is initiated in CD4 single positive thymocytes, as suggested by the genetic lineage tracing of $Foxp3^+$ T [Zhou et al., 2009]. This stage of thymocytes maturation has been linked to negative selection occurring in the thymus. For this reason, this finding supports the claim that Tregs develop during negative selection, concurrently with the autoreactive $Foxp3^-CD4^+$ T cells that escape negative selection [Jordan et al., 2001, Sprent and Kishimoto, 2002].

Tregs are essential for ensuring natural tolerance and preventing autoimmunity, by controlling the activation and proliferation of auto-reactive lymphocytes [Sakaguchi et al., 1995, Sakaguchi et al., 2001, Sakaguchi, 2004]. Accumulating evidences have shown that Tregs have the potential to suppress many kind of immune response. During immune response against pathogens, Tregs modulate the immune response preventing associated immune pathology [Cahill et al., 1997, Belkaid et al., 2002, Hori et al., 2002, Suvas et al., 2004]. Also, they prevent rejection of transplants [Taylor et al., 2001, Kingsley et al., 2002, Graca et al., 2002] and modulate the immune response against tumors [Onizuka et al., 1999, Ohue and Nishikawa, 2019]. Tregs ensure tolerance to commensal bacteria [Nagano et al., 2012, Nutsch and Hsieh, 2012] and promote maternal tolerance to the fetus during pregnancy [Aluvihare et al., 2004].

Several mechanisms of suppression perpetuated by Tregs have been identified. Tregs can directly inhibit other immune cells either by secreting anti-inflammatory cytokines, or by expressing co-inhibitory molecules such as cytotoxic T lymphocytes antigen 4 (CTLA4) and lymphocyte activation gene 3 protein (LAG3). Also, they are able to indirectly inhibit other lymphocytes, by modulating the activity of APCs.

It was believed that Tregs constitute a distinct lineage of cells simultaneously committed to Foxp3 expression and regulatory activity [Sakaguchi, 2005, Sakaguchi et al., 2008]: in this view, tolerance robustness was reduced to the putative robustness of Foxp3 differentiation program. This idea was progressively undermined. Experimental evidences have shown that Foxp3 differentiation program is plastic and unstable and, among Tregs, different populations of cells have been named.

Thymic Tregs (tTreg) differentiate from thymic precursors, during the single positive (SP) $CD4^+$ $CD8^-$ stage, when thymic selection takes place and upon a strong signal related to their TCR specificity [Fontenot et al., 2005]. These cells exit the thymus already expressing Foxp3 and with the ability of preventing lymphocytes proliferation. Tregs differentiation happens also in the periphery: in this case Foxp3 expression and regulatory function is induced in mature peripheral naive CD4 T cells, as a consequence of exposure to antigens in the periphery [Curotto de Lafaille and Lafaille, 2009,

Bilate and Lafaille, 2012]. These cells are called peripheral Tregs (pTregs). pTregs have been claimed to be important in maintaining tolerance, particular to self-antigens not expressed in thymus and to help tumor cells to escape from immune surveillance [Yamaguchi and Sakaguchi, 2006]. For both thymic and perypheral Tregs the differentiation program has been proposed to take place in two steps [Burchill et al., 2008, Schallenberg et al., 2010]. The first step is the up-regulation of CD25, the α-chain of the high-affinity trimeric IL-2 receptor, induced through TCR signaling. Up-regulation of CD25 makes Foxp3$^-$CD25$^+$ thymocytes more receptive to IL-2 stimulation, which induces the Foxp3 up-regulation (second step). The full extent of differences and similarities between tTreg and pTregs populations is yet to be defined. Helio and neuropilin-1 have been used as markers to distinguish tTreg (Helios$^+$ Nrp1$^+$) and pTreg (Helios$^-$ Nrp1$^-$) [Yadav et al., 2012, Weiss et al., 2012]. It has been shown that Foxp3 differentiation program is unstable in both tTreg and pTreg [Duarte et al., 2009, Zhou et al., 2009]. Under inflammation [Zhou et al., 2009, Yurchenko et al., 2012, Mellor and Munn, 2011] and in lymphodeficient conditions [Duarte et al., 2009, Komatsu et al., 2009] Tregs can lose Foxp3 expression and be reprogrammed to conventional T cells, mostly self-reactive. However, under physiologic conditions, Tregs show stable phenotype [Rubtsov et al., 2010].

Stable expression of Foxp3 is required for Tregs to maintain suppressive function [Williams and Rudensky, 2007]. Many molecular mechanisms that underpin Foxp3 expression are known. Foxp3 gene has three conserved non-coding sequence (CNS) regions, that serve as enhancer regions, and a conserved region defined as promoter which is up-stream the transcriptional start site (TSS). These regions are bound by several transcription factors downstream the TCR, the IL-2 and the TGF-β signalling pathways, influencing the induction, consolidation and tuning of Foxp3 transcription. Foxp3 expression requires continuous TCR signalling which causes downstream NFAT (Nuclear factor of activated T cells) to bind to the Foxp3 promoter and to the CNS1 [Tone et al., 2008]. NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) regulates Foxp3 expression downstream of TCR stimulation, by binding to CNS3, which leads to open the Foxp3 promoter region [Ruan et al., 2009, Zheng et al., 2010, Long et al., 2009]. NF-kB also binds to the Foxp3 promoter and to the CNS2 [Long et al., 2009, Zheng et al., 2010].

Downstream the TCR signalling pathway, also the activator protein 1(AP1) binds to the Foxp3 promoter [Mantel et al., 2006], the cyclic AMP response element-binding protein (CREB) binds to the CNS2 [Kim and Leonard, 2007] and the Nr4a proteins bind to the promoter [Sekiya et al., 2013]. The binding of a RUNX1-CBFβ complex (runt-related transcription factor 1, core-binding factor subunit β complex) to CNS2 is fundamental for the maintenance of Foxp3 expression in Tregs [Kitoh et al., 2009a] and allow for Foxp3 binding to CNS2 [Zheng et al., 2010]. IL-2 stimulation triggers downstream Jack-STAT5 (Janus Kinase-signal transducer activator of transcription 5) signalling, with consequent binding of the STAT5 dimers to the promoter and CNS2, which activate Foxp3 transcription [Yao et al., 2007, Villarino et al., 2007]. Extra-thymic Tregs development relies, along with TCR engagement and IL-2 stimulation, on TGF-β induced smad2/3 (small mothers against decapentaplegic) signalling, through the binding to CNS1 [McKarns and Schwartz, 2005].

Based on the molecular knowledge, models of the gene regulatory network (GRN) underlying the CD4 T cells differentiation program, in response to heterogeneous environments, have been proposed. Gene regulatory network models focus on single cells and represent, through logic rules, the collections of regulators that interact with each other and other substances inside a cell, to govern gene expression levels. These models investigate how gene expressions are affected from external environmental inputs. In particular, the model developed in [Naldi et al., 2010, Abou-Jaoudé et al., 2015], in which gene regulatory network together with signaling pathways drive the CD4 T cell differentiation program, found the canonical CD4 T cells phenotypes, including Tregs, as stable states of the state transition graph. Remarkably, for what concerns the stability of the cell subtypes with respect to environmental changes, in this model Tregs appear to be context-dependent and more plastic than the other CD4 T cell subpopulations. Along these canonical populations, and coexisting with them, other transiently hybrid phenotypes have been found, which express combined features of the canonical ones, including Foxp3 expression, in a context-dependent way.

1.8 Epigenetic control of Foxp3 expression

As already mentioned, three conserved non coding regions, other than a promoter, have been identified within the Foxp3 locus. These regions have been shown to play important role in ensuring stability to Foxp3 expression [Zheng et al., 2010, Schlenner et al., 2012].

Tregs have been shown to display full demethylation of a conserved CpG-rich region within the first intron of the Foxp3 locus (the CNS2), as well as histone acetylations modifications [Floess et al., 2007, Polansky et al., 2008, Huehn et al., 2009].

The demethylation of the CNS2 facilitates the recruitment of several transcription factors to the CNS2 itself. Among the ones described, there are RUNX1-CBFβ complex, CREB/ATF and Ets-1 that bind to the CNS2; STAT5 that binds to Foxp3 promoter and to the CNS2 and Foxp3 itself [Kitoh et al., 2009b, Kitagawa et al., 2013, Sekiya et al., 2016, Kim and Leonard, 2007, Polansky et al., 2010]. Particularly, Foxp3 binding to CNS2 appears to happen after and being dependent on CNS2 demethylation. Foxp3 binding to CNS2 also depends on RUNX1-CBFβ [Zheng et al., 2010], with whom it forms large complexes [Ono et al., 2007]. In this sense the demethylation of the Foxp3 locus has been proposed to provide epigenetic memory of Foxp3 expression, because it maintains the Foxp3 locus accessible to the transcription factors that then trans-activate Foxp3 transcription. The complete demethylation of this region, called the Tregs-specific demethylated region (TSDR), has been claim to be the key mechanism through which stable Foxp3 expression is achieved in CD4 T cells [Floess et al., 2007, Huehn et al., 2009, Polansky et al., 2008, Zheng et al., 2010] and methylation status of the TCDR has been used for the identification of stable Tregs [Toker et al., 2013].

Demethylation at the TSDR is not required for initiation of Foxp3 expression, but it is needed for its long-term maintenance [Polansky et al., 2008, Huehn et al., 2009, Zheng et al., 2010] and happens when Foxp3 expression is already sustained [Bending et al., 2018]. Furthermore TSDRs are fully demethylated in stable Tregs, while they are fully methylated both in conventional CD4 T cells and in *in vitro* induced Tregs [Huehn et al., 2009]. Forced TSDR demethylation confers stability to Foxp3 expression in *in vitro*-induced Tregs [Polansky et al., 2008]. Finally, ex-Foxp3 $CD4^+$ T cells, generated in lymphopenic

conditions, show a partially demethylated TSDR [Miyao et al., 2012]. Interestingly, among them some are able to reacquire Foxp3 expression upon activation and become fully demethylated at the TSDR. Others remain Foxp3$^-$ and exhibit fully methylated TSDR upon activation [Miyao et al., 2012].

CpG-reach regions that are preferentially demethylated in Tregs have been found in other genomic regions, outside the Foxp3 locus. These regions are also called TSDRs, they have been found to be distributed in genes that belong to the Treg signature genes and they are important for Treg differentiation and function. These genes include Ctla4, Il2ra, Ikzf4 and Tnfrsf18 [Ohkura et al., 2012].

1.9 Foxp3 dynamics *in vivo*

As already mentioned, in lymphodeficient condition and under inflammation, Foxp3$^+$ cells have been observed to lose Foxp3 expression [Duarte et al., 2009, Komatsu et al., 2009]. When these cells were transferred into lymphodeficient host, a fraction of them became pathogenic, producing inflammatory cytokines and infiltrating tissues [Duarte et al., 2009]. However, 10$\%$ of them reacquired Foxp3 expression within 4 weeks [Komatsu et al., 2009].

In the attempt to investigate whether Foxp3 loss could be observed in lymphoreplete conditions, two laboratories have generated two different transgenic mouse models to perform fate mapping studies. Zhou et al. generated the Foxp3$^{\text{GFP-Cre}}$ $\times$ ROSA26YFP transgenic mouse by crossing the mice expressing the GFP-Cre fusion protein under the control of the Foxp3 promoter on a bacterial artificial chromosome (BAC), with the reporter mice which express the YFP, under the control of the ROSA26 promoter, after the excision of the loxP-flanked cassette [Zhou et al., 2009]. Some years later, Miyao *et al.* generated the Foxp3$^{\text{GFP-Cre}}$ $\times$ ROSA26RFP transgenic mouse, in which differently from the previous one, the GFP-Cre is expressed under the control of the endogenous Foxp3 locus [Miyao et al., 2012]. In both mice, cells that express Foxp3 are permanently labelled with YFP or RFP, respectively. These mice allow for assessing the frequency of cells that have expressed Foxp3 at one point and then have down-regulated it: cells that up-regulate Foxp3 become first GFP$^+$, then GFP$^+$ YFP$^+$ or GFP$^+$ RFP$^+$, while exFoxp3 cells are GFP$^-$ YFP$^+$ (or GFP$^-$ RFP$^+$). In both cases 10 to 20 $\%$ of CD4 T cells were found to be exFoxp3.

When exTregs recovered from lymphoreplete condition were transferred into lymphodeficient host, a portion of them became pathogenic. Also, in case these exTregs belonged to a diabetogenic TCR transgenic mouse, they managed to transfer diabetes into lymphodeficient host [Zhou et al., 2009]. Yet, when exTregs were transferred into lymphodeficient host, 10% of them reacquired Foxp3 expression within 4 weeks [Komatsu et al., 2009].

Nevertheless, in case the genetic fate-mapping was performed in an inducible way in adult mice, less than 5% of Tregs lost Foxp3 expression during 5 months [Rubtsov et al., 2010]. In fact Rubtsov et al. generated the Foxp3$^{\text{GFP-Cre-ERT2}}$ $\times$ ROSA26YFP transgenic mouse. In this mouse when the GFP-Cre-ERT2 is expressed, it stays in the cytosol. Only upon treatment with tamoxifen it enters the nucleus, allowing for excision of the loxP-flanked cassette.

1.10 Outline of the thesis

The aim of this thesis is to investigate theoretically and quantitatively the mechanisms that ensure tolerance robustness. In physiologic condition tolerance is maintained thanks to processes that involve two complementary levels: on the one hand, at a cellular level, Foxp3 expression in Tregs is stably maintained along time; on the other hand, at a multicellular level, Tregs population is maintained in balance with the Teffs population they suppress. For this reason our approach also followed two complementary levels.

The thesis investigates the possibility that Tregs can be maintained as a stable population, even in case Foxp3 expression depends on the input the cells receive from the other immune cells populations, in a density-dependent way. Also, the work questions whether the stability of Foxp3 expression, observed in physiologic condition, can be due to the context, rather than to a programmed developmental process. Using experimental data available from literature, we studied whether data support the possibility that Tregs identity is context-dependent, rather that committed.

Beside the present general introduction and a general discussion, the thesis is organized in three chapters. Each one follows the structure of a paper.

In Chapter 2, a new model of Treg and Teff population dynamics, mediated by APCs, is developed, as an extension of the cross regulation model. This model

accounts for loss of Foxp3 expression in Tregs, in case they lack sufficient pro-Foxp3 stimuli. According to the model, Tregs that lose Foxp3 expression convert into Teffs. Stability and bifurcation analysis is performed. In this chapter is shown that there are parameter regimes in which, for slow rate of Foxp3 loss, not only Tregs population can be stably maintained, but also their robustness in response to perturbation is increased, if compared to to the original cross regulation model.

Chapter 3, moving from the theoretical result of the chapter 2, focuses on the cellular level. It investigates how the assumption of context dependent Foxp3 loss can be realized at a cellular level in CD4 T cells. In particular, it focuses on the time scale at which the Foxp3 loss would occur. A stochastic model for Foxp3 expression in CD4 T cells is developed, in which gene expression can be reversible and context-dependent, as well as committed. Two potential populations of Tregs are considered: committed Tregs, that in no case can lose Foxp3 expression, and plastic Tregs, whose Foxp3 expression depends on the context. By tuning the model parameters, the model accounts for the possibility that either one of the two populations exists or that they coexist, in any proportion. A meta-analysis is performed, by fitting the model to experimental data on in vivo Foxp3 expression, in different experimental contexts. The analysis founds that experimental data are compatible with Tregs being a homogeneous pool of CD4 T cells, in which Foxp3 expression is either stable or labile depending on the context, in particular depending on Teffs number and proportion. The rate of loss of Foxp3 expression in peripheral Tregs in absence of pro-Foxp3 stimuli is quantified.

Chapter 4 studies whether the slow loss of Foxp3 expression in Tregs can be the consequence of epigenetic dynamics. An extended version of the stochastic model of Foxp3 expression, introduced in Chapter 3, is developed, that also accounts for epigenetic dynamics. A meta-analysis is performed, to check whether both stability and lability of Foxp3 expression can be explained as a combination of fast and context-dependent transcription/translation dynamics together with a slow and context-independent epigenetic dynamics. Data fitting suggests that indeed this could be the case.

The final discussion tries to put together the results of the three chapters, in a critical way, highlighting point of force of the thesis, as well as weakness and pitfalls.

In all the models presented in this work we tried to avoid complexity as much

as possible, trying to capture the essential features of the mechanisms that, case by case, we were investigating.

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2

Tolerance is compatible with context-dependent Foxp3 expression in Tregs. Theoretical insight from T cells population dynamics.

Statement: All the work included in this chapter is my own, carried out under the supervision of Jorge Carneiro and Jocelyne Demengeot.

Abstract

Immunological tolerance toward body components and tissues is a robust property of the adaptive immune system. It relies on the development and maintenance of populations of functional Regulatory T cells (Tregs), able to prevent the clonal expansion of auto-reactive CD4 T Effector cells (Teffs). If not controlled by specific Tregs, auto-reactive Teffs that circulate in the periphery undergo activation and clonal expansion, causing autoimmunity.

In healthy individuals, persistence of Tregs is ensured by density-dependent interactions that Tregs make with Antigen Presenting cells (APCs) and Teffs that they control. Depending on the initial conditions of the T cell populations, the

same interactions can also result in Tregs extinction, giving rise to autoimmunity. This claim was proposed in the past by the cross regulation model for APCs-mediated CD4 T cells interactions and validated using *in vivo* and *in vitro* experimental data. According to this view, tolerance and autoimmunity represent two alternative stable states for the immune system.

Tregs are characterised as a subset of CD4 T cells enriched in auto-reactivity which selectively express the transcription factor Foxp3, whose stable expression is necessary for their regulatory function. In the last years, an increasing number of experiments have shown loss of Foxp3 expression in CD4 T cells, in conditions such as lymphopenia and under inflammation. The lability of Foxp3 expression *in vivo* poses the problem of how Tregs can be maintained as a functional population and, more generally, how tolerance is robustly maintained.

Here we extended the cross regulation model accounting for context-dependent Foxp3 loss and subsequent conversion of Treg into Teff. We show that tolerance is maintained provided that Foxp3 loss is sufficiently slow. Furthermore, introduction of Foxp3 lability determines a qualitative change in the tolerance stable state. As a result, robustness of tolerance in response to perturbation is increased. We also show that thymic output results in enhanced tolerance robustness, whereas thymic involution causes a decrease in the amplitude of the perturbations the system can cope with, without breaking tolerance.

2.1 Introduction

The presence of auto-reactive lymphocytes in the periphery, that have escaped thymic negative selection, is well documented [Ramsdell et al., 1989, Heath et al., 1992]. These cells, can undergo clonal expansion and cause autoimmunity, if they are not controlled by specific/cognate regulatory T cells, as experimental evidences have shown [Sakaguchi et al., 1995, Annacker et al., 2000, Annacker et al., 2001, Almeida et al., 2002].

Several experiments of adoptive transfers have been performed in the past years, in which immunodeficient mice were reconstituted with CD4 Teffs and/or CD4 Tregs, sorted from immunocompetent mice. The aim of these experiments was to check for development of immunopathology in the host over time and to quantify the number of equilibrium of T cells reached in the host animal. Im-

munodeficient mice that were transferred with Teffs cells alone, developed systemic immunopatology that led to death, caused by uncontrolled proliferation of Teffs which infiltrate tissues [Sakaguchi et al., 1995, Annacker et al., 2001]. On the contrary, when Tregs were transferred alone, they did not cause death nor autoimmunity. Co-transfer of Tregs together with Teffs, depending on their respective proportions, resulted in either of the two scenario. Particularly, in mice in which tolerance was not broken upon co-transfer, the total proliferation of T cells was controlled: the equilibrium reached in the host was characterized by a smaller number of T cells, compared with the case of Teffs transferred alone. Moreover, the number of Tregs at equilibrium increased with the one of activated Teffs in the host [Almeida et al., 2002, Annacker et al., 2001].

Indeed Tregs damp down the activation and clonal expansion of auto-reactive T cells. These experiments have also shown that Tregs maintenance and proliferation depends on Teffs, in the sense that Tregs feed on the populations of Teffs they suppress.

In healthy individuals mechanism are operating to ensure the stable and robust maintenance of specific Tregs population, able to control the activation and proliferation of auto-reactive Teffs in the periphery, without affecting the capacity of the immune system to fight against pathogens.

Carneiro and León [León et al., 2000, Carneiro et al., 2007], in previous theoretical research, have been able to recapitulate, through mathematical modeling, these fundamental properties of both immunity and tolerance. The cross regulation model (CRM) interprets both immunity and tolerance, as the result of non-linear and density-dependent population dynamics, occurring among Teffs, Tregs and mediated by APCs of similar specificity. The model has been validated using both *in vivo* and *in vitro* experimental data and is able to explain the immune response against tumors, together with tumor control or expansion [Carneiro et al., 2007, Leon et al., 2007].

For what concerns the duality tolerance-autoimmunity, observed in the adoptive transfer experiments just mentioned, the CRM explains it in terms of bistability occurring in the system in which populations of auto-reactive Teffs (the ones that can cause autoimmunity) and Tregs of similar specificity interact, mediated by APCs presenting auto-antigens.

The cross regulation model supports parameter regimes in which the system

is characterised by two stable equilibria. One referred as tolerance state, in which Tregs and Teffs populations coexist, with Tregs controlling proliferation of cognate Teffs, and the autoimmune state, in which Tregs are extinct and auto-reactive Teffs proliferation is not controlled. Moreover, in the case of autommune state, Teffs proliferation is determined by APCs availability.

It is well known that Tregs selectively express the TF Foxp3, which is necessary for Tregs development and regulatory function [Hori, 2003, Fontenot et al., 2003, Khattri et al., 2003]: individuals that lack Foxp3 expression in CD4 T cells, develop severe and multi-organs immuno-pathology and die early after birth. For long time the robust maintenance of Tregs in healthy individuals was attributed to the robust expression of Foxp3 in Tregs. In fact, in physiologic conditions, Foxp3 expression is stably maintained for months and across several rounds of cell division [Rubtsov et al., 2010].

Nevertheless, in the last years it has been shown that, in different conditions *in vivo*, such as in lymphodeficient condition [Komatsu et al., 2009, Duarte et al., 2009] and under inflammation [Zhou et al., 2009, Yurchenko et al., 2012, Mellor and Munn, 2011], $Foxp3^+$ cells can lose Foxp3 expression. Also, upon loss of Foxp3 expression, these cells become pathogenic, infiltrating tissues and expressing inflammatory cytokines [Duarte et al., 2009]. The lability of Foxp3 expression *in vivo* poses therefore the problem of how it is possible to maintain Tregs as population. More in general, Foxp3 labilty poses the question of how can natural tolerance mediated by $Foxp3^+$ regulatory T cells be robust, if Foxp3 gene expression has been shown to be context-dependent and potentially labile.

This question has not been addressed so far and constitute a reason for theoretical study. The Cross regulation model, as it stands, does not address the possibility of Foxp3 loss. Here, based on the Cross Regulation model, we extended the model accounting for context-dependent lability of Foxp3. Introducing the possibility for $Foxp3^+$ cells that lack the environmental stimuli for Foxp3 maintenance, to convert into Teff, we then used the model to address the following issues. Does the system still display bi-stability? Or does the it collapse to the autoimmune state? Can tolerance be maintained, can it be robust, even if Foxp3 expression is labile?

We show that our model maintains the essential features of the cross regu-

lation model, and that as long as the rate at which Tregs lose Foxp3 expression, in absence of pro-Foxp3 stimuli, is slow enough, the system displays bi-stability. Finally we show that there is an interval of values for the Foxp3 loss rate that correspond to enhanced robustness of Tregs, with respect to their ability to cope with perturbation, without breaking tolerance.

2.2 The model

The model is an extension of the cross regulation model, which has been extensively described and analyzed in [León et al., 2000, León et al., 2001, León et al., 2003, León et al., 2004, Carneiro et al., 2005, Carneiro et al., 2007]. In this section we recapitulate the principles and assumptions of the original model, adding also the novel assumption of Foxp3 expression loss in unstimulated Tregs.

2.2.1 General biological principles and model assumptions

The model adopts two general biological principles of cell population dynamics, valid in any multicellular organism. The first one states that any cell lineage needs recurrent interactions with other cells in order to persist as a population. In absence of interactions cells die for apoptosis. Furthermore, the turnover of the cells in any lineage is mediated by interactions among cells. These interactions are either direct, such as contact inhibition, or indirect, such as competition for limited survival factors and growth factors, be they provided by molecules (such as cytokines) or by engagement with other cells (such as TCR engagement with MHC-peptide complex displayed by APCs).

The model describes the dynamics of peripheral CD4 Teffs and Tregs and their interaction, mediated by APCs. It assumes that APCs display MHC-peptide complexes that can be recognized by the T cell receptors on the lymphocytes surface. Furthermore, Teffs, depending on their specificity, meaning on the set of antigens they recognize, can either induce autoimmunity or mount immune response against pathogens. Finally, Tregs suppress Teffs with similar specificity preventing their proliferation and clonal expansions.

The model is based on the following assumptions:

- Many TCRs recognize the same peptide and many peptides are recognized by the same TCR [Sewell, 2012]. Therefore, APCs in the body can be considered as heterogeneous populations, each one presenting a given set of peptides. Each APC population is considered homogeneous in terms of recognition and conjugation with T cell populations (hereafter referred as cognate populations);
- Similarly, Tregs and Teffs are classified as different populations according to their clonal specificity and, in particular, according to their ability to recognize and interact with the same APCs population. We will therefore refer to triplet of Tregs, Teffs and APCs populations able to interact among themselves (hereafter referred as cognate populations);
- Each APCs population is in stationary state: we are considering physiological condition (no immunological challenge) in which, despite of their turnover, APCs are expected to be constant;
- Teffs and Tregs are assumed to be exported in the periphery as such, by the thymus, with functional TCR;
- Resting T cells slowly die for apoptosis;
- T cells activation, which is responsible for their proliferation, needs interaction with APCs that present cognate antigen. T cells activation also depends on the interactions that Teffs and Tregs make with each other;
- Interactions are indirect, such as competition for limited cognate APCs, and direct, given the molecular processes that require proximity among T cells and APCs. The model assumes the simplest form of this interaction that ensures a certain degree of specificity: T cells and APCs interact by forming multicellular conjugates;
- Teffs proliferation is promoted by productive interactions with cognate APCs, while is inhibited in case the interaction occurs together with cognate Tregs;
- Tregs proliferation is promoted by productive interactions with cognate APCs together with Teffs;
- Absence of productive interactions for Tregs, promotes loss of Foxp3 expression in Tregs and subsequent conversion into Teffs.

Assumptions are summarized in the reaction diagram in Figure 2.1.

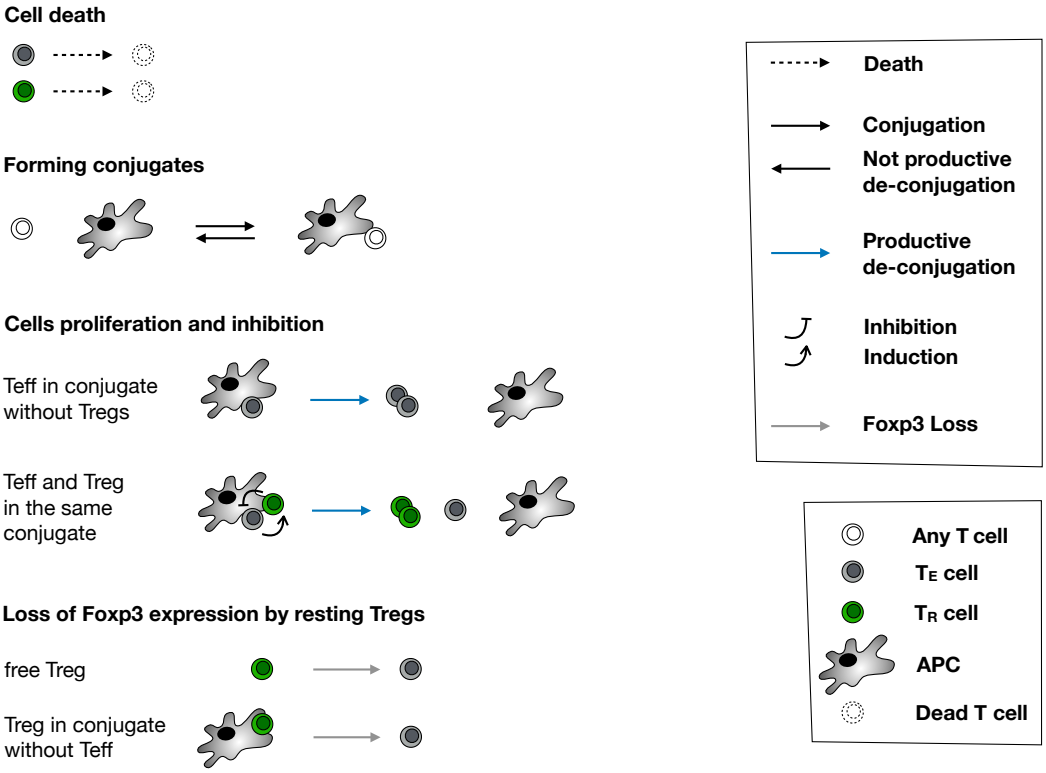

Figure 2.1: **Main rules of the model.** Reaction diagram indicating events and reactions underlying APCs mediated Tregs and Teffs dynamics.

2.2.2 Mathematical formulation of the model

The system is constituted by a set of ordinary differential equation, describing the time evolution of the densities of Tregs (R) and Teffs (E), mediated by a single cognate population of APCs, at fixed density A. Equations are the following, both characterized by a proliferation term, a death term and a conversion term:

$$\frac{dR}{dt} = p_R R_a(R,E) - d_R R - c_R R_u(R,E), \tag{2.1}$$

$$\frac{dE}{dt} = p_E E_a(R,E) - d_E E + c_R R_u(R,E). \tag{2.2}$$

In equations 2.1 - 2.1, R_a and E_a are the density of activated Tregs and Teffs in multicellular conjugate with cognate APCs, respectively, while p_R and p_E are the respective proliferation rates. Activated Tregs are the ones in conjugate together with at least a Teff, while activated Teffs are the one in conjugate without any Treg. Parameters d_R and d_E represent the respective death rates. Finally R_u indicates the density of un-activated Tregs (being either free or in conjugate without any Teff) and c_R is the rate at which they lose Foxp3 expression and convert into Teffs.

The densities R_a, R_u and E_a are functions of R and E and are computed in two steps. The first one consists in the calculation of the density of Tregs and Teffs in conjugates. The second step consists in computing their distribution in multicellular conjugates, together with cognate APCs. We start from the assumption that the time scale at which any T cell conjugates and de-conjugates from an available site on APC (conjugation and non-productive de-conjugation in Figure 2.1, which happen at rates c and d) is much faster than the time scale at which populations densities change (productive de-conjugation, in the same figure). Under this assumption we can, therefore, consider the density C of T cells in conjugates to be at equilibrium (quasi-steady-state approximation). This equilibrium density depends on the total densities of T cells, on the total density of APCs sites and on the constant of equilibrium, in the following way:

$$C = \frac{1 + K(S+T) - \sqrt{(1+K(S+T))^2 - 4K^2ST}}{2K}, \tag{2.3}$$

where $T = R + E$ is the total density of T cells and S is the total density of APC conjugation sites given by $S = nA$, with n being the number of sites per APC and A the total density of APCs. Finally K is the constant of equilibrium for conjugates, given by the ratio between the conjugation rate and the sum of the two de-conjugation rates (productive and not productive ones).

Then, the density of Tregs and Teffs in conjugate, can be respectively computed as following:

$$R_c = C\frac{R}{T}. \quad E_c = C\frac{E}{T}. \tag{2.4}$$

We can define the probabilities that a site is occupied by either a Treg (ϱ) or a Teff (ε), respectively as:

$$\varrho = \frac{R_c}{S}, \quad \varepsilon = \frac{E_c}{S}. \tag{2.5}$$

Finally the density of activated Tregs and Teff are respectively:

$$R_a = R_c\left(\frac{2\varepsilon}{2-\varrho}\right), \tag{2.6}$$

$$E_a = E_c\left(1 - \frac{2\varrho}{2-\varepsilon}\right). \tag{2.7}$$

2.3 Results

2.3.1 Regulation can take place even in presence of context-dependent Foxp3 lability.

We analyzed the steady states of the system and their dependency on key model parameters. Particularly, we focused on the total density S of APCs sites and on the rate c_R at which un-stimulated Tregs lose Foxp3 expression.

We found that the model recapitulates a crucial feature of the original cross regulation model. In particular, for sufficiently large values of the density S of APCs sites ($S > S^*$), the system displays bi-stability. In other words the system is characterized by two stable equilibria: the autoimmune equilibrium, in which Teff cells population out-competes Tregs, and the tolerance equilibrium in which the

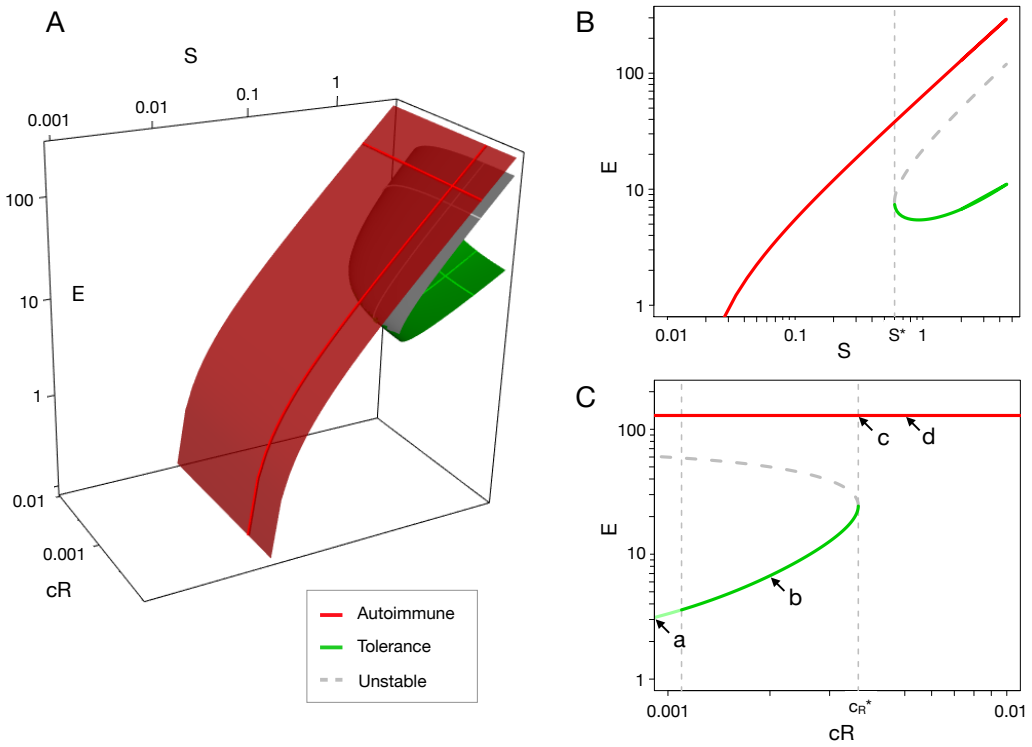

Figure 2.2: **Bi-stability of the system is preserved for adequately large density of APCs site ($S > S^*$) and within a range of values of Foxp3 loss rate ($0 \leq c_R \leq c_R^*$) for unstimulated Tregs.** A: Bifurcation diagram showing the density of Teff cells of equilibrium (E) as a function of the density of available APCs sites (S) and of the rate of Foxp3 loss by unstimulated Tregs (c_R). The red surface corresponds to the stable autoimmune equilibrium of the system, characterized by the absence of Tregs, the green surface corresponds the the stable healthy equilibrium, characterized by the coexistence of Tregs and Teff, the former regulating the latter. Finally grey surface corresponds to the unstable equilibrium. Curves on the surface are draw for $c_r = 0.002$ and $S = 2$. Values of the parameters are: $p_r = 1.0, p_e = 1.3, d_r = 0.02, d_e = 0.02, c = 1, d = 1$.B and C: 2D sections of the bifurcation diagram. B: density of Teff cells of equilibrium (E) as a function of the density of available APCs sites (S), for $c_R = 0.002$. Vertical dashed line, in correspondence to $S = 0.6$, indicates the saddle-node bifurcation. C: density of Teff cells of equilibrium (E) as a function of the rate of Foxp3 loss by unstimulated Tregs (c_R), for $S = 2$. Vertical lines indicates, respectively, the change in healthy equilibrium from stable focus to stable node as c_R increases, occurring at $c_R = 0.0011$, and the saddle-node bifurcation, at $c_R^* = 0.0036$.

two cognate populations coexist, Teffs being under the control of Tregs (Figure 2.2 A and B).

Bi-stability implies that any of the two equilibria can be reached by the system depending on whether the initial condition lies in each of the respective equilibrium basin of attraction (see green and red regions in Figure 2.3). The basin of attraction of the tolerance equilibrium highlights all the perturbations in both population densities that the system can cope with, without breaking tolerance.

We found that bi-stability is lost for limited APC availability. In other words, in case of rare antigen presentation, the unique stable equilibrium is the autoimmune one, which is characterized by the presence of Teffs alone.

In summary, if Tregs are committed to Foxp3 expression ($c_R = 0$) bi-stability is ensured as long as the total density S of APCs sites is big enough, as shown by the original cross regulation model. Nevertheless, in case Tregs lose Foxp3 expression in a context-dependent way, there is an entire range of values for the Foxp3 loss rate $0 \leq c_R < c_R^*$, for which bi-stability is still preserved (Figure 2.2 C). For faster Foxp3 loss rate by un-stimulated Tregs ($c_R \geq c_R^*$), the system no longer displays bi-stability and it admits only the autoimmune equilibrium (see Figure 2.2 C, and Figure 2.3 bottom).

2.3.2 Foxp3 lability can result in increased Tregs population robustness

We found that within the range of values of c_R that allow for bi-stability, the stable equilibrium of tolerance changes its characteristics. In absence of Foxp3 loss ($c_R = 0$) and for small values of the parameter c_R, the tolerance equilibrium is a stable focus, while for higher values of c_R the equilibrium becomes a stable node. The difference between a stable focus and a stable node mainly concerns the response of the system to the same perturbation of the equilibrium. In other words, if we consider a perturbation of the equilibrium that leaves the system within the basin of attraction of the equilibrium, the return to the equilibrium either follows oscillations (in case of stable focus) or happens much smoothly (in case of stable node). This result can be appreciated in Figure 2.4. Looking at the phase plane of the system, the trajectories within the basin of attraction of the tolerance equilibrium (dashed curves within the green area) present over-shootings and dangerous decreases in Teffs population for $c_R = 0$, which no longer appear for

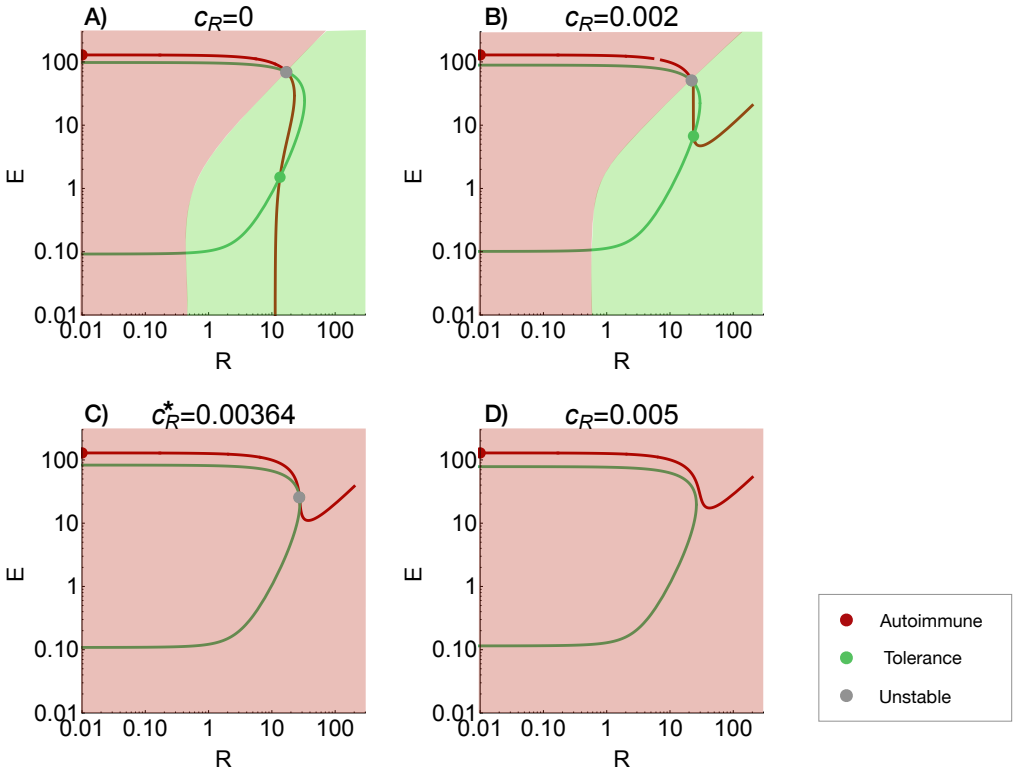

Figure 2.3: **Tolerance can be maintained for Foxp3 loss rate $0 \leq c_R < c_{R*}$.** Phase plane of the system for different values of the Foxp3 loss rate c_R. Selected values for c_R, are the same highlighted in figure 2.2 B, with arrows and small letters. Red lines represent the null-clines of the density E of Teffs, green lines represent the null-clines of the density R of Tregs. Equilibria of the system, found in correspondence of the intersections of null-clines, are depicted by dots. In each graph, red dot represents the stable autoimmune equilibrium, red area the corresponding basin of attraction; green dot represents the stable tolerance equilibrium, green area the corresponding basin of attraction. Grey dot depicts the un-stable saddle-node equilibrium. Values of the other parameters are: $p_r = 1.0, p_e = 1.3, d_r = 0.02, d_e = 0.02, S = 2, c = 1, d = 1$.

greater values of c_R (*e.g.* $c_R = 0.002 < c_R^*$).

2.3.3 Thymic output favours tolerance

We next explored the consequences of constant thymic influx in the system, in presence of context-dependent Foxp3 expression. To do that we added a constant positive source term to each of the equations of the system 2.1-2.2, s_R and s_E respectively:

$$\frac{dR}{dt} = p_R R_a - d_R R - c_R R_u + s_R, \tag{2.8}$$

$$\frac{dE}{dt} = p_E E_a - d_E E + c_R R_u + s_E, \tag{2.9}$$

where $s_R = \sigma$ and $s_E = \sigma\, k$. The parameter k allows for differential thymic influx, between Tregs and Teffs population. In particular, values of k bigger than 1, imply a higher input in the periphery from the thymus of Teff cells, in compared with Tregs.

We then studied the phase plane of the system, for different values of the source parameter σ and in correspondence to the previously studied value of the Foxp3 rate loss ($c_R = 0.002$), which ensures bi-stability to the system and robustness of tolerance. We found that the introduction of the thymic source, even if the contribution of Teffs exceeds the one of Tregs ($k = 1.2$), change the autoimmune equilibrium. In the presence of thymic output, in fact, the autoimmune equilibrium is characterized by the presence of Tregs, which nevertheless, remain outnumbered by cognate Teffs and are not able to control their proliferation. The change in the autoimmune state is caused by the change in shape of the null-cline of Tregs, whose intersection with the Teffs null-cline determines the steady state of the system (as shown in figure 2.5).

As the thymic output increases, the basin of attraction of the tolerance state, increases as well. Also, for the critical value $\sigma^* = 0.07$ of the thymic source, a saddle-node bifurcation occurs in which the autoimmune steady state disappears. In this case, and for higher values of thymic output, the only stable equilibrium for the system, remains the tolerance one, in which proliferation of autoreactive Teffs is controlled by cognate Tregs.

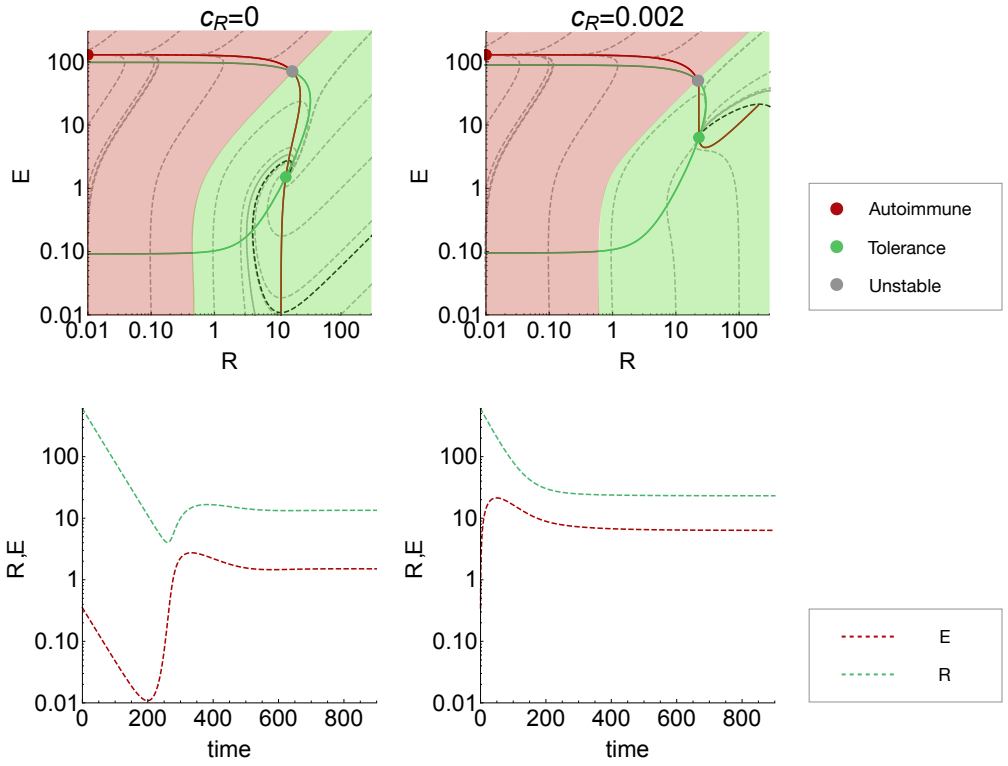

Figure 2.4: **Robustness of tolerance is enhanced for slow rate c_R of Foxp3 loss.** Up: Phase plane, basins of attraction and trajectories of the system, in case of committed Tregs ($c_R = 0$, on the left) and for slow Foxp3 loss rate ($c_R = 0.002$, on the right). As in Figure 2.3 red line represents the E null-cline, red dot the autoimmune equilibrium, red area its basin of attraction; green line represents the R null-cline, green dot the tolerance equilibrium where Teffs are under the control of Tregs, green area its basin of attraction. In dashed grey are represented some trajectories: oscillations can be observed in the tolerance basin of attraction in case $c_R = 0$, where the equilibrium is a focus, for slow rate of loss the trajectories within do not oscillate and goes much smoothly toward the equilibrium, which is a node. Bottom: time course of the system in response to the same perturbation from the tolerance equilibrium, in case of committed Tregs ($c_R = 0$, on the left) and for slow Foxp3 loss rate ($c_R = 0.002$, on the right).

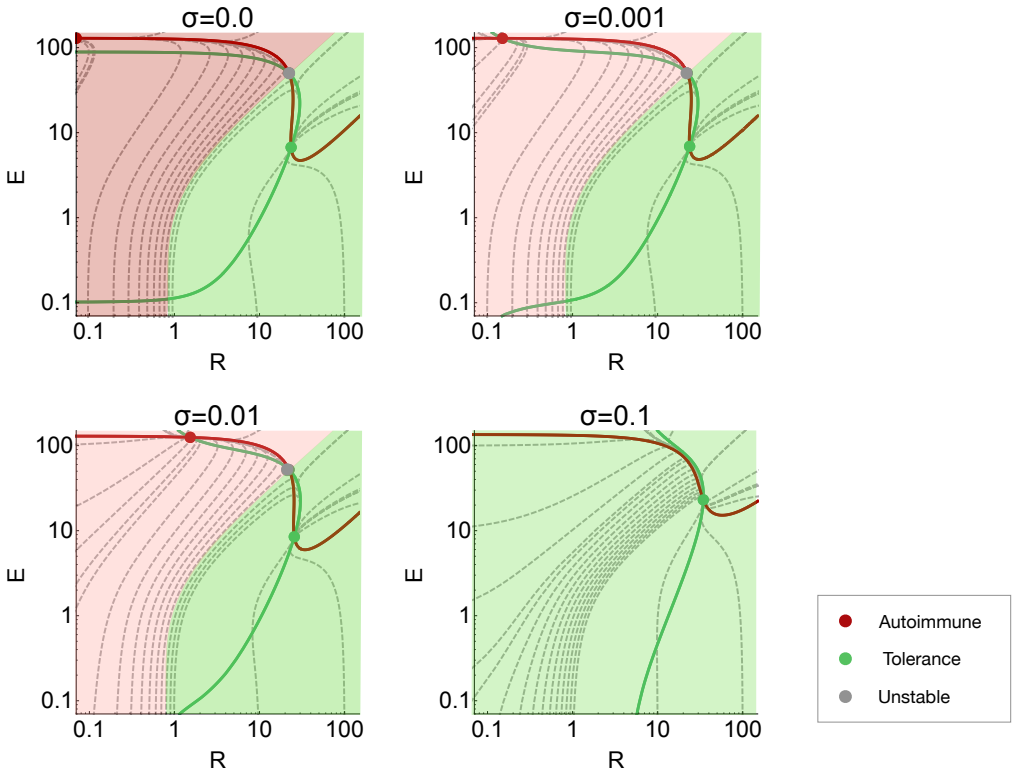

Figure 2.5: **Thymic output inhances tolerance.** Phase plane of the system, in case of slow Foxp3 rate loss, for increasing values of the constant thymic source σ. Red lines represent the null-clines of the density E of Teffs, green lines represent the null-clines of the density R of Tregs. Equilibria of the system, found in correspondence of the intersections of null-clines, are depicted by dots. In each graph, red dot represents the stable autoimmune equilibrium, red area the corresponding basin of attraction; green dot represents the stable tolerance equilibrium, green area the corresponding basin of attraction. Grey dot depicts the un-stable saddle-node equilibrium. Note that in presence of thymic output, in the autoimmune stable equilibrium Tregs are not completely extinct .Values of the other parameters are: $p_r = 1.0, p_e = 1.3, d_r = 0.02, d_e = 0.02, S = 2, c = 1, d = 1, c_R = 0.002, k = 1.2$.

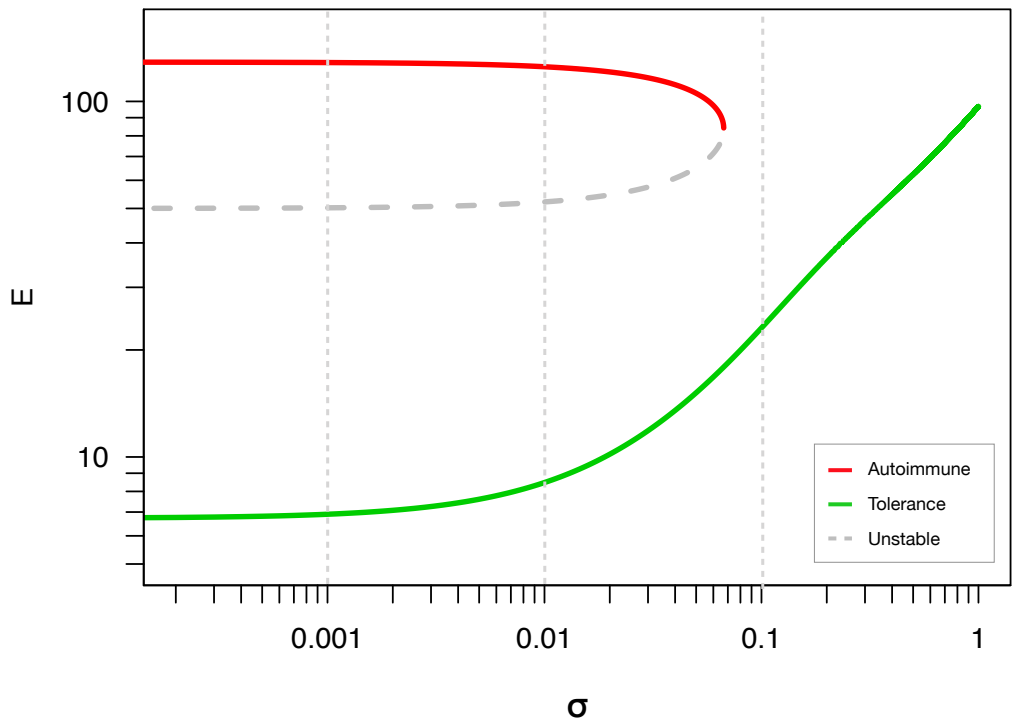

Figure 2.6: **Thymic output enhances tolerance.** Bifurcation diagram of the system showing the density of Teff cells of equilibrium (E) for increasing values of the constant thymic source σ. Red line corresponds to the stable autoimmune equilibrium of the system, in which Tregs are outnumbered; green line corresponds to stable tolerance equilibrium, in which Tregs control Teffs proliferation; grey dashed line corresponds to the unstable saddle-node equilibrium. For $\sigma = 0.07$ a saddle-node bifurcation occurs, in which the autoimmune and the unstable equilibria collide and annihilate each other. Values of the other parameters are: $p_r = 1.0, p_e = 1.3, d_r = 0.02, d_e = 0.02, S = 2, c = 1, d = 1, c_R = 0.002, k = 1.2$.

2.4 Discussion

In this chapter we investigated the theoretical effect of context-dependent Foxp3 expression in Tregs on Tregs population maintenance. To do this, we extended the previous developed cross regulation model [León et al., 2001, Carneiro et al., 2007], introducing the possibility of Foxp3 expression loss. In particular, because Foxp3 expression maintenance in Tregs needs continuous TCR and IL-2 stimulation [Levine et al., 2014], we introduced the rule that Tregs that lack either TCR stimulation or IL-2, would lose Foxp3 expression according to the rate c_R.

2.4.1 Enhanced tolerance robustness

We found the existence of parameter regimes in which, as long as the Foxp3 loss rate is slow enough, some fundamental characteristics of the original cross regulation model (reviewed in chapter 1) are maintained.

In this parameter regimes, the bi-stability of the system is maintained: the system supports two stable states, the tolerance steady-state in which Tregs and Teffs populations coexist with the farmer controlling the latter, and the autoimmune steady state, in which Tregs population are outnumbered and only Teff persist. This result implies that, even in presence of context-dependent Foxp3 loss, tolerance can be maintained.

Also, the existence of bi-stability depends on APCs population density. In particular bi-stability of the system is ensured as long as antigen presentation is not rare. On the contrary, for rare antigen presentation, meaning for low density of APCs, only the cognate Teffs population is sustained. In other words, our analysis suggests that even in presence of context-dependent Foxp3 loss, the system supports the self *versus* non-self discrimination.

Moreover, we found that, for an entire interval of the rate c_R of Foxp3 loss, the equilibrium of coexistence becomes a stable node, while for no loss (as in the original CRM) or extremely slow loss rate, the same equilibrium is a stable focus. This means that, differently from the original model, in which after a perturbations homeostasis is restored through oscillation, when Foxp3 loss rate belongs to the said interval, homeostatic restoration happens much more smoothly.

Avoiding oscillation after a perturbation from the healthy steady state of co-

existence could be fundamental for tolerance robustness. In fact, both overshootings and decreases in either population densities can cause the system to collapse toward the autoimmune steady state, especially in presence of stochasticity. Indeed during the oscillations, a stochasticity can cause the trajectory to jump the the autoimmune basin of attraction. Alternatively, stochasticity together with oscillations in trajectories, can cause the extinction of any of the two populations.

In case stochasticity causes any of the two populations to get extinct, the expected outcome is the consequent collapse of the system toward the autoimmunity steady state. If Tregs collapse first, the system reaches the autoimmune equilibrium, characterised by the presence of auto-reactive Teffs alone, sustained by the cognate APCs. Nevertheless, in case Teffs collapse first, the remaining Tregs are destined to die out, without the necessary growth factor produced by the cognate Teffs. The result is the entire collapse of a the given niche of auto-reactive Teffs and Tregs. The consequence of said extinction is, most likely, autoimmunity. In fact, as we discussed in chapter 1, the immune system has evolved in a way that auto-reactive Teffs clones, which have escaped negative selection in the thymus, are ”coupled” with Tregs of similar specificity. In the periphery, the population dynamics then determines the two populations to reach the coexistence equilibrium, thank to the APCs presenting their cognate antigen. We also mentioned that Tregs are mainly produced in the thymus early in development, during a time window characterised by a special wave of Tregs production, which then declines over time [Nishizuka and Sakakura, 1969, Sakaguchi et al., 1982, Modigliani et al., 1996]. Nevertheless, lymphopoiesis continues throughout the entire life of individuals. For this reason, in case a niche of auto-reactive Tregs and Teffs gets extinct after the perinatal period, it is still likely that other clones of similar specificity, meaning auto-reactive, will be produced by the thymus. This clones, in the periphery, will find APCs able to activate them. Nevertheless, in adulthood, given the decreased lymphocytes thymic productions, the auto-reactive Teffs most likely will not find, in the periphery, counterpart Tregs able to control them. Which will lead to their clonal expansion and subsequent autoimmunity.

On the one hand, in absence of continuous thymic output, the amount of cognate auto-reactive Tregs and Teffs that are in the periphery, determines which

of the two states is achieved, either the tolerant or the autoimmune. On the other hand, the presence of thymic output, increases the extension of the basin of attraction of the tolerance state, at the cost of the basin of attraction of the autoimmune state, which instead decreases. With increased thymic influx, the autoimmune state converts into an equilibrium in which, despite not being predominant, Tregs coexist with Teffs. Finally for even higher thymic influx, and even if the thymus exports more Teffs than Tregs, the autoimmune steady-state completely disappears. In other words, while in case of huge thymic influx, as the one expected in perinatal period, auto-reactive lymphocytes populations that exit the thymus are likely to evolve toward the tolerant state, when the thymic output declines, it is more likely, although not guaranteed, that they will evolve toward auto-immunity.

2.4.2 Slow dynamics to be investigated

In case of lability of Foxp3 expression, we found that bi-stability is maintained as long as the context-dependent loss of expression is a slow process. The requirement for a critical slow Foxp3 loss rate, c_R, to ensure tolerance, is a non trivial theoretical result. In fact, we do not know the characteristic time of of Foxp3 loss *in vivo*. Estimating such characteristic time and exploring the possible mechanisms through which the aforesaid slowness can be achieved need further study and they will be argument of the next two chapters.

2.5 Methods

The null-clines and the equilibria of the system of odes (figures 2.3, reflPhase-PlanesTimeCourse) were numerically computed and plotted using Mathematica [Wolfram Research, Inc.,]. In case of the system including the thymic output (Figure 2.5), null-clines and equilibria were computed using XPPAUT [Ermentrout, 2007], exported into a text file, then imported and plotted using Mathematica. Trajectories, were computed and plotted using the ode solver of Mathematica. 3D and 2D bifurcation diagrams of the system (figure 2.2), were numerically computed and plotted using R. 2D bifurcation diagrams were also computed and analyzed using XPPAUT, the exported into text files, to be finally

imported and plotted in R. Bifurcation diagram in figure 2.6 was numerically computed and plotted using R.

2.6 Supplemental Materials

The functions that describe the densities of activated Teffs and Tregs (see equations 2.6 and 2.7) have been originally described in [León et al., 2001]. They are *ad hoc* functions that are able to capture the non-linear interactions between Tregs and Teffs in multicellular conjugates. These equations allow the model with two conjugation sites per APC to display bi-stability and reproduce the same qualitative behavior in the bifurcation diagrams observed in models twith higher number of conjugation sites per APCs.

As an example, in figure 2.7 is shown the bifurcation diagram, describing the density of Teffs (E) as a function of the density of the available APCs sites (S). Figure 2.7 refers to the model with three conjugation sites per APCs. In this case, under the assumption that the total density S of available sites is much higher than the number of sites that form a multicellular conjugate ($S \gg 3$), the multinomial distribution can be used to approximate the density of activated Teffs and Tregs, as following:

$$R_a = R_c(2-\varepsilon)\varepsilon, \tag{2.10}$$

$$E_a = E_c(1-\varrho)^2. \tag{2.11}$$

Comparison between figures 2.7 and 2.2B shows the same qualitative behavior. The saddle-node bifurcation and the consequent establishment of the tolerance equilibrium, occurs for smaller density of available APCs sites, if compared with the previous case, indicating a higher efficiency of Tregs.

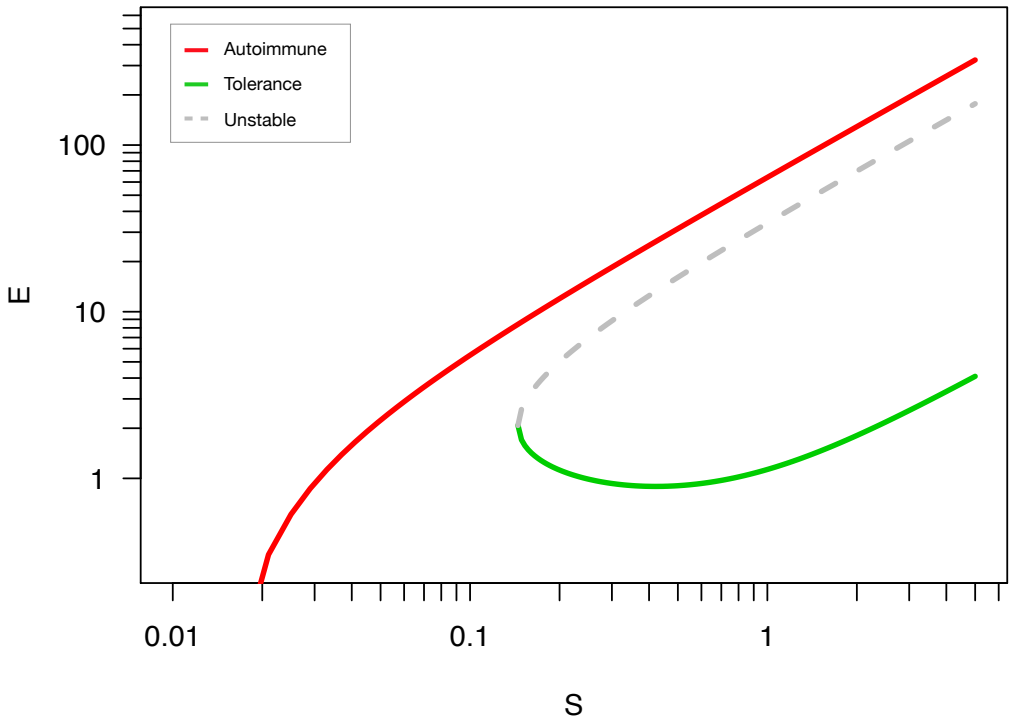

Figure 2.7: **Bifurcation diagram of the model with three conjugation sites per APC.** Bifurcation diagrams of the system showing the density of Teff cells of equilibrium (E) as a function of the density of available APCs sites (S) for the model with three conjugation sites. Red line corresponds to the stable autoimmune equilibrium of the system, in which Tregs are outnumbered; green line corresponds to stable tolerance equilibrium, in which Tregs control Teffs proliferation; grey dashed line corresponds to the unstable saddle-node equilibrium. A saddle-node bifurcation occurs for $S = 0.15$.

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3

A quantitative study of Foxp3 expression dynamics in murine CD4 T cells

Statement: All the work included in this chapter is my own, carried out under the supervision of Jorge Carneiro and Jocelyne Demengeot.

Abstract

Regulatory T cells are essential for immune tolerance to body tissues. They selectively express Foxp3, a transcription factor necessary for their development and function. Experiments performed to address Foxp3 dynamics in mice, tracking its expression in cell cohorts in different experimental conditions, show that Foxp3 expression can be either stably maintained over several months (in lymphoreplete condition) or can be lost within few weeks (in different lymphodeficient conditions). So far, it has not been possible to reconcile all these observations.

Here we seek a unifying explanation for Foxp3 expression across the experiments. We developed a stochastic model for Foxp3 expression in CD4 T cells. In this model, Foxp3 expression can be fully reversible or irreversible, depending on parameter settings. We fitted the model to the available data sets in the literature, describing the temporal evolution of $Foxp3^+$ cell frequency in cohorts of cells that were either $Foxp3^+$ or $Foxp3^-$ at a given time. In this way, we identified the most

consistent and parsimonious model parameter sets.

We found that the experimental data could not be coherently reproduced without postulating the existence of a large subpopulation of CD4 T cells(not less than 80%)that in no case can up-regulate Foxp3. From meta-analysis we also conclude that there is neither need to call for commitment to Foxp3 expression to explain Foxp3 stability, nor for heterogeneity within Foxp3 expressing cells to explain Foxp3 lability. Finally, Foxp3 stability positively correlates with absolute number and frequency of Foxp3$^-$ cells in the host. We conclude that the experimental data are compatible with Tregs being a homogeneous pool of CD4 T cells, in which Foxp3 expression is stable or labile depending on the context.

3.1 Introduction

Regulatory T cells (Tregs) are essential for immune tolerance to body tissue by damping down the activation and clonal expansion of auto-reactive CD4 effector T cells. Tregs constitute a subpopulation of CD4 T cells, enriched in self-reactivity [Kim et al., 2007], and they selectively express the transcription factor Foxp3. Foxp3 expression is necessary for Tregs development and for their regulatory function [Fontenot et al., 2003, Hori, 2003]. Any mutation that leads to Foxp3 loss of function is enough for individuals, both mice and humans, to suffer multi-organ auto-immunity and die early after birth.

In the last years several experiments have been performed in different laboratories to address Foxp3 dynamics and stability *in vivo* in mice. These experiments follow *in vivo* cohorts of either CD4$^+$Foxp3$^+$ or CD4$^+$Foxp3$^-$ T cells, under different experimental conditions, to asses the frequency of cells that, respectively, maintain or acquire Foxp3 expression over time. According to these observations, Foxp3 expression can be either stably maintained over several months, in lymphoreplete condition, or can be lost within few weeks, in lymphodeficient condition.

Experiments that follow cohort of Foxp3$^+$cells in lymphoreplete condition are performed by using the inducible Cre-Lox system in mice that have a fully developed immune system. In these mice, upon tamoxifen administration, Foxp3$^+$ cells are labeled genetically and permanently, as well as their daughter cells. In this case, none of the labeled cells lose Foxp3 expression after four months

[Rubtsov et al., 2010]. Also, when CD4$^+$Foxp3$^+$ Tregs are purified from a lymphoreplete mouse and adoptively transferred into a wild type mouse, 8% of them lose the expression within 4 weeks and this frequency is maintained for the following two months [Komatsu et al., 2009].

Experiments that follow cohort of Foxp3$^+$ cells in lymphodeficient condition are performed upon adoptive cells transfer: Foxp3$^+$ cells are purified from a lymphoreplete mouse and transferred into a lymphodeficient Rag2$^{-/-}$ host [Duarte et al., 2009, Komatsu et al., 2009]. In this case, half of the followed cells lose Foxp3 expression within four weeks, and become pathogenic upon a second transfer into a lymphodeficient host. Also, in the attempt to understand the cause of lability of Foxp3 expression in lymphodeficient conditions, adoptive transfer experiments into lymphodeficient hosts (either Rag2$^{-/-}$ or TCR$\beta^{-/-}$) have been complemented by either sustained administration of IL-2 i.v. [Duarte et al., 2009] or by co-transferring, together with CD4$^+$Foxp3$^+$ Tregs cells, also CD4$^+$Foxp3$^-$ T cells, in different proportions [Duarte et al., 2009, Komatsu et al., 2009]. In both cases the frequency of cells maintaining Foxp3 expression over time increases in a dose dependent way. Remarkably, depending on the dose of transferred Foxp3$^-$ T cells, even the frequency of Foxp3$^+$ cells that maintain Foxp3 expression characteristic of the lymphoreplete condition could be rescued [Komatsu et al., 2009].

Finally, experiments that follow cohorts of Foxp3$^-$ cells are performed upon adoptive transfer in lymphodeficient host, either Rag2$^{-/-}$, TCR$\beta^{-/-}$ or CD3$\epsilon^{-/-}$. Total peripheral cells are purified from a lymphoreplete mouse in physiologic condition and transferred, either alone or together with Foxp3$^+$cells, into lymphodeficient host mice. In these cases, almost none of the followed cells acquire Foxp3 expression in the periphery within four weeks. No significant acquisition of Foxp3 expression in the periphery is observed, no matter whether Foxp3$^-$ cells are transferred alone or whether they are co-transferred, together with Foxp3$^+$cells, even in the proportion that keeps Foxp3 maintenance as good as in the lymphoreplete condition [Duarte et al., 2009, Komatsu et al., 2009, Paiva et al., 2013]. However, higher frequency of acquisition of Foxp3 expression in the periphery (10%) is observed in case the cohort of Foxp3$^-$ cells is constituted by the so called exTregs [Komatsu et al., 2009]. ExTregs are CD4$^+$Foxp3$^-$ cells that once have been sorted from physiologic condition as Foxp3$^+$ and then have lost Foxp3 expression within four weeks upon a first adoptive transfer into

lymphodeficient condition. Also, when peripheral Foxp3$^-$ cells are enriched in recent thymic emigrants, then within four weeks around 6% of cells acquire Foxp3 expression in the periphery [Paiva et al., 2013].

Taken together these experiments place the issue of context-dependency for what concerns the stability of Foxp3 expression, at least for a subset of Foxp3$^+$ CD4 T cells. Yet, a coherent interpretation that reconciles all the observations is still lacking. Over the past years there have been several claims, often inconsistent within each other. Tregs have been claimed to constitute a distinguished lineage, in which Foxp3 expression is stable across many rounds of cell divisions *in vitro* and *in vivo*, in several conditions [Sakaguchi, 2004, Rubtsov et al., 2010]. Foxp3 expression has been otherwise claimed to be context-dependent [Duarte et al., 2009, Zhou et al., 2009, Bailey-Bucktrout and Bluestone, 2011]; also, Foxp3$^+$ cells have been claimed to be heterogeneous for what concerns their ability to stably express the gene: it has been proposed that within CD4 T cells sorted as Foxp3$^+$, the majority are committed to the Treg lineage, while a minor population just transiently express the Foxp3 gene [Komatsu et al., 2009]. Finally, within the "heterogeneity model", it has been proposed that the committed state of Tregs is achieved by DNA demethylation of the Foxp3 locus, irrespective of ongoing Foxp3 expression [Miyao et al., 2012]. Basic questions remain still open. What controls lability of Foxp3 expression in the environment of a mouse? Are Foxp3$^+$ cells heterogeneous regarding their capacity to stably maintain Foxp3 expression and are they all susceptible to Foxp3 expression loss? Finally, are all CD4 T cells inducible to become Tregs *in vivo*, upon appropriate stimuli?

Here we address these questions through a quantitative approach. We developed a model for Foxp3 expression in CD4 T cells that accounts for both Foxp3 commitment and stochastic and context-dependent reversible Foxp3 expression. Indeed, depending on model parameters, the gene expression can be fully reversible or irreversible. We used this model to perform a meta analysis on available experimental data on Foxp3 dynamics, aiming at identifying the conditions for persistence of cellular Foxp3 expression.

By fitting the model to experimental data, we found that the model can coherently reproduce them all, as far as the ratio between Foxp3 transcriptional activation and repression rates is tuned across the experiments.

Meta analysis suggests that a large subpopulation of CD4 T cells, ranging from 85 to 90% of CD4 T cells, in no case can be induced to up-regulate Foxp3 expression *in vivo*, not even transiently. From the meta-analysis we also conclude that there is no need to call neither for commitment to Foxp3 expression nor for heterogeneity within Foxp3 expressing cells to explain the stability and lability of Foxp3. In fact, the data can be explained under the assumption that up to 28% within Foxp3$^+$ CD4 T cells in lymphoreplete conditions are committed to Foxp3 expression. Nevertheless, our analysis suggests that the most likely scenario is that less than 7% within Tregs are indeed committed to Foxp3 expression. Remarkably the ensemble of data is compatible with Tregs being a homogeneous pool of CD4 T cells, in which Foxp3 expression is stable or labile depending on the context.

Particularly, stability of Foxp3 expression correlates with high number and high frequency of CD4$^+$Foxp3$^-$ T cells in the host.

3.2 Results

3.2.1 A stochastic model able to describe Foxp3 dynamics in CD4 T cell cohorts.

We developed a stochastic model for Foxp3 expression in a CD4 T cell in which Foxp3 expression can be reversible or irreversible, depending on model parameters. According to the model, any CD4 T cell can be found in one out of four mutually exclusive states, indicated as Z_0, Z_1, Z_2 and Z_3 and depicted in Figure 3.1. A cell that is either in state Z_0 or in state Z_1 is negative for Foxp3 expression: Foxp3 is not transcribed/translated and/or the protein has been already degraded. A cell is in either state Z_2 or Z_3 if the Foxp3 gene is transcribed, translated and the protein is expressed.

In order to account for possible commitment to Foxp3 expression, the model assumes that the entire pool of CD4 T cells can be divided into up to three distinguished compartments.

A first compartment is constituted by the cells in state Z_3 and represents the fraction α of the total pool of CD4 T cells. These cells are Foxp3$^+$ and are committed to Foxp3 expression.

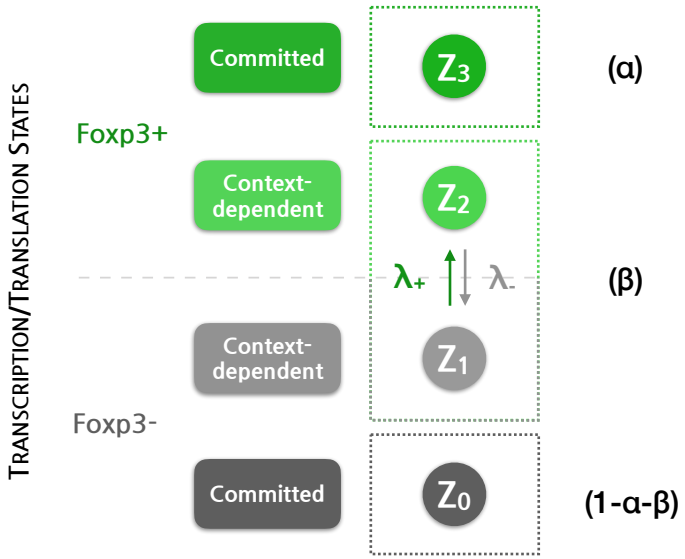

Figure 3.1: **Stochastic model of Foxp3 expression in a CD4 T cells, able to describe Foxp3 dynamics in CD4 T cell cohorts.** Cartoon of the stochastic model. According to the model any CD4 T cell can be found in any of the four mutually exclusive states, here represented by a circle. A cell in state Z_0 or Z_1 is negative for Foxp3 protein; a cell in state Z_2 or Z_3 is expressing Foxp3. Moreover, state Z_0 is characteristic of a CD4 T cell that in no case can express Foxp3, state Z_3 is characteristic of a cells that is committed to Foxp3 expression, therefore in no case can lose it. Stochastic transitions between states can happen to any cell that is in either state Z_1 or Z_2, according to constant rates. Respectively λ_+ is the rate of Foxp3 up-regulation (transition from state Z_1 to state Z_2), while vice-versa λ_- is the rate of Foxp3 gene repression and protein degradation. Rates are assumed to depend on the context in which the cell is embedded. The model assumes that, within the entire pool of CD4 T cells, a fraction α is found in state Z_3(Foxp3 committed cells), a fraction β is found in one state out of Z_1 or Z_2, the remaining $1-\alpha-\beta$ being in state Z_0(cells that cannot express Foxp3). Rates λ_+ and λ_- as well as fractions α and β are model parameters, whose possible values need to be determined, based on experimental evidences.

A second compartment represents the fraction β of CD4 T cells and it is constituted by the cells which are either in state Z_1 or Z_2. Therefore the cells in such compartment can be either expressing the gene (if in state Z_2) or not (if in state

Z_1). Also, depending on the inputs received from the environment and translated in terms of model parameters, the cells in the second compartment are assumed to be able to acquire and/or lose Foxp3 expression over time, in the periphery. A transition from a Z_1 to Z_2 represents Foxp3 gene transcription and translation, until the cell becomes positive for the Foxp3 protein. Conversely, the transition from Z_2 to Z_1 takes into account the inhibition of transcription and translation as well as the degradation of both the RNA and the protein, until the cell is negative for the Foxp3 protein. The cells in such second compartment are modeled by a continuous time Markov chain model. Transitions happen stochastically with average context-dependent constant rates, λ_+ for Foxp3 up-regulation and λ_- for Foxp3 down-regulation. Each constant rate is the expected value of a specific exponential distribution and its value can be tuned as will.

The remaining $1-(\alpha+\beta)$ portion of CD4 T cells is constituted by CD4 T cells that are in state Z_0. These cells are Foxp3$^-$ T cells that do not express Foxp3, regardless of the context.

Based on these assumptions, the model is able to describe the frequency over time of Foxp3$^+$ cells within a cohort of sorted Foxp3$^+$ cells from lymphoreplete condition. Such frequency, which in other words represents the frequency of cells that maintain Foxp3 expression over time, is indicated by $m(t)$ and can be expressed as following:

$$m(t) = \frac{\alpha + eq\,\beta\big(e^{-\vartheta t}(1-\varrho)+\varrho\big)}{\alpha+eq\,\beta}, \tag{3.1}$$

where

$$\vartheta = \lambda_+ + \lambda_- \tag{3.2}$$

represents the decay rate of Foxp3 expression within the Foxp3$^+$ cells that belong to the second compartment of the model and ϱ is defined as

$$\varrho = \frac{\lambda_+}{\lambda_+ + \lambda_-}. \tag{3.3}$$

Finally, the variable eq in equation 3.1 is the frequency of Foxp3$^+$ cells at equilibrium in lymphoreplete condition, restricted to the second compartment of CD4 T cells, the one that are assumed to be able to up- and down-regulate Foxp3

expression, depending on the context. Reasonings for the definition of ϑ and ϱ are explained in more details in material and methods section 3.4.1, where the solutions are fully derived.

Let us stress the fact that, due to the relations among $\lambda_+, \lambda_-, \varrho, \vartheta$, which imply:

$$\lambda_+ = \varrho\,\vartheta, \qquad \lambda_- = (1-\varrho)\,\vartheta, \tag{3.4}$$

the solution 3.1, it is not just a simple exponential decay. To understand this statement, it can be helpful to look at three extreme cases. In case either $\lambda_+ \ll \lambda_-$ or $\lambda_+ \simeq \lambda_-$, the expression for $m(t)$ is a single exponential decay, with rate of decay equal to θ. Nevertheless, in case $\lambda_+ \gg \lambda_-$, the decay disappears and the solution becomes a plateau. In particular:

$$\varrho \simeq 1 \qquad \lambda_+ \gg \lambda_- \qquad m(t) \simeq 1 \tag{3.5}$$

$$\varrho \simeq 0 \qquad \lambda_+ \ll \lambda_- \qquad m(t) \simeq \frac{\alpha + e^{-\vartheta t} eq\,\beta}{\alpha + eq\,\beta} \tag{3.6}$$

$$\varrho \simeq 0.5 \qquad \lambda_+ \simeq \lambda_- \qquad m(t) \simeq \frac{\alpha + eq\,\beta\big(e^{-\vartheta t}0.5 + 0.5\big)}{\alpha + eq\,\beta}. \tag{3.7}$$

The model describes also the frequency over time of Foxp3$^+$ cells within a cohort of sorted Foxp3$^-$ cells from lymphoreplete condition. Because it represents the frequency of Foxp3$^-$ cells that acquire Foxp3 expression in the periphery, we indicated this frequency by $a(t)$:

$$a(t) = \frac{\big(1 - e^{-\vartheta t}\big)(1-eq)\beta\varrho}{1-(\alpha + eq\beta)}. \tag{3.8}$$

Finally, the model can represent the frequency $aex(t)$ of cells that acquire Foxp3 expression in the periphery within a cohort of Foxp3$^-$ cells entirely constituted by cells that belong to the second compartment. In other words, if we assume that there is a subset of CD4$^+$Foxp3$^-$ T cells that in no case can up-regulate Foxp3 expression, when computing the frequency $aex(t)$, we are restricting our attention to the subset of Foxp3$^-$ that are able, in principle and depending on the

inputs received by the environments, to up-regulate Foxp3 in the periphery. This frequency can be expressed as following:

$$aex(t) = \varrho - e^{-\vartheta t}\varrho. \tag{3.9}$$

More detailed description of the solutions can be found in material and method section 3.4.1.

For each of these frequencies $m(t), a(t), aex(t)$ of Foxp3$^+$ within specific cohort of CD4T cells, we computed the corresponding equilibrium:

$$m_\infty = \frac{\alpha + eq\beta\varrho}{\alpha + eq\beta}, \qquad a_\infty = \frac{(eq-1)\beta\varrho}{1-(\alpha+eq\beta)} \qquad aex_\infty = \varrho. \tag{3.10}$$

In summary, using a stochastic model for Foxp3 expression in CD4 T cell, we were able to describe the frequency of Foxp3$^+$ CD4 T cells over time, within specific cohort of CD4 T cells. The possible cohorts were constituted by either the total Foxp3$^+$ CD4 T cells, or the total Foxp3$^-$ CD4 T cells or the subset of Foxp3$^-$ CD4 T cells able to up-regulate the gene, if opportunely stimulated.

3.2.2 Experiments assessing Foxp3 expression dynamics in the periphery can be reconciled under a single stochastic framework.

We used the stochastic model for Foxp3 expression, described in the previous section, to quantitatively reproduce the experimental data collected from literature, that address Foxp3 dynamics in mice.

The experiments used in our analysis are extensively described in material and methods section 3.4.2. These experiments track the frequency of Foxp3$^+$ cells within cell cohorts of either Foxp3$^+$ or Foxp3$^-$ CD4 T cells, in ten different experimental conditions. In this way the frequency of cells that maintain or acquire Foxp3 expression over time, is assessed. The different experimental settings comprise, first, the case in which Foxp3 expression is followed in cohorts of Foxp3$^+$ cells in lymphoreplete mice. Also, Foxp3 expression is assessed in cohorts of CD4 T cells sorted from lymphoreplete mice and then adoptively transferred, either alone or together with complementary populations, into lymphodeficient mice that otherwise would lack these populations of lymphocytes.

Experimental results are presented in Figure 3.2, in dots. The frequency of cells that either maintain (in blue) or acquire (in yellow) Foxp3 expression over time vary across different experimental conditions.

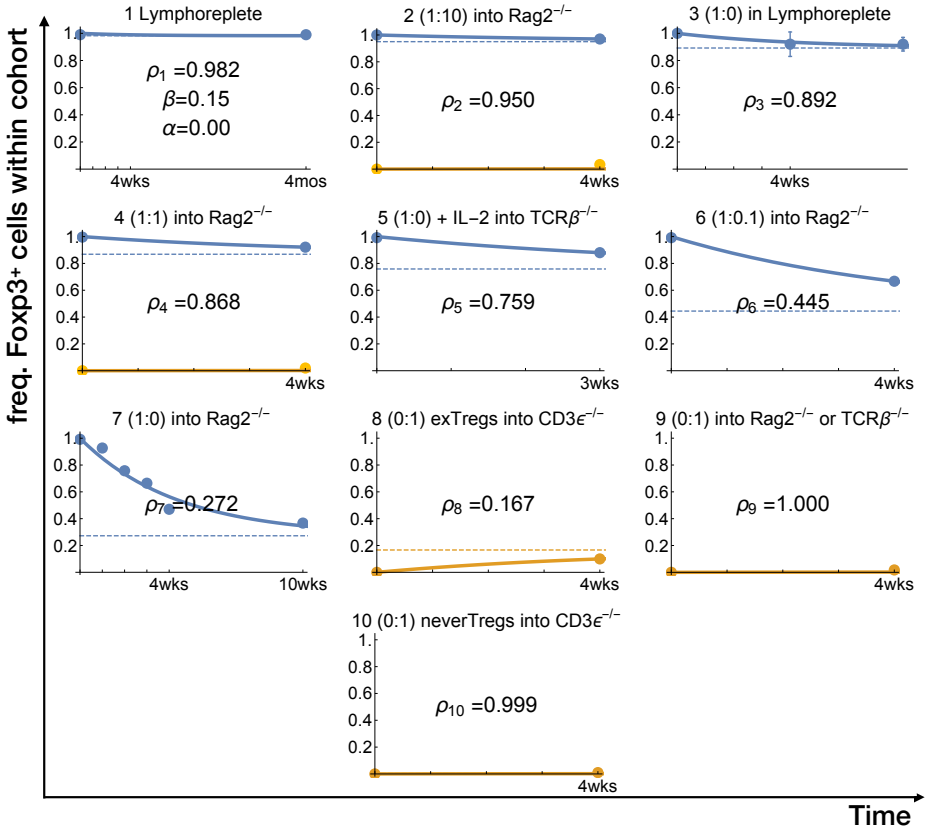

Figure 3.2: **Model predictions reproducing the experimental data on Foxp3 in vivo dynamics.** Frequency of Foxp3$^+$ cells within cohort of cells followed *in vivo* over time, in different experimental conditions. Dots represent experimental data (experiments are described in details in materials and methods section), lines are the model predictions from one representative parameter set out of the equally good sets found. Solid lines are the temporal solutions of the stochastic model, dashed lines represent the predicted value of equilibrium. In blue, the experimental data in which maintenance of Foxp3 expression over time, is assessed, in yellow, the experimental data in which acquisition of Foxp3 expression assessed over time. In each experiment is reported the value of the rate ${}^i\rho$ obtained through the fitting. Other parameter values are $\alpha = 0, \beta = 0.15$.

We should stress the fact that most of the experimental data included in the

analysis are constituted by only two time points, with few exceptions. However, the experiment in [Duarte et al., 2009], here labeled as 7, in which Foxp3^{+} cells are sorted from the Foxp3-GFP reporter lymphoreplete mouse and transferred into lymphodeficient Rag2$^{-/-}$ host mouse, provides us with kinetics of the frequency of cells that lose Foxp3 expression over time. This experiment is the most informative for inferring the rate of decay of Foxp3 expression.

In order to understand the reasoning behind the meta-analysis we performed, it can be useful to recall that Foxp3 expression in CD4 T cells is known to depend on continuous T cell receptor stimulation [Levine et al., 2014] by cognate antigen presenting cells, as well as continuous IL-2 stimulation, mainly produced by activated T effector cells [Malek, 2003, Malek, 2008]. Also, TGF-β stimulation, produced by several lineages of leukocytes, including Tregs, has been shown to play a role in up-regulation of Foxp3 expression, at least *in vitro* [Chen et al., 2003, Davidson et al., 2007, Yao et al., 2007]. Therefore, because of the different compositions of the host immune cells populations across the experiments analyzed, it is reasonable to assume that the average source and availability of IL-2 and TGF-β might vary across experiments and be peculiar of any given experimental condition. On the other hand, the composition of antigen presenting cells population can be considered the same across the different hosts. When we mention the average source and availability of IL-2 and TGF-β we are referring to the one computed both across the space of interaction among the cells and over the time window of each experimental observation. Therefore, in our analysis we categorize the different hosts as different environments. Each environment is characterized by peculiar average properties that provide an average amount of pro-Foxp3 inputs to any circulating CD4 T cells, which can be translated in terms of model parameter values.

To asses these parameter values, we fitted the solutions in equations 3.1, 3.8, 3.9 to the ensemble of experimental data, by minimizing the sum of the square of the residuals (details in Materials and method section 3.4.3). We assumed the transcription/translation activation and repression rates to depend on the specific experimental setting. Therefore we defined the following rates:

$$ {}^{i}\lambda_{+},\ {}^{i}\lambda_{-} \qquad i = 1, ..., 10 \tag{3.11} $$

where the index i refers to the i-th experiment. Looking for the most consistence

and parsimonious parameter sets, able to quantitatively reproduce all the experimental data, we distinguished the model parameters as experimental-context-dependent and independent. Parameters that are independent from the specific experimental setting, whose values are constant across all the experiments, are:

$$\alpha, \beta, \tag{3.12}$$

$$\vartheta = {}^{i}\lambda_{+} + {}^{i}\lambda_{-} \qquad i = 1, ..., 10 \tag{3.13}$$

In fact, α and β represent the fraction of CD4 T cells that are committed to Foxp3 expression and the ones that can lose and acquire Foxp3 expression over time, respectively. These fractions are characteristics of the followed cell cohort which, in all the experiment analyzed, belong to mice in physiologic condition. Also, the model parameter ϑ can be considered unique across the experiment given that, as mentioned before, there is only one experimental setting (experiment 7) that provides us with information about the kinetics of Foxp3.

For each experimental setting, we define instead the following experimental context-dependent parameters:

$${}^{i}\varrho = \frac{{}^{i}\lambda_{+}}{{}^{i}\lambda_{+} + {}^{i}\lambda_{-}} \qquad i = 1, ..., 10 \tag{3.14}$$

The parameter ${}^{i}\varrho$ represents the frequency of equilibrium of the Foxp3^{+} cells within the second compartment of CD4 T cells. Note that the experiment labelled as 1, assesses the frequency of CD4 T cells that maintain Foxp3 expression over time in lymphoreplete condition [Rubtsov et al., 2010]. In other words, the variable eq, appearing in equations 3.1, 3.8, corresponds to ${}^{1}\rho$.

We found that the model is able to reproduce all the experimental data, by tuning the ratios ${}^{i}\varrho$, $i = 1, ..., 10$, between activation and repression rate of transcription across experiments, as shown in Figure 3.2, and for optimum values of α, β and ϑ, that are kept constant across the experiments.

We found an ensemble of 2483 parameter sets that, based on the sum of the square of residuals, explain equally well the experimental data. The distribution, across these possible sets of parameters, of the Foxp3 down-regulation rate associated with the experiment 7, ${}^{7}\lambda_{-}$, is characterized by a median value of one event for month. We found the same median value for the up-regulation

rate of Foxp3 in lymphoreplete condition, ${}^1\lambda_+$. On the contrary, the median rate of Foxp3 expression loss in lymphoreplete condition, ${}^1\lambda_-$, was found close to zero meaning that, in lymphoreplete condition, CD4 T cells never lose Foxp3 expression. Similarly, for the case in which Foxp3$^+$ T cells are transferred alone in lymphodeficient condition, we found the rate of Foxp3 up-regulation, ${}^7\lambda_+$, was almost zero. Cumulative distributions are shown in Figure 3.3.

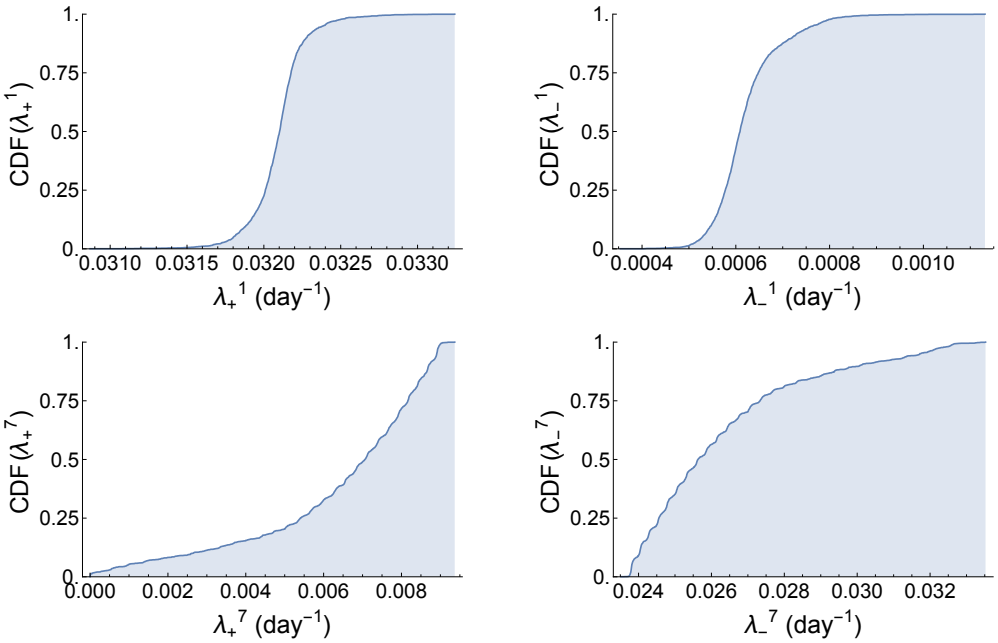

Figure 3.3: **Cumulative distribution of Foxp3 activation and repression rates in lymphoreplete and lymphodeficient conditions.** (Top) Cumulative distribution functions (CDF) of ${}^1\lambda_+$ and ${}^1\lambda_-$, Foxp3 activation and repression rates in experiment 1, across the optimal parameter set found. (Bottom) Cumulative distributions of ${}^7\lambda_+$ and ${}^7\lambda_-$.

3.2.3 There is no need to assume commitment to Foxp3 expression.

We found that, in order to coherently reproduce the experimental data on Foxp3 dynamics in cohorts of CD4 T cells, there is no need to call for commitment to Foxp3 expression, not even for a specific subset of CD4 T cells. Infact values of the model parameter α, indicating the fraction of CD4 T cells committed to Foxp3

expression in lymphoreplete condition, compatible with the experimental data, ranged from 0 to 4.2%. Also the third quartile was found to be just 1% of the total CD4 T cells pool. This percentage corresponds to 13% of Foxp3 committed cells within Foxp3$^+$ sorted from a lymphoreplete mouse, as shown in Figure 3.4.

In summary, although experimental data are compatible with the assumption that up to 28% of Foxp3$^+$cells being committed to Foxp3 expression, our meta-analysis of the experimental data showed that the most likely scenarios is that very few cells are indeed committed (less than 13% of Foxp3$^+$ in lymphoreplete condition).

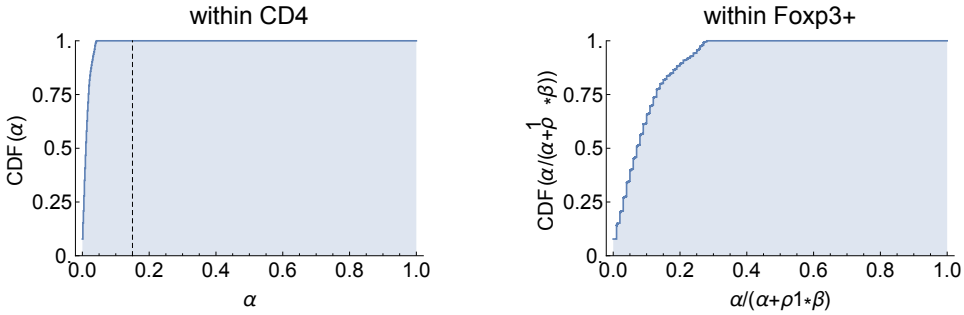

Figure 3.4: **Up to 4.2% of CD4 T cells can be committed to Foxp3 expression, although data are most likely compatible with no commitment at all.** Cumulative distribution function of the model parameter indicating the frequency of Foxp3 committed cells in physiologic condition, compatible with experimental data. Left: CDF of α, the frequency committed cells within the entire pool of CD4 T cells. Right: CDF of $\alpha/(\alpha + {}^1\rho + \beta)$, the frequency of committed cells within Foxp3$^+$ cells.

3.2.4 A large sub-population of CD4 T cells in no case is induced to up-regulate Foxp3 expression *in vivo*.

We also found that, in order for the model to fit the experimental data of Foxp3 dynamics *in vivo*, it was necessary to relax the assumption that any CD4 T cells is able, under opportune conditions, to up-regulate Foxp3 gene in the periphery. On the contrary, in order to coherently explain all the experimental data, both in lymphodereplete and lymphodeficient, it was necessary to assume the existence of cells that in no case can express the gene. These cells represent the $1 -$

$\alpha - \beta$ fraction of CD4 T cells. Our analysis suggested that these cells, which are CD4 T cells in state Z_0 according to our model, represent at least 84.5% of the entire CD4 T cells pool. Compatible frequencies for these cells ranged, according to our analysis, from 84.5% up to 89.8%, with median value of 85.2%. In Figure 3.5 is shown the cumulative distribution of the frequency that could explain the experimental data.

3.2.5 Foxp3 stability positively correlates with Teff and Treg in the host.

Finally, we searched for correlations between the model parameter ${}^i\rho$, and observables that change across the experiments. Parameter ${}^i\rho$ was defined in equation 3.14 as the ratio between Foxp3 activation and repression rates. We defined it as a context-dependent parameter and we showed it is able to explain the experimental data. We found that high Foxp3 stability positively correlates with high number and high frequency of $CD4^+Foxp3^-$ T cells in the animal host in which Foxp3 dynamics is followed, at time zero of experiments. In fact, if we restrict our analysis to the experiments in which stability of Foxp3 expression is addressed (experiments with labels from 1 to 7), the Spearman's rank correlation coefficient between the values of ρ and the absolute number of $Foxp3^-$ cells is equal to 0.9. Also, the Spearman's rank correlation coefficient between the values of ρ and the frequency of $Foxp3^-$ cells in the host at time zero of the ex-

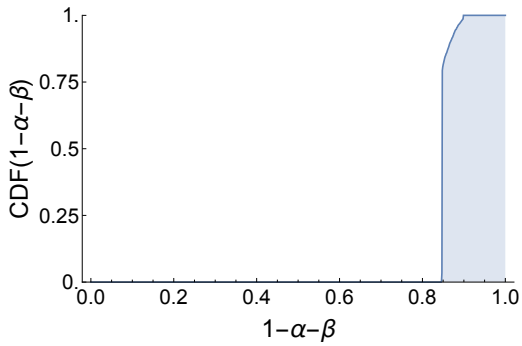

Figure 3.5: **A large subpopulation of CD4 T cells in no case can be induce to express Foxp3.** Cumulative distribution function of the frequency $1 - \alpha - \beta$ of CD4 T cells that, compatibly with the ensemble of experimental data, cannot express Foxp3 in physiologic condition.

periment is 0.94. We could not find a strong positive correlation between ρ and the absolute number of Foxp3$^+$ cells, although it is positive and equal to 0.21.

Figure 3.6 (top) shows the values of ${}^i\rho$ across experiments in which stability of Foxp3 is assessed: the value of the ratio ρ decreases monotonously as the label of the experiment increases. Figure 3.6 (bottom) shows the ranks of the model parameter ρ according to increasing values as a function of the ranks of a given experimental observables. Ranks increase according to the value of the parameter they refer to. The considered observables are the following: the frequency of Foxp3$^-$ cells at time zero (left), the absolute number of Foxp3$^-$ cells at time zero (center), the absolute number of Foxp3$^+$ cells at time zero (right) are shown. An increasing monotonous trend indicates a positive correlation with ρ. Activated CD4$^+$Foxp3$^-$ T cells are known to be the main *in vivo* producers of the T-cell growth factor IL-2 [Malek, 2008] (CD8$^+$ T cells and dendritic cells are also IL-2 producers [Malek, 2008] however, given the experimental settings considered in our analysis, neither of the two cell populations are expected to vary across the experiments). For this reason while computing the correlation between ρ and either the frequency or the number of Foxp3 $^-$ cells, we did not include the experiment labeled as 5, in which sustained IL-2 administration complements the cells transfer.

In summary, we found that stability of Foxp3 expression strongly correlates with the context, particularly with high number and frequency of CD4$^+$Foxp3$^-$ cells in the host. Also number of Foxp3$^+$ cells in the host positively correlates with Foxp3 stability.

Label	ϱ		% Foxp3-		# Foxp3-	
	Value	Rank	Value	Rank	Value	Rank
1	0.99	6	0.88	5	30 000 000	5.5
2	0.94	5	0.90	6	1 000 000	4
3	0.89	4	0.87	4	30 000 000	5.5
4	0.85	3	0.5	3	175 000	3
6	0.40	2	0.09	2	25 000	2
7	0.26	1	0	1	0	1

Label	ϱ		# Foxp3+	
	Value	Rank	Value	Rank
1	0.99	7	4 500 000	6
2	0.94	6	100 000	1
3	0.89	5	4 900 000	7
4	0.85	4	175 000	2.5
5	0.72	3	250 000	4.5
6	0.40	2	250 000	4.5
7	0.26	1	175 000	2.5

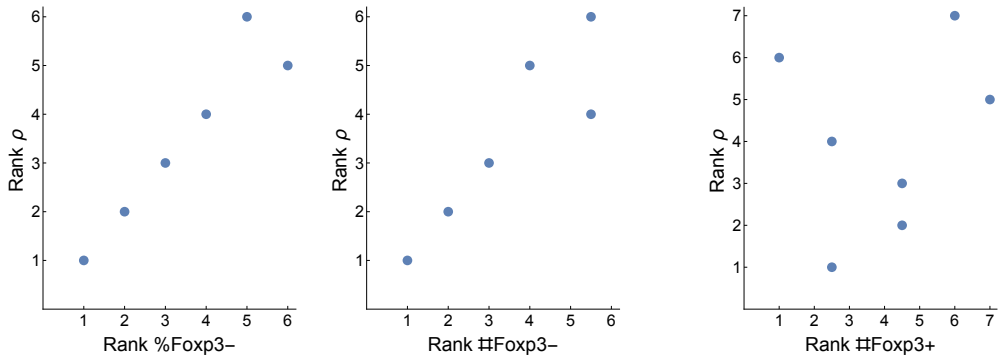

Figure 3.6: **Foxp3 stability positively correlates with Teff and Tregs in the host.** (Top) Values and ranks of the model parameter ρ and of the observables across the experiments in which stability of Foxp3 is assessed. Observables considered are frequency and absolute number of Foxp3$^-$ (left) or Foxp3$^+$ (right) CD4 T cells at time zero across experiments, indicated by corresponding labels. Higher ranks correspond to higher values of the parameter. (Bottom) Ranks of the model parameter ρ *versus* ranks of experimental observables (frequency and absolute number of Foxp3$^-$ or Foxp3$^+$) across experiments.

3.3 Discussion

In this chapter we looked for a coherent interpretation of Foxp3 expression dynamics in CD4 T cells *in vivo* in mice. In particular, we aimed at address the conditions for persistence of Foxp3 expression in CD4 T cells. For this, we tried to understand whether commitment to Foxp3 expression is a necessary assumption in order to explain stable Foxp3 expression and, if not, what, beside commitment, controls stability and lability of Foxp3 expression in the environment of a mouse. Also, we asked whether we could draw conclusions about the hetero-

geneity of Foxp3$^+$ cells, regarding their ability to maintain stable Foxp3 expression. Similarly, we investigated whether any conclusion could be drawn about the heterogeneity of Foxp3$^-$ CD4 T cells, regarding their ability to up-regulate Foxp3, under appropriate stimulation.

We addressed these questions by developing a stochastic model for Foxp3 expression in CD4 T cells, in which Foxp3 expression can be fully reversible, irreversible or heterogeneous, depending on model parameters. We then used this model to perform a meta-analysis on available experimental data, which track the frequency of Foxp3 expressing cells over time in CD4 T cell cohort *in vivo*, addressing Foxp3 stability and dynamics in different experimental conditions.

3.3.1 A single framework for different experimental contexts.

We showed that all the experimental data could be quantitatively reproduced under a single framework. Data used for the analysis included the experiments in lymphoreplete condition, where Foxp3 expression is stably maintained over months, the ones in lymphodeficient conditions, where Foxp3 expression can be lost within few weeks, and also compelled several intermediate cases.

3.3.2 Heterogeneity of CD4 Foxp3$^-$ T cells.

Meta-analysis showed that in order for all the experiments to be coherently reproduced, it is necessary to assume heterogeneity within Foxp3$^-$ cells that can be sampled from mice in physiologic condition. In particular, analysis suggested that the majority of CD4 T cells, spanning from 84.5 to 89.8$\%$ of them, in no case happen to be induced to express Foxp3 *in vivo*.

The presence of a large subpopulation of CD4 T cells that, in lymphoreplete condition, are prevented to up-regulate Foxp3 can be biologically explained in different ways. The first explanation can be in terms of T cell receptors (TCR) repertoire of CD4 T cells. Foxp3 expression, in fact, has been shown to require continuous TCR engagements together with IL-2 stimulation to be maintained [Levine et al., 2014]. Also *in vitro* Foxp3 induction in CD4 naive T cells has been shown to rely on TCR stimulation, together with IL-2 and TGF-β [Chen et al., 2003, Davidson et al., 2007, Yao et al., 2007]. It has also been shown that, despite of the negative selection toward self-reactive lymphocytes

that takes place in the thymus during lymphocytes development, Tregs have a TCR repertoire skewed toward self-reactivity [Kim et al., 2007]. It is therefore reasonable to assume that, because of their repertoire, not all the CD4 T cells can get in the periphery enough TCR engagement together with the necessary cytokines stimulations, for Foxp3 to be stably expressed, while only a small fraction of them (not more then 20%), get the amount of needed stimulation.

Other explanations, which are not in conflict with the previous one, can be given either in terms of differentiation state of the cells or in terms of maturation. Indeed, the majority of CD4 T cells, due to their differentiation program, have a gene expression profile that inhibits Foxp3 expression; also, recent thymic emigrant CD4 T cells, that have been shown to be the preferential precursor of Tregs that differentiate in the periphery, have also been shown to lose, over time, the potential to up-regulate Foxp3 [Paiva et al., 2013].

3.3.3 Homogeneous Tregs population.

On the other hand, there is no need to call for heterogeneity within Foxp3$^+$ cells. Indeed the ensemble of experimental data is compatible with Tregs being a homogeneous pool of CD4 T cells in which Foxp3 expression is stable or labile depending on the context. Particularly, Foxp3 stability strongly positively correlates with frequency and absolute number of Foxp3$^-$ CD4 T cells in the host. Weak positive correlation was also found with the absolute number of Foxp3$^+$ cells, in accordance with the notion that neither maintenance of Foxp3 expression nor re-acquisition of Foxp3 by exTregs depend on TGF-β [Komatsu et al., 2009].

Although not necessary for explaining the experimental data, the existence of Foxp3$^+$ cells that are committed to Foxp3 expression cannot be excluded. In fact, data also supported the possibility that up to 28% of Foxp3$^+$ cells are committed to Foxp3 expression, while the remaining ones express the gene as far as the context provides the opportune stimuli. Yet the frequency of cells committed to Foxp3 expression, represents a minority of Foxp3$^+$ cells. These results are summarized in Figure 3.7, left.

3.3.4 A coherent view of Foxp3 dynamics in different contexts.

The analysis provided us with a new key to reading coherently the experiments we analyzed, which is summarized in Figure 3.7, right. We analyzed the distribution of $\alpha + \beta$ (the frequency of CD4 T cells that either are or can be Foxp3$^+$) together with the distribution of ${}^1\rho$ (the frequency of equilibrium, in lymphoreplete condition, of Foxp3$^+$ cells within the compartment of cells that, based on the context, are able to up- or down- regulate the gene).

Based on this analysis, we concluded that in lymphoreplete condition all the cells that can up-regulate Foxp3, end up expressing the gene. When Foxp3$^+$ cells are followed over time, whether in lymphoreplete condition (experiment 1) or after being transferred into lymphodeficient host (experiment 7), the followed cohort is the same. This cohort is sampled from the compartments highlighted in Figure 3.7, right, through the vertical green line. What changes is then the context in which the cohort is followed over time and which provides more or less amount of pro-Foxp3 inputs, mainly depending on frequency and number of cognate Foxp3$^-$ CD4 T cells. Indeed, as the number of Foxp3$^-$ cells in the host, or its ratio with respect to Foxp3$^+$ cells, decrease, the same happens to the frequency of Foxp3$^+$ cells that maintain Foxp3 expression over time, as shown in Figure 3.7, top right.

From the same analysis it follows that within any cohort of total peripheral Foxp3$^-$ CD4$^+$ T cells, sorted from lymphoreplete conditions, (sampled from the compartments highlighted with vertical dark gray line in Figure 3.7, right) a negligible proportion of them (around 0.3$\%$) belongs to the compartment of cell able to up-regulate the gene (vertical light gray line). For this reason, we observe poor acquisition of Foxp3 expression within this cohort of cells, no matter the context in which cells are followed. As an example, it should be noted that even in the context that ensures a degree of stability to Foxp3$^+$ cells that is comparable to the physiologic case (experiment 2, showed in Figure 3.2), nevertheless the acquisition of Foxp3 expression by total peripheral Foxp3$^-$CD4$^+$ T cells is the same as in lymphodeficient condition (experiment 9). In the same line, we have also noted that the value of ${}^9\rho$ was poorly informative in the sense that could freely span from 0 to 1, without changing the quality of the fitting (see Materials and methods section). This result means that, no matter how much the environment provides pro-Foxp3 inputs to total peripheral Foxp3$^-$CD4$^+$ T cells (and therefore

no matter how big the correspondent ratio ρ is), the frequency of cells able to express the gene is so small that the up-regulation will be negligible.

This is not the case for the experiment 8, which assesses the frequency of Foxp3 induction in cohort of exTreg. In this case, the cell cohort is constituted by cells whose Foxp3 expression is influenced by the context (compartment highlighted with vertical light grey line in Figure 3.7, right). We observe indeed Foxp3 induction, within 4 weeks, in 10% of the cells and the value of ${}^{8}\rho$, differently from ${}^{9}\rho$ while similar to ${}^{i}\rho$, $i = 1, 2, ..., 7$, is very informative (see Materials and methods section).

We should keep in mind that a possible differential proliferation among Foxp3^{+}CD4^{+}, Foxp3^{-}CD4^{+} T cells and exTregs could be a confounding factor.

For instance, in experiments performed in lymphodeficient conditions, where Foxp3^{+} T cells are transferred alone and a population of Foxp3^{-} cells accumulates over time, growing in frequency, we cannot conclude whether this population is mainly due to Foxp3 loss, or to preferential proliferation of a small population that has loss Foxp3 expression. The second possibility has been proposed [Komatsu et al., 2009, Miyao et al., 2012]. Nevertheless, to confirm this possibility, a quantitative link between the CFSE proliferation and the respective change in frequencies should be established.

3.3.5 A slow dynamics yet to be explained.

What still remains to be explained is the ”slow” dynamics through which Foxp3^{+}CD4^{+} T cells lose Foxp3 expression, when transferred alone in lymphodeficient host. Our analysis showed that the median time needed for Foxp3 expression loss, by Foxp3^{+} cells in this condition, is in fact around four weeks. Because the half-life of the Foxp3 protein has been estimated to be of few hours [Morawski et al., 2013], the same being for RNA half-life (1.46 hrs) [Bending et al., 2018], the observed dynamics cannot be explained in terms of Foxp3 protein and RNA degradations, once the transcription is over. We instead need to call for other processes, like epigenetics. In fact, epigenetic signatures of the Foxp3 locus, which determine chromatin remodeling and accessibility of the locus to the transcription factors and machinery, are inherited and maintained across cell generations. For this reason their modifications and dynamics are slow if compared with transcription and translation dynamics. This analysis is

object of the next chapter.

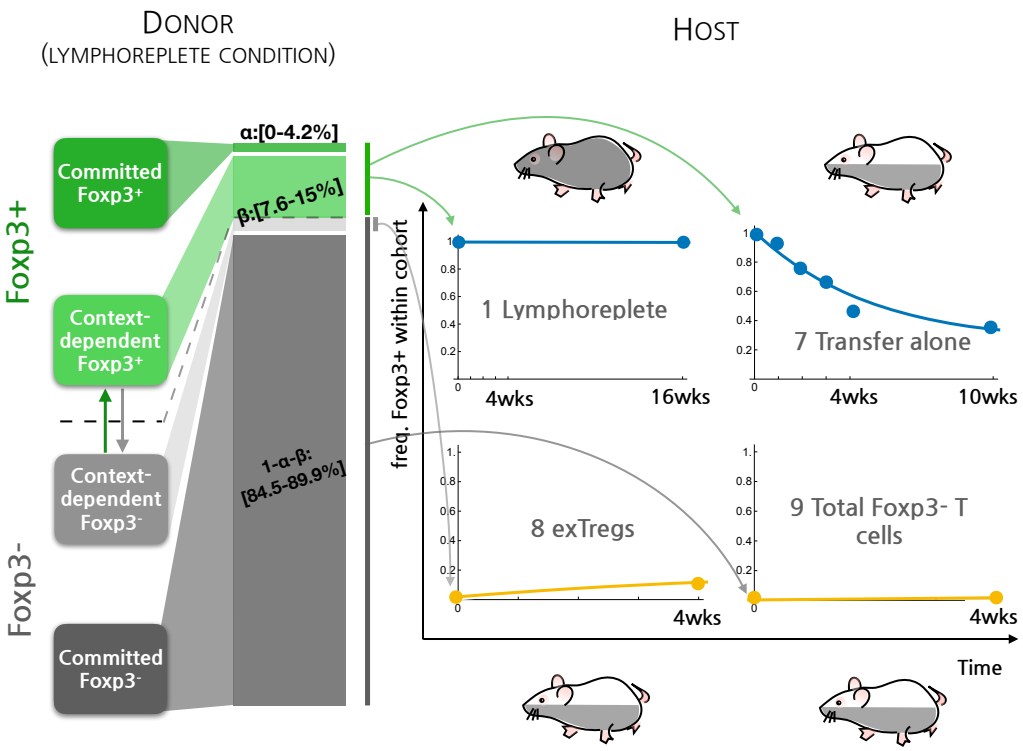

Figure 3.7: **A new key to reading coherently the different experiments we analysed.** Left: According to our meta-analysis, the pool of CD4 T cells in a lymphoreplete mouse can be divided into, at least, two compartments. One constituted by cells that in no case can up-regulate Foxp3 ("committed Foxp3$^-$"), a second one of cells that, depending on the context, can express or not the gene ("context-dependent"). A third compartment (constituted by "committed Foxp3$^+$" cells) is also compatible with data, but it is not necessary to explain the data observed. Intervals of admissible values for the fraction of each compartment (α, β and $1-\alpha-\beta$) are shown. Right: Illustrations of the compartment/s from which cell cohorts are sampled for the tracking of Foxp3 expression, in different experiments. Full colored mouse indicates lymphoreplete mouse, partially colored mice indicate lymphodeficient hosts that have received lymphocytes by adoptive transfer.

3.4 Materials and methods

3.4.1 Stochastic model of Foxp3 expression in a CD4 T cell.

The model introduced in the main text describes the probability over time for a CD4 T cell to be in any of the four states, given the following model parameters: α is the frequency of Foxp3 committed CD4 T cells, β is the frequency of CD4 T cells that can be either positive or negative for Foxp3 and are able to change their phenotype over time, λ_+, λ_- are the rates of acquisition and loss of Foxp3 expression of the cells belonging to the fraction β of CD4 T cells.

We defined the probability vector:

$$\underline{z}(t) = (z_1(t), z_2(t)), \tag{3.15}$$

where $z_k(t)$, $k \in \{1, 2\}$, is the probability, restricted to the cells in the fraction β of CD4 T cells, of being in state Z_k at time t. The time evolution of $\underline{z}(t)$, is described by the following system of ordinary differential equations :

$$\dot{\underline{z}} = \underline{z}\,\underline{\Lambda}, \tag{3.16}$$

where $\dot{\underline{z}}$ indicates the time derivative of the vector $\underline{z}(t)$ of probabilities and $\underline{\Lambda}$ is the infinitesimal generator matrix and it is a 2×2 matrix defined on $\mathbb{R}^+$ as following:

$$\underline{\Lambda} = \begin{pmatrix} -\lambda_+ & \lambda_+ \\ \lambda_- & -\lambda_- \end{pmatrix}. \tag{3.17}$$

Given the rates of transitions and the initial probability distribution

$$\underline{z}(0) = (1 - p_2, p_2), \tag{3.18}$$

where p_2 is the probability of being Foxp3$^+$ at time zero for a cell in the fraction β, the solution of the Markov chain model is unique and it is the following:

$$\underline{z}(t) = \underline{z}(0) \cdot e^{\underline{\Lambda} t}, \tag{3.19}$$

where $e^{\underline{\Lambda} t}$ is the exponential matrix and $\cdot$ indicates the scalar product. The probability of the cell being Foxp3$^+$ at time t, which is the second component of the

solution in equation 3.19, can be explicitly written as following:

$$f(t,p_2)=\frac{e^{-t(\lambda_++\lambda_-)}\big((-1+e^{t(\lambda_++\lambda_-)})\lambda_++p_2(\lambda_++\lambda_-)\big)}{\lambda_++\lambda_-} \tag{3.20}$$

By substituting in equation 3.20

$$\vartheta=\lambda_++\lambda_-,\qquad \varrho=\frac{\lambda_+}{\lambda_++\lambda_-} \tag{3.21}$$

which means defining:

$$\lambda_+=\varrho\vartheta,\qquad \lambda_-=(1-\varrho)\vartheta, \tag{3.22}$$

the solution for the probability of the cell in the fraction β being Foxp3⁺ at time t simplifies as:

$$f(t,p_2)=e^{-\vartheta t}(p_2-\varrho)+\varrho. \tag{3.23}$$

Finally, remembering that the initial conditions $p_2=1$ means that the probability of a a cell in the fraction β to be Foxp3⁺, is equal to 1, we obtain the definitions for $m(t)$, $a(t)$, $aex(t)$, reported in the main text.

In particular, the probability over time for a general CD4 T cell to be Foxp3⁺ at time t, given that it was Foxp3⁺ at time $t=0$ is:

$$m(t)=\frac{\alpha+eq\beta\big(f(t,p_2=1)\big)}{\alpha+eq\beta}=\frac{\alpha+eq\beta\big(e^{-\vartheta t}(1-\varrho)+\varrho\big)}{\alpha+eq\beta}, \tag{3.24}$$

where eq is the frequency of Foxp3+ cells at equilibrium in lymphoreplete condition, restricted to the second compartment of CD4 T cells. The same probability, given that at time $t=0$ the cell was Foxp3⁻ is:

$$a(t)=\frac{(1-eq)\beta\big(f(t,p_2=0)\big)}{(1-\alpha-\beta)+(1-eq)\beta}=\frac{\big(1-e^{-\vartheta t}\big)(1-eq)\beta\varrho}{1-(\alpha+eq\beta)}. \tag{3.25}$$

The same probability, given that at time $t=0$ the cell was Foxp3⁻ and particularly in state Z_2, is:

$$aex(t)=f(t,p_2=0)=\varrho-e^{-\vartheta t}\varrho. \tag{3.26}$$

As mentioned in the main text, we assumed that any CD4 T cell of the fraction β in a given host, due to the immune cell population composition of the host, is subjected to a mean field of pro-Foxp3 inputs per time units, as antigen presentation by cognate antigen presenting cells and cytokines stimulations by other CD4 T cells. The field of inputs over the time window of each experiment is reflected in the model parameters. We also assumed that every time that a cell up-regulate or down-regulate Foxp3 expression, it does not impact on the mean field: no transition that happen over the time of any given experiment can change the average characteristics of the field of inputs in which the very cell is embedded. This means that the model parameter values are constant over the time of any experiments and reflect the average characteristic of the experimental setting. Under these assumptions, the probability over time for a cell to be in any state of the model can me compared with the frequencies of CD4 T cells that are in any state of the model. For this reason, we can use the equations 3.24, 3.25, 3.26 as a measure of the frequency of Foxp3$^+$ cells within, respectively, a cohort of Foxp3$^+$ cells from lymphodeficient condition, a cohort of Foxp3$^-$ cells from lymphodeficient condition and a cohort of exFoxp3$^+$ cells.

3.4.2 Experimental data on Foxp3 stability *in vivo* collected from literature.

Here we report the description of the experiments collected from literature and whose data were included in our analysis. These experiments track over time the frequency of cells expressing Foxp3 within cohorts of cells that were either Foxp3$^+$ or Foxp3$^-$ at time zero of the observation. When assembling the data set, we adopted a necessary condition which would ensure a fair comparison among experimental data in different contexts. We chose experiments in which the cell cohorts were sorted (or labelled) as total peripheral CD4$^+$ T cells, either Foxp3$^+$ or Foxp3$^-$, from (or in) adult lymphoreplete Foxp3 reporter mice. Also, we included experiments in which those same cohort were followed over time upon a second transfer. Data from experiments performed under the same experimental conditions and reported by different papers, were pooled together, by averaging analogous time points, and were then marked with the same label.

Inducible labelling, upon tamoxifen administration, of Foxp3$^+$ cells, in

lymphoreplete mouse.

Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$ $\times$ R26-YFP mice from 6 to 8 weeks of age received three doses of tamoxifen (8 mg each) at days 0, 1, and 3, by oral gavage. Mice were analysed at day 14 and at 5 months after treatment. Frequency and number of Foxp3^{+} cells within CD4^{+}YFP^{+} T cells recovered from spleen or lymph nodes, were assessed (n=7). Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$ $\times$ R26YFP mice were generated by the authors of the paper by breeding knock in Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$ mice with the ROSA26YFP mice [Rubtsov et al., 2010]. In the knock in Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$ mice the cassette containing an internal ribosome entry site (IRES), followed by DNA sequence encoding a triple fusion protein eGFP-Cre-ERT2 (enhanced green fluorescent protein, with Cre recombinase and mutated human estrogen receptor ligand-binding domain) is inserted into the 3‘ untranslated region (UTR) of the Foxp3 gene. Rosa26-YFP mice the harbour the loxP site-flanked STOP cassette in the ubiquitously expressed ROSA26, followed by a DNA sequence encoding the yellow fluorescent protein. In these mice, when the fusion protein GFP-CreERT2 is expressed, it resides in the cytosol. Treatment with tamoxifen, allows for the GFP-CreERT2 protein to enter the nucleus and to excide the floxed STOP cassette. It causes therefore constitutive and heritable expression of YFP in cells that were expressing Foxp3 at the time of tamoxifen administration.
Label: 1
Reference: [Rubtsov et al., 2010](Fig. 1B)

Adoptive cells transfer of peripheral Foxp3^{+} cells, into lymphoreplete mouse.

Ly5.2/Thy1.1 mice received i.v. 1×10^5 CD4^{+}eGFP^{+} T cells sorted from Foxp3eGFP Ly5.1/Thy1.2. Mice (n=5,6) were analyzed at 4 and 8 weeks post transfer, assessing the frequency of eGFP^{-} cells in CD4^{+}Ly5.1^{+}Thy1.2^{+} donor T cells, pooled from lymph nodes and spleen. Before staining for Ly5.1, Thy1.2, and CD4, the recovered cells were enriched for donor T cells by depleting Thy1.1^{+}, Ig^{+}, and adherent cells by panning. B6.Ly5.1 congenic mice were used at 5-12 weeks of age.
Label: 3
Reference: [Komatsu et al., 2009](Fig. 1B)

Adoptive cells transfer of peripheral Foxp3$^+$ cells, into lymphodeficient mouse + sustained IL-2 i.p. administration.
TCR$\beta^{-/-}$ mice received i.v. 2.5×10^5 Thy1.1$^+$ CD4$^+$ Foxp3-GFP$^+$ cells. Mice were also injected daily with recombinant IL-2 i.p., during 3 weeks. Mice were analyzed 3 weeks after transfer, assessing the frequency of Foxp3$^-$ cells in CD4$^+$CD3$^+$ T cells recovered in spleen or pooled lymph node.(n=4)
Label: 5
Reference: [Duarte et al., 2009] (Fig 2D)

Adoptive cells transfer of peripheral Foxp3$^+$ alone into lymphodeficient mouse. (Data from two papers)
(First pool of data) Rag2$^{-/-}$ mice received i.v. 2.5×10^5 Thy1.1$^+$ CD4$^+$ Foxp3-GFP$^+$ cells. Mice were analyzed at weeks 1, 2, 3, 4 and 10 after transfer, assessing the frequency of Foxp3$^-$ cells in CD3$^+$ Thy1.1$^+$ cells, recovered from spleen or lymph nodes.
Label: 7
Reference: [Duarte et al., 2009] (Fig 1E)
(Second pool of data) CD4$^+$eGFP$^+$ T cells were sorted from Foxp3^{eGFP} Ly5.2 mice and adoptively transferred into Rag2$^{-/-}$ mice. Mice were analyzed 4 weeks after transfer, assessing the frequency of GFP$^+$ cells within the Foxp3$^+$ (Ly5.1$^-$) donor-derived CD4$^+$TCRβ^+ cells recovered from lymph nodes. Mice were used at 5 to 12 weeks of age.
Label: 7
Reference: [Komatsu et al., 2009] (Fig. 1A)

Adoptive cells transfer of peripheral Foxp3$^+$ together with Foxp3$^-$ cells at Foxp3$^+$: Foxp3$^-$ ratio, into lymphodeficient mouse. (Data from two papers)
(First pool of data) Rag2$^{-/-}$ mice received 2.5×10^5 Thy1.1$^+$ CD4$^+$ Foxp3-GFP$^+$ cells together with the same number or 10 times less Thy1.2$^+$ CD4$^+$ Foxp3-GFP$^-$ cells. Mice were analyzed 4 weeks after transfer, assessing the frequency of Foxp3$^-$ cells gated in CD3$^+$ Thy1.1$^+$ cells in the spleen of mice host mice (n=4 for each group).
Labels: 4, 6
Reference: [Duarte et al., 2009] (Fig 2A)

(Second pool of data) CD4$^+$eGFP$^+$ and eGFP$^-$ T cells were sorted from Foxp3^{eGFP} Ly5.2 and Foxp3^{eGFP} Ly5.1 mice, respectively, and adoptively transferred into Rag2$^{-/-}$ mice, mixed at a 1:1 or 1:10 ratio (1×10^5 eGFP$^+$ plus 1×10^5 or 1×10^6 eGFP$^-$). Mice were analyzed 4 weeks after transfer. Lymph nodes cells were stained for CD4, TCRβ and Ly5.1. Frequency of GFP$^+$ cells within the Foxp3$^+$ (Ly5.1$^-$) or Foxp3$^-$ (Ly5.1$^+$) donor-derived CD4$^+$TCRβ^+ cells recovered from each host mouse, was assessed. Mice were used at 5 to 12 weeks of age.
Labels: 2, 4
Reference: [Komatsu et al., 2009] (Fig. 1A)

Adoptive cells transfer of peripheral Foxp3$^-$ cells, recovered at 4 weeks after a previous transfer of Foxp3$^+$ cells (exFoxp3$^+$), into a lymphodeficient mouse.
CD3$\varepsilon^{-/-}$ mice received i.v. 1×10^5 Foxp3$^-$ T cells from Foxp3^{eGFP} (exFoxp3$^+$) recovered at 4 weeks after a previous transfer of Foxp3$^+$ cells into CD3$\varepsilon^{-/-}$. Mice were analyzed 4 weeks after the second transfer. Cells recovered from lymph nodes were stained for CD4 and Ly5.1 and analyzed for eGFP expression, assessing the frequency of re-induced Foxp3$^+$ T cells in CD4$^+$Ly5.1$^+$ donor cells. B6.CD3$\varepsilon^{-/-}$mice were used at 5 to 12 weeks of age.
Label: 8
Reference: [Komatsu et al., 2009] (Fig 3C)

Adoptive cells transfer of peripheral Foxp3$^-$ cells, into lymphodeficient mouse (data from two papers)
(First pool of data) TCR$\beta^{-/-}$ mice received i.v. 3×10^5 CD4$^+$CD8$^-$Foxp3$^-$ cells purified from pooled lymph nodes isolated from Foxp3^{GFP} reporter mice. Mice were analyzed 4 weeks after transfer, assessing the frequency of Foxp3$^+$ cells within CD4$^+$TCR$^+$ lymphocytes recovered from pooled branchial, inguinal, and axillary lymphnodes, or pooled mesenteric lymph nodes, or spleen(6 independent experiments of n=6 each).
Label: 9
Reference: [Paiva et al., 2013] (Fig. S1)
(Second pool of data) Rag2$^{-/-}$ mice received i.v. 1×10^5 CD4$^+$eGFP$^-$ T cells sorted from Foxp3^{eGFP} Ly5.1 mice. Mice were analyzed 4 weeks after transfer.

Lymph nodes cells were stained for CD4, TCRβ and Ly5.1. Frequency of GFP$^+$ cells within the Foxp3$^+$ (Ly5.1$^-$) donor-derived CD4$^+$TCRβ^+ cells recovered from each host mouse, was assessed. Mice were used at 5 to 12 weeks of age.
Label: 9
Reference: [Komatsu et al., 2009] (Fig. 1A)

Adoptive cells transfer of peripheral Foxp3$^-$ cells, recovered at 4 weeks after a previous transfer of Foxp3$^-$ cells (stillFoxp3$^-$), into lymphodeficient mouse

CD3$\varepsilon^{-/-}$ mice received i.v. 1×10^5 Foxp3$^-$ T cells (stillFoxp3$^-$) from Foxp3^{eGFP}Ly5.1 mice recovered at 4 weeks after a previous transfer of Foxp3$^-$ cells into CD3$\varepsilon^{-/-}$. Mice were analyzed 4 weeks after the second transfer. Cells recovered from lymph nodes were stained for CD4 and Ly5.1, and analyzed for eGFP expression. Frequency of induced Foxp3$^-$ T cells in CD4$^+$Ly5.1$^+$ donor T cells was assessed. Mice were used at 5 to 12 weeks of age.
Label: 10
Reference: [Komatsu et al., 2009] (Fig 3C)

Label	Experimental setting	Donor	Host	Initial proportion (Foxp3$^+$: Foxp3$^-$)	Initial cells number (Foxp3$^+$, Foxp3$^-$)	Cells recovered from	Time points (days)	Reference
1	Inducible labelling of Foxp3$^+$ cells, into lymphoreplete mouse	-	Foxp3$^{eGFP\text{-}Cre\text{-}ERT2}$ × R26-YFP	$(1:7)$	$(4.5\times10^6, 3\times10^7)$	LNs, Spleen	$T_1=\{1,126\}$	[Rubtsov et al., 2010](Fig. 1B)
3	Adoptive cells transfer of peripheral Foxp3$^+$ cells, into lymphoreplete mouse	Foxp3^{eGFP}KI (Ly5.1/Thy1.2)	Foxp3^{eGFP}KI (Ly5.2/Thy1.1)	$(1:6.5)$	$(4.9\times10^6, 3\times10^7)$	Lns,Spleen	$T_3=\{0,28,56\}$	[Komatsu et al., 2009](Fig. 1B)
5	Adoptive cells transfer of peripheral Foxp3$^+$ cells, into lymphoreplete mouse + sustained IL-2 i.p. administration	Foxp3^{eGFP}KI Thy1.1	TCR$\beta^{-/-}$	$(1:0)$	$(2.5\times10^5, 0)$	LNs, Spleen	$T_5=\{0,21\}$	[Duarte et al., 2009](Fig. 2D)
	Adoptive cells transfer of peripheral Foxp3$^+$ cells, alone or together with Foxp3$^-$ cells, in portion (Foxp3$^+$:Foxp3$^-$), into lymphodeficient mouse:							
2	(1:10)	Foxp3^{eGFP}KI (Ly5.2, Ly5.1)	Rag2$^{-/-}$	$(1:10)$	$(10^5, 10^6)$	LNs	$T_2=\{0,28\}$	[Komatsu et al., 2009](Fig. 1A)
4	(1:1)	Foxp3^{eGFP}KI (Thy1.1, Thy1.2)	Rag2$^{-/-}$	$(1:1)$	$(2.5\times10^5, 2.5\times10^5)$	Spleen	$T_4=\{0,28\}$	[Duarte et al., 2009](Fig. 2A)
		Foxp3^{eGFP}KI (Ly5.2, Ly5.1)	Rag2$^{-/-}$	$(1:1)$	$(10^5, 10^5)$	LNs		[Komatsu et al., 2009](Fig. 1A)
6	(1:0.1)	Foxp3^{eGFP}KI (Thy1.1, Thy1.2)	Rag2$^{-/-}$	$(1:0.1)$	$(2.5\times10^5, 2.5\times10^4)$	Spleen	$T_6=\{0,28\}$	[Duarte et al., 2009](Fig. 2A)
7	(1:0)	Foxp3^{eGFP}KI (Thy1.1)	Rag2$^{-/-}$	$(1:0)$	$(2.5\times10^5, 0)$	LNs, Spleen	$T_7=\{0,7,14,...$	[Duarte et al., 2009](Figg. 1E, 2A)
		Foxp3^{eGFP}KI (Ly5.2)	Rag2$^{-/-}$	$(1:0)$	$(10^5, 0)$	LNs	$21,28,70\}$	[Komatsu et al., 2009](Fig. 1A)
	Adoptive cells transfer of peripheral Foxp3$^-$ cells,							
9	into lymphodeficient mouse	Foxp3^{eGFP}KI (Ly5.1)	Rag2$^{-/-}$	$(0:1)$	$(0, 10^5)$	LNs	$T_{11}=\{0,28\}$	[Komatsu et al., 2009](Fig. 1A)
		Foxp3^{eGFP}KI	TCR$\beta^{-/-}$	$(0:1)$	$(0, 3\times10^5)$	LNs, Spleen		[Paiva et al., 2013] (Fig. S1)
8	Adoptive cells transfer of peripheral Foxp3$^-$ cells, recovered at 4 weeks after a previous transfer of Foxp3$^+$ cells (exFoxp3$^+$), into a lymphodeficient mouse	Foxp3^{eGFP}KI	CD3$\varepsilon^{-/-}$	$(0:1)$	$(0, 10^5)$	LNs	$T_9=\{0,28\}$	[Komatsu et al., 2009](Fig. 3C)
10	Adoptive cells transfer of peripheral Foxp3$^-$ cells, recovered at 4 weeks after a previous transfer of Foxp3$^-$ cells (stillFoxp3$^-$), into a lymphodeficient mouse	Foxp3^{eGFP}KI	CD3$\varepsilon^{-/-}$	$(0:1)$	$(0, 10^5)$	LNs	$T_9=\{0,28\}$	[Komatsu et al., 2009](Fig. 3C)

Table 3.1: **Summary of the 10 different experimental setting included in the analysis.** Label attributed to each experiment; experimental setting description; donor mice, in case of adoptive transfer; host mice in which the cohorts of cells are followed; proportion Foxp3$^+$ and Foxp3$^-$ CD4 T cells present in the host at time zero of experiment; absolute number of Foxp3$^+$ and Foxp3$^-$ CD4 T cells present in the host at time zero of experiment; organs from which analysed cells were recovered; frequency and number of Foxp3$^+$ and Foxp3$^-$ cells present in the host at time zero of the experiments; available experimental time points, measured in days; references.

3.4.3 Fitting of the model to the experimental data

Let us indicate the model prediction for the frequency over time of Foxp3$^+$ cells within the cohort of cells followed in the i-th experimental setting as following:

$$ {}^{i}f(t) = {}^{i}f(t, \alpha, \beta, \vartheta, {}^{i}\varrho), \qquad i = 1, 2, ..., 10. \tag{3.27} $$

We defined the following score function:

$$ S = \sum_{i=1}^{10} \sum_{\tau \in T_i} \left({}^{i}f(\tau) - {}^{i}\hat{f}(\tau) \right)^2, \tag{3.28} $$

given by the sum of the residuals between the model prediction ${}^{i}f(t)$ and the i-th experimental data set ${}^{i}\hat{f}(\tau)$, where T_i for $i = 1, ..., 10$, is the set of the discrete time points τ of the i-th experiment.

To ensure a complete analysis of the values for the frequency of Foxp3 committed cells that are compatible with the ensemble of experimental data, we fixed values for the parameter α, uniformly distributed within the interval

$$ I_\alpha = [0, 0.15]. \tag{3.29} $$

For each of these fixed values, we minimized the score function S with respect to the parameters $\beta, \vartheta,\ {}^{1}\varrho,\ {}^{2}\varrho, ...,\ {}^{10}\varrho$. The minimization was subjected to the following constraint:

$$ 0.10 \leq \alpha + \beta\ {}^{1}\varrho \leq 0.15, \tag{3.30} $$

which translates the well known result that in physiologic condition 10-15$\%$ in CD4 T cells are found to be Foxp3$^+$. We explored the parameter space choosing random initial values for the parameters ranging in specific intervals:

$$ \beta \in I_\beta = [0, 1], \quad \vartheta \in I_\vartheta = [0, 3], \quad {}^{i}\varrho \in I_{\,{}^{i}\varrho} = [0, 1],\ i = 1, 2, ..., 10. \tag{3.31} $$

The "Principal Axis" algorithm [Brent, 2013] was used to minimize the score function S. To avoid local minima, we fixed a threshold th $= 0.01878$ for the maximum score value allowed, given that the square of the residuals in case of the experiment 7 fitted alone was equal to 0.014784.

The algorithm converged efficiently, finding global minimum for S in correspondence of optimal distributions for the parameters. Optimum values for ϑ and for ${}^{i}\varrho$, $i = 1, 2, ..., 8$ were found regardless of their initial values, spanning in their respective ranges, as shown in Figure 3.8. However, the score function S was found to be not much sensitive to values of the ratios ${}^{9}\varrho$ and ${}^{10}\varrho$, as distributions of initial values and optimum values are very similar. We discuss this result in the conclusion section. Finally, concerning the fractions α and β, the algorithm converged only for fixed values of α below 0.042 and for initial values of β included in the interval $[0.06, 0.59] \subset I_{\beta}$. The cumulative distribution functions for the initial and optimum values of the model parameters parameters showed in Figure 3.8, concerns the distributions of 2483 different runs of the optimization algorithm.

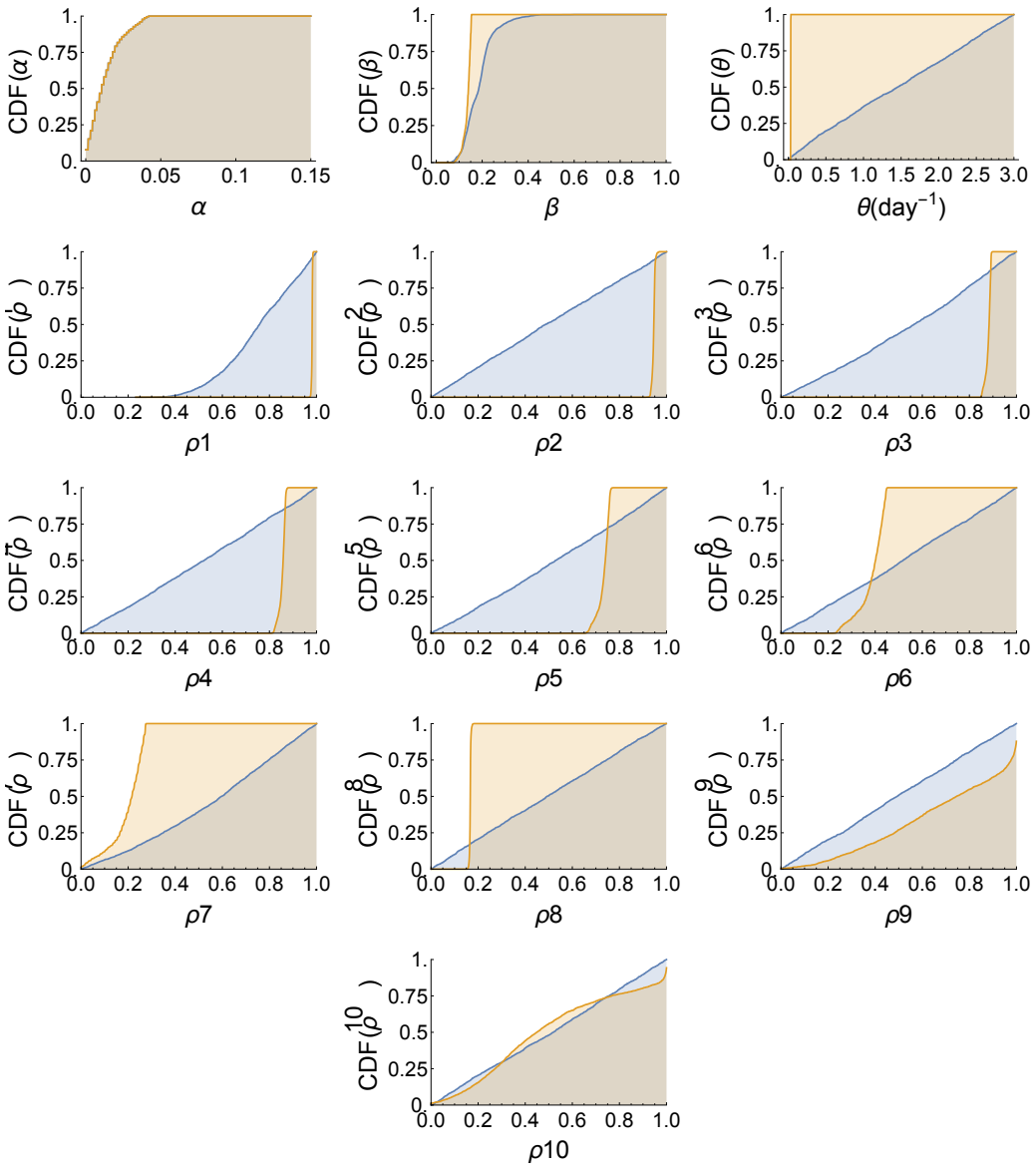

Figure 3.8: **Convergence toward minimum for the score function S with respect to model parameters.** Cumulative distribution functions of the initial values (blue lines) of the model parameters provided to the algorithm in order to minimize the score function S and of the optimum values (yellow lines) that minimize S. In each plot, parameters range within their respective initial interval $I_\alpha, I_\beta, I_\vartheta,\ {}^{i}I_{\varrho},\ i = 1, 2, ..., 10$.

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4

Slow epigenetic dynamics of Foxp3 locus can explain Foxp3 dynamics in murine CD4 T cells in different contexts. A quantitative approach.

Statement: All the work included in this chapter is my own, carried out under the supervision of Jorge Carneiro and Jocelyne Demengeot.

Abstract

Foxp3 expression is necessary for Tregs development and regulatory function. While in physiologic condition Tregs show stable Foxp3 expression, in several other experimental conditions, Foxp3 expression can be lost over time, following a slow dynamics. This dynamics cannot be explained as the result of transcription, translation and protein degradation.

Here we propose that both stability and lability of Foxp3 expression is the result of the combination of the fast and context-dependent transcription activation and repression dynamics together with a slow epigenetic dynamics, that depend on the transcriptional state of the cell, rather than on the context in which the cell

is.

We developed a stochastic model for Foxp3 expression in CD4 T cells that accounts for transcriptional, translational and epigenetic dynamics and in which Foxp3 expression can be reversible or irreversible. Model parameters that control the dynamics of either transcription or epigenetic remodeling of the chromatin, can be tuned in a context-dependent or -independent way. We fitted the model to experimental data collected from the literature, describing the temporal evolution of Foxp3$^+$ cells frequency in Tregs cohort, in different experimental conditions.

We found that the different dynamics can be all coherently and quantitatively reproduced, by tuning the ratio between fast Foxp3 transcriptional activation and repression rates across the experiments, while epigenetic rates were kept slow and constant across the experiments. Also, model predicts that in physiologic condition, where Tregs are stable, the vast majority of Tregs has an active state of chromatin in the Foxp3 locus. This characteristic ensures on the one hand, the stability of Foxp3 expression in lymphopenic conditions, where the context provides stimuli that enhance Foxp3 gene transcription, prevent its repression; on the other hand it explains the slow dynamics of Foxp3 expression loss, observed in those lymphopenic condition where Foxp3 transcription is prevented. Indeed the dynamics of Foxp3 loss, observed in lymphopenia, coincides with the epigenetic remodeling dynamics in Foxp3$^+$ T cell predicted by our model.

4.1 Introduction

The forkhead box protein 3 (Foxp3) is a transcription factor, mainly expressed in the subset of CD4 T cells, called Regulatory T cells (Tregs). Foxp3 gene expression is necessary for Tregs development and confers them suppression activity [Fontenot et al., 2003, Hori, 2003, Khattri et al., 2003]. In physiologic condition for mice, Foxp3 expression in CD4 T cells has been observed to be stably maintained *in vivo* over several rounds of cells divisions, persisting for several months [Rubtsov et al., 2010]. *In vivo* Foxp3 stability over time has also been observed in Foxp3$^+$ cohort of CD4 T cells, upon adoptive transfer into normal lymphoreplete mice [Komatsu et al., 2009]. Even in lymphodeficient mice in which normal proportion of Foxp3$^+$ and Foxp3$^-$ CD4 T cell have been reconstituted, by co-transferring both populations, Foxp3 expression is maintained, at least for four

weeks [Komatsu et al., 2009]. Yet, loss of Foxp3 expression by Foxp3^{+} CD4^{+} T cells and consequent *in vivo* accumulation of the so called exTregs in the periphery of mice, has been experimentally observed in several contexts, such as lymphopenia and under inflammation [Duarte et al., 2009, Komatsu et al., 2009, Mellor and Munn, 2011, Yurchenko et al., 2012].

Through meta-analysis on experimental data that assessed Foxp3 dynamics *in vivo* in different experimental conditions, we have already suggested that, at least for a consistent fraction of Tregs, Foxp3 stability or lability is determined by the context in which Tregs are embedded (see chapter 3). In particular Foxp3 stability in Tregs strongly positively correlates with the frequency of Foxp3^{-} CD4 T cells within the total CD4 T cells compartment, as well as with absolute number of Foxp3^{-} CD4 T cells within the host (see chapter 3). This result is in accordance with the knowledge that Foxp3 expression needs, together with T cell receptor stimulations [Levine et al., 2014], continuous IL-2 stimulation, mainly produced by activated Foxp3^{-} CD4 T cells [Malek, 2008], to be maintained.

When occurring, the process of loss of Foxp3 expression over time within cohort of Foxp3^{+} T cells follows a rather slow dynamics. For instance, in the case in which Foxp3^{+} T cells are purified from Foxp3-GFP reporter mouse and then adoptively transferred into Rag2$^{-/-}$ mouse, we have estimated the average time a Treg is able to maintain Foxp3 expression, in the periphery and in absence of pro-Foxp3 stimuli, to be four weeks. On the other hand the half-life of Foxp3 protein has been experimentally estimated to be of 2-3 hours [Morawski et al., 2013], as well as the one of Foxp3 RNA [Bending et al., 2018]. For this reason, the slow dynamics that characterizes Foxp3 loss in lymphopenic condition cannot be explained by the time expected for Foxp3 transcripts and protein to be degraded, after Foxp3 transcription is over.

The experimental observations of loss of Foxp3 expression, by cells in environment that do not provide enough pro-Foxp3 stimuli, hould already rule out the possibility that Foxp3 directly regulates itself through a positive feedback loop. Beside that, there are not very convincing evidences of Foxp3 enhancing its own expression.

Yet, it has been widely described that CD4 T cells use epigenetic and the remodeling of the chromatin structure to imprint gene induction events that occurred in the progenitor cells [Reiner, 2005]. Concerning Tregs, CD4^{+}CD25^{+}

T cells have been shown to display full demethylation of a conserved CpG-rich region within the Foxp3 locus in one of its enhancers, as well as histone modifications [Floess et al., 2007]. In particular, it has been proposed that the complete demethylation of this region, called the Tregs-specific demethylated region (TSDR), is the key mechanism through which stable Foxp3 expression is achieved in CD4 T cells [Floess et al., 2007, Miyao et al., 2012].

Epigenetic signatures of the gene locus, which determine chromatin remodeling and accessibility of the locus to the transcription factors and machinery, are inherited and maintained across cell generations. Epigenetic signatures dynamics include the changes in epigenetic marks that make the locus more or less competent for transcription and which then become inherited in the next generations. For this reason these dynamics can be considered slow, if compared with transcription and translation activation and repression dynamics.

Here we want to put forward the idea that prolonged Foxp3 expression in a CD4 T cell, due to a context that provides enough pro-Foxp3 stimuli, can induce epigenetic remodeling of the locus that, in turns, ensures stable Foxp3 expression. In this view, the process occurs without epigenetic remodeling being directly induced by external inputs provided by the context. In particular, we propose that both the stability of Foxp3 expression observed in lymphoreplete conditions, as well as its lability, observed in limphopenia, and encompassing all the intermediate cases of mice whose immune system populations of CD4 T cells have been partially reconstituted by adoptive transfers, can be explained as the result of a combination of two processes: a fast and context-dependent transcription/translation activation and repression of the Foxp3 gene, combined with a slow and context-independent epigenetic remodeling of the Foxp3 locus.

4.2 Results

4.2.1 A stochastic model for gene expression accounting for epigenetic remodeling and transcription is able to describe Foxp3 dynamics in CD4 T cell cohorts.

We decided to explore whether the different dynamics of Foxp3 expression observed *in vivo* in different experimental contexts can be explained by combin-

ing fast and context-dependent transcription dynamics together with slow and context-independent chromatin remodeling of the gene locus.

Model assumptions and description.

For this, we developed a stochastic model for gene expression at the Foxp3 locus in a CD4 T cells, that accounts for transcriptional, translational and epigenetic dynamics.

According to the model, which is an extension of the model developed in the previous chapter, any CD4 T cell can be found in one out of a set of mutually exclusive states. Each state is indicated by Z_{ij}. The subscript i refers to the transcriptional/translational state of the cell and can acquire values in $\{0, 1, 2, 3\}$. If $i \in \{0, 1\}$ the cell is Foxp3$^-$, if $i \in \{2, 3\}$, the cell is Foxp3$^+$. The subscript j refers to the epigenetic status of the chromatin at the Foxp3 locus. In this regard, states are distinguished by the collection of epigenetic marks that either prevent accessibility of the locus from the transcription machinery, or that makes the locus fully competent for transcription. Also, we assumed that the locus can be found in an intermediate condition, in which either expression or repression of the gene can occur. In nature there is an almost-continuum of different epigenetic states, two of which (the extreme ones) are clearly identifiable It is our choice to discretize the continuum into three states and we refer to those states by the subscripts j=0 (for closed chromatin), j=1 (for the intermediate state), j=2 (for open chromatin). A cartoon of the model is depicted in figure 4.1.

The model assumes that a fraction β of the entire pool of CD4 T cells can change their status over time: these cells can acquire or lose Foxp3 expression and undergo modification of their epigenetic marks. Any transition from one state to another one happens stochastically, according to a characteristic constant rate which is the expected value from a exponential distribution. We indicated these rates by Greek letters. In particular we assumed that if the locus is not accessible by the transcription machinery, the gene is silenced and transcription is not possible. Nevertheless, the locus undergoes stochastic remodeling, switching from state with j=0 to j=1 and vice-versa, according to rates ε_{01} and ε_{10}. In case the locus is at least partially accessible (j=1), gene transcription is possible, as well as its repression. Transition from Foxp3$^-$ to Foxp3$^+$ includes transcription of the gene and translation and occurs according to the rate τ_+. Vice-versa, the

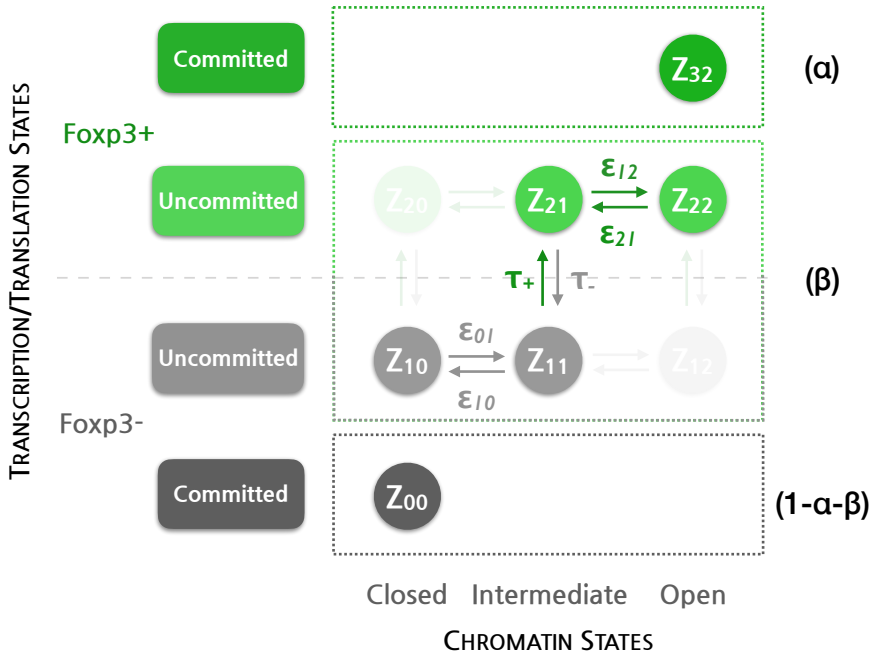

Figure 4.1: **A stochastic model for gene expression at the Foxp3 locus in a CD4 T cells, accounting for epigenetic remodeling and transcription.** Cartoon of the stochastic model. Circles represent the mutually exclusive states in which any CD4 T cell can be found. Arrows, where present, represent the stochastic transitions between states, happening according to constant rates indicated by Greek letters: $\varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}$ are the epigenetic remodeling rates, while τ_+ and τ_- are, respectively, transcription activation and repression rates. Dashed lines separate three compartments, among which state transitions are precluded. In parenthesis, the fraction of CD4 T cells belonging to each compartment.

opposite transition, which occurs according to the rate τ_-, includes repression of the transcription and degradation of transcripts and proteins. Moreover, we assumed that in case of ongoing transcription, the locus can undergo further chromatin remodeling (according to the constant rate ε_{12}), becoming fully activated. For Foxp3$^+$ cells whose chromatin at Foxp3 locus is fully activated (cells in state Z_{22}), the model assumes that repression of the gene is prevented. Then, for the gene to be shut down, it is necessary first a transition to epigenetic state characterized by j=1 (state Z_{21} of cell), which occurs with rate ε_{21}.

Finally, the model accounts for the existence of cells that do not change their state over time. We called these cells committed and we considered two types of them. We assumed that a portion α of CD4 T cells are committed to Foxp3 expression, with a fully activated chromatin: their state was named Z_{32}. The remaining $1-\alpha-\beta$ fraction of CD4 T cells are committed to be Foxp3$^-$, with a locus that is fully incompetent for transcription. Their state was namedZ_{00}.

We assumed that the rates τ_+, τ_- depend on the context in which the cell is embedded. Indeed we expect the rates of Foxp3 transcription activation and repression to depend on the immune cell populations composition in the host and on the subsequent inputs the cell receives from the environment (in terms of IL-2 and TGF-β availability, mainly). Instead, we assumed that the rates $\varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{21}$ of epigenetic remodeling, are context independent. In other words, the value of the epigenetic remodeling rates is conserved across environments, whether constituted by lymphodeficient or lymphoreplete mice, and encompassing all the the lymphodeficient hosts in which the immune system CD4 populations have been partially reconstituted by adoptive transfers.

The fractions α and β are model parameters that we assumed to be context-independent and that need to be quantified. However, we had previously estimated α to range from 0 to 4.2$\%$ of the total CD4 T cells compartment, and β to range from 7.6$\%$ to 15.4$\%$ (see chapter 3).

Transient behavior and equilibrium.

The model is able to reproduce the frequency of cells $m(t)$ that maintain Foxp3 expression over time, within a cohort of Foxp3$^+$ CD4 T cells that belong to a

mouse in lymphoreplete condition:

$$m(t) = \frac{\alpha + \bar{f}_\infty \beta f(t)}{\alpha + \bar{f}_\infty \beta}, \tag{4.1}$$

where $\bar{f}_\infty$ represents the frequency at equilibrium of Foxp3$^+$ cells, in lymphoreplete condition, within the uncommitted CD4 T cells, meaning within the CD4 T cells that are assumed to up-regulate or down-regulate Foxp3 depending on the context. Also, $f(t)$ is the frequency of Foxp3 positive cells within the uncommitted CD4 T cells at time t. The expression for $f(t)$ is a linear combination of three exponential functions plus a constant:

$$f(t) = x_0 + \sum_{j=1}^{3} x_j e^{r_j t}, \tag{4.2}$$

where the functions $x_j (j = 0, ..., 3)$ depend on the model rates and on the initial distributions of the uncommitted cells within the states Z_{10}, Z_{11}, Z_{21}, Z_{22}; while the decay rates $r_j (j = 1, ..., 3)$ are the three roots of a polynomial $q(x)$ of degree three, whose expression depends on the rates only. Both the expressions for $f(t)$, which is the solution of a continuous time Markov chain model, and the one for $q(x)$ are fully derived in Method section. Equation 4.1 can be used to quantify the frequency of cells that maintain Foxp3 expression over time, in any cohort of Foxp3$^+$ cells, either followed in lymphoreplete condition or sorted from lymphoreplete conditions and then adoptively transferred in another host.

The model predicts also that, once the rates are fixed, and irrespective of the initial distributions across the four uncommitted states, waiting long enough, the system shall reach a stable equilibrium, resulting in a fixed frequency of Foxp3$^+$ cells within the uncommitted T cells, defined as following:

$$f_\infty = \frac{\varepsilon_{01}(\varepsilon_{21} + \varepsilon_{12})\rho}{\varepsilon_{01}(\varepsilon_{21} + \varepsilon_{12}\rho) + \varepsilon_{10}\varepsilon_{21}(1 - \rho)}, \tag{4.3}$$

where ρ is the ratio between Foxp3 transcriptional activation and repression, characteristic of the environment in which the cells are followed, and defined

as:

$$\rho = \frac{\tau_+}{\tau_+ + \tau_-}. \tag{4.4}$$

The expression in 4.3, which represents the frequency of cells in either state Z_{21} or Z_{22} within the uncommitted CD4 T cells, can be also used to quantify the portion at equilibrium of uncommitted Foxp3$^+$ cells that have a fully active state of the chromatin, meaning the portion of uncommitted Tregs in state Z_{22}:

$$\frac{\varepsilon_{12}}{\varepsilon_{12} + \varepsilon_{21}}. \tag{4.5}$$

Under the assumption that epigenetic remodeling rates of the Foxp3 locus do not change across hosts, the fraction in equation 4.5 refers to the uncommitted Tregs with fully open chromatin found in any mouse in physiologic condition (that we assume being at equilibrium).

4.2.2 There is a family of solutions, that combine context-independent epigenetic remodeling with context-dependent transcription, able to explain Foxp3 *in vivo* dynamics.

We fitted the model to experimental data, available from the literature, describing the temporal evolution of Foxp3$^+$ cell frequency in cohort of cells that were Foxp3$^+$ in physiologic condition, at a given time. In all these experiments, the cohorts of cells were followed *in vivo* either in physiologic condition [Rubtsov et al., 2010] or in lymphodeficient host (Rag2$^{-/-}$ or TCR-$\beta^{-/-}$) after being adoptively transferred, either alone or together with CD4$^+$Foxp3$^-$ population in different proportions [Duarte et al., 2009, Komatsu et al., 2009]. Depending on the experimental context, the frequency measured of Foxp3$^+$ within the followed cohort over time, changes: in lymphoreplete condition, Foxp3 expression is kept in all the followed cells during four months, while on the contrary in lymphodeficient condition, 40% of cells lose Foxp3 expression within four weeks . All the experimental data and the model fittings are shown in figure 4.9 of supplemental material section.

We used the model to check whether Foxp3 dynamics *in vivo* could be explained as the result of the combination of a slow context-independent epigenetic remodeling of the Foxp3 locus, together with a much faster and context-

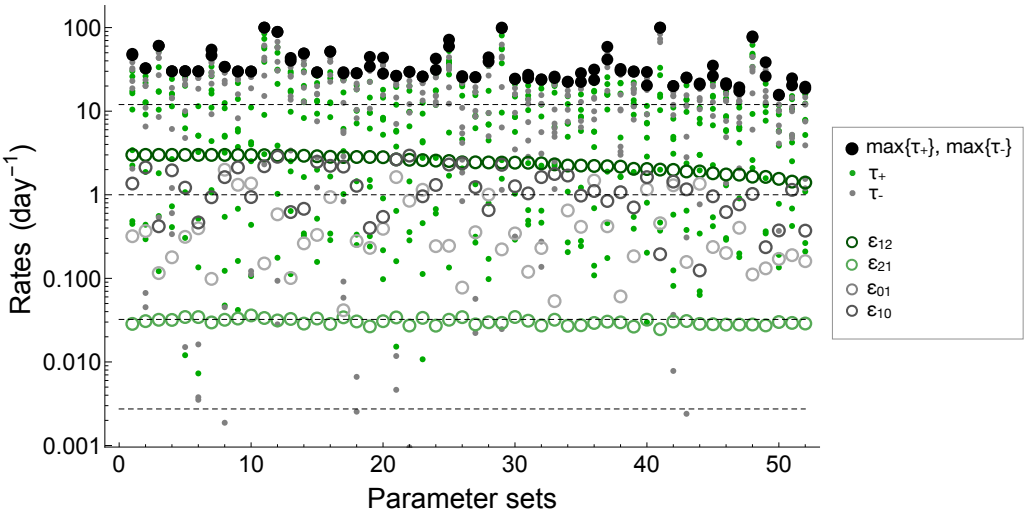

Figure 4.2: **Slow epigenetic rates and fast transcriptional rates.** Values of the rates that are compatible with the experimental data of Foxp3 dynamics *in vivo*. Each column represents one parameter set (any of the set fits equally well all the 10 experiments considered in our analysis). Rates of transcription/translation activation and repression are represented, respectively, by green and grey dots. Black dots correspond to the fastest transcription rates (both activation and repression). Epigenetic remodeling rates for Foxp3$^+$ cells are represented by green open circles (dark green for ε_{12}, light green for ε_{21}). Epigenetic remodeling rates for Foxp3$^-$ cells are represented by gray open circles. Horizontal dashed grey lines highlight the value of rate corresponding to, respectively, from bottom to top, one event per year, one event per month, one event per day, twelve events per day. Parameter sets are sorted according to decreasing value of the epigenetic remodeling rate ε_{12}.

dependent transcription and translation dynamics. To do that, while performing the fitting, we imposed all the epigenetic rates to be constant across experiments, while transcription/translation rates could change their value across experiments, if needed. Also, the frequency of committed Tregs and the one of uncommitted CD4 T cells (respectively α and β) were fixed as context-independent.

In each fitting, we also imposed the fastest epigenetic rate to be much slower than both the fastest transcription activation and repression rates. This constraint on the one hand, forces transcription activation and repression to be faster than epigenetic remodeling. On the other hand it allowed the search for parameters to include the possibility that, in some hosts, the context fully prevents either the gene transcription (in which case $\tau_+ \ll 1$) or, on the contrary, the shut down of the transcription (in which case $\tau_- \ll 1$).

Under these assumptions, we looked for optimum values for the rates, able to quantitatively reproduce the data. We found an entire family of possible solutions (figure 4.2) that can explain equally well all the experimental data.

The highest values for transcription rates (either activation of repression) were found in the two extreme contexts, namely in lymphoreplete condition and in the case of Tregs transferred alone in lymphodeficient host. In fact, respectively, the median value of activation rates τ_+^1 in lymphoreplete condition was 26.6 day^{-1}, and the median value of repression rate in lymphodeficient condition, τ_-^7, was equal to 29.86 day^{-1} (see transcription rates in figure 4.3).

Conversely, the rate τ_+^7 of activation of Foxp3 transcription in lymphodeficient host was found with much slower median value of 0.25 day^{-1} (and a minimum value close to zero). Also, the rate τ_-^1 of Foxp3 transcription repression in lymphoreplete condition, was found much slower if compared with the correspondent rate of activation τ_+^1, yet the median value was 8.43 day^{-1}, with minimum values found close to zero (see figure 4.3).1 We found slow and context-independent epigenetic remodeling rates able to reproduce all the experimental data. We observe that the distributions of the remodeling rates for Foxp3$^-$ CD4 T cells (ε_{01} and ε_{10}) were much spread, if compared with the ones of Foxp3$^+$ CD4 T cells (see figure 4.2 and epigenetic rates in figure 4.3). This result held particularly for the rate ε_{21}, which distribution was found to be very narrow around the value 0.03 day^{-1}. Finally although being both slow, the values for the rate ε_{12} were consistently found higher (with median value of 2.44 day^{-1}) that the ones for the

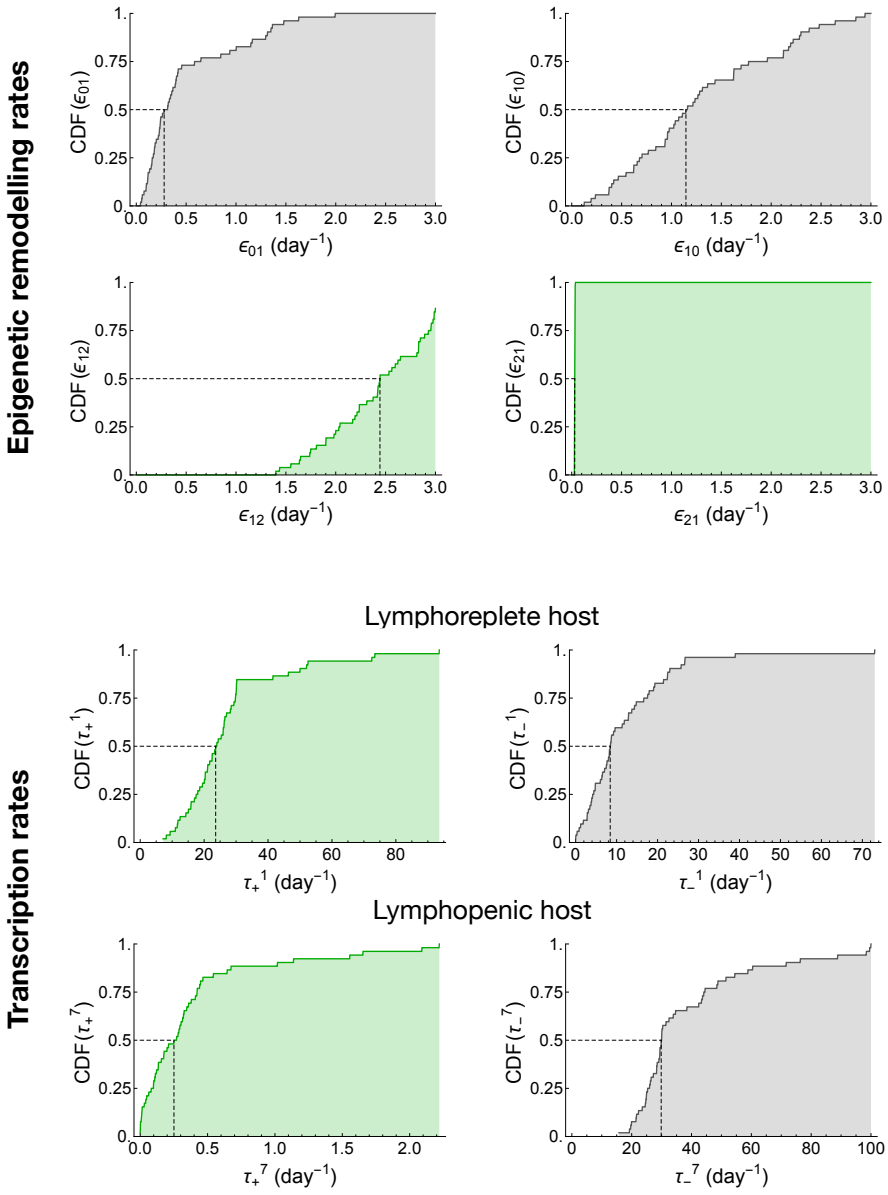

Figure 4.3: **Cumulative distribution functions of fast transcription and slow epigenetic rates.** Cumulative distribution functions (CDF) of the transition rates found by fitting the data to the ensemble of experimental data on Foxp3 dynamics *in vivo*, in ten different experimental settings. (Top) Slow epigenetic remodeling rates, assumed to be constant across all the experiments (context-independent rates). (Bottom) Fast transcription/translation activation and repression rates in two different and extreme contexts: lymphoreplete host and lymphodeficient host (Tregs adoptively transferred alone into lymphodeficient host mice).

rate ε_{21}.

To summarize, we found a family of solutions that, combining a slow and context-independent epigenetic remodeling of the Foxp3 locus together with much faster context-dependent transcription and translation dynamics, are able to reproduce coherently all the experimental data here gathered on Foxp3 dynamic *in vivo* in mice, in different experimental contexts.

4.2.3 Slow context-independent epigenetic explains the dynamics of loss of Foxp3, while being compatible with Foxp3 stability in physiologic condition.

In all the sets of model parameters compatible with experimental data, we found the value of the fraction in equation 4.5, equal to 99%. This result means that the vast majority of uncommitted cells whithin a cohort of Foxp3$^+$ T cells sorted from physiologic condition is characterised by fully active state of the chromatin.

Also previous results, found in the simpler version of the stochastic model analyzed in chapter 3, regarding the fraction of CD4 T cells committed to Foxp3 expression (α) and the fraction of cells to which Foxp3 expression is precluded ($1-\alpha-\beta$), still held in the results of the current model fittings. In particular, the possible values, compatible with the data, for the fraction α of Foxp3 committed cells within the CD4 T cells compartment ranged between 0.0% and 4.2%, which correspond to a maximum of 28% of Foxp3 committed cells within Tregs (see figure 4.4). Also, the frequency β of CD4 T cells, that can acquire or lose Foxp3 expression in a context-dependent way, was found to range between 7.9% and 15.4% (see figure 4.5).

It can readily be imagined that each combination of α and β of a particular parameter set, results in a specific portion of Foxp3 committed cells, within the total Foxp3 expressing cells. We looked at the kinetics of the sub-population of Foxp3$^+$ cells across the three states defined by the model (namely Z_{32}, Z_{22}, Z_{21}), in the model solutions that reproduce experimental data, in lymphoreplete and lymphodeficient condition. No matter the portion of committed cells within Tregs (cells in state Z_{32}) and the one of uncommitted with fully active state of chromatin (cells in state Z_{22}), we always observed the same temporal behavior, as shown in figure 4.6. Particularly, in lymphodeficient conditions, when indeed a dynamics of Foxp3 loss is experimentally observed, we found that this very tem-

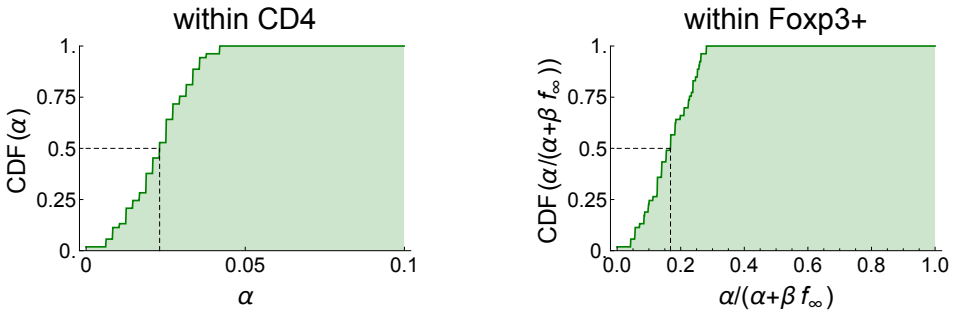

Figure 4.4: **Frequency of committed Tregs.** Left: Cumulative distribution functions (CDF) of the frequency α of committed Tregs within the CD4 T cells compartment. Rigth: CDF of the frequency of committed Tregs within the CD4$^+$Foxp3$^+$ T cells.

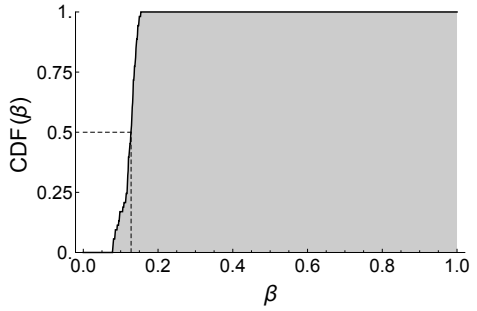

Figure 4.5: **Frequency of context-dependent CD4 T cells.** Cumulative distribution functions (CDF) of the frequency β of context-dependent CD4 T cells within CD4 T cells pool.

poral dynamics is entirely explained by the population of uncommitted cells with fully active Foxp3 locus. These cells, over time and according to the slow rate ε_{21}, lose the epigenetic signature that confers them the active state of chromatin, and then, quickly and because of the context, lose Foxp3 expression. Indeed, the state Z_{22} characterized by intermediate state of the chromatin, always constitutes a transitory state: accordingly, the corresponding sub-population never accumulates.

As a matter of fact, it is interesting to note that the value of the rate ε_{21}, which was found to be narrowly distributed around the value 0.03 day^{-1} (figure 4.3) and which describes the transition from active to intermediate state of the chromatin, is the characteristic rate of decay of a single exponential function that best fits the experimental data in lymphodeficient condition (see in figure 4.6). In this regard, it is worth noting that the mentioned experiment is the only one that, among the ones included in our analysis, provides information on kinetics. By fitting the data with linear combinations of exponential functions, we found that those data cannot be distinguished from a single exponential decay of the form:

$$y(t) = a + (1 - a)e^{-rt}, \tag{4.6}$$

with rate of decay $r = 0.03$, as we tested through the Aikake information criteria [Akaike et al., 1973, Bozdogan, 1987].

In summary, we found that the dynamics of Foxp3 expression loss, when observed, can be fully explained by a slow epigenetic remodeling of the chromatin at the Foxp3 locus that occurs in uncommitted Foxp3$^+$ cells in a context-independent way. This phenomenon, also occurs in contexts in which Foxp3 expression is stable, and it is compatible with Foxp3 expression stability. In this case, the system is at equilibrium, and the context-dependent frequent activation of transcription counterbalances the context-independent epigenetic remodeling.

4.2.4 Foxp3 mean residence time relates to frequency of committed Tregs

As we shown, the experimental data used in our analysis, support an infinite families of solutions, in which the frequency of committed Tregs can range from 0 to 28% of the total peripheral Tregs. Unfortunately (see figure 4.6), given the exper-

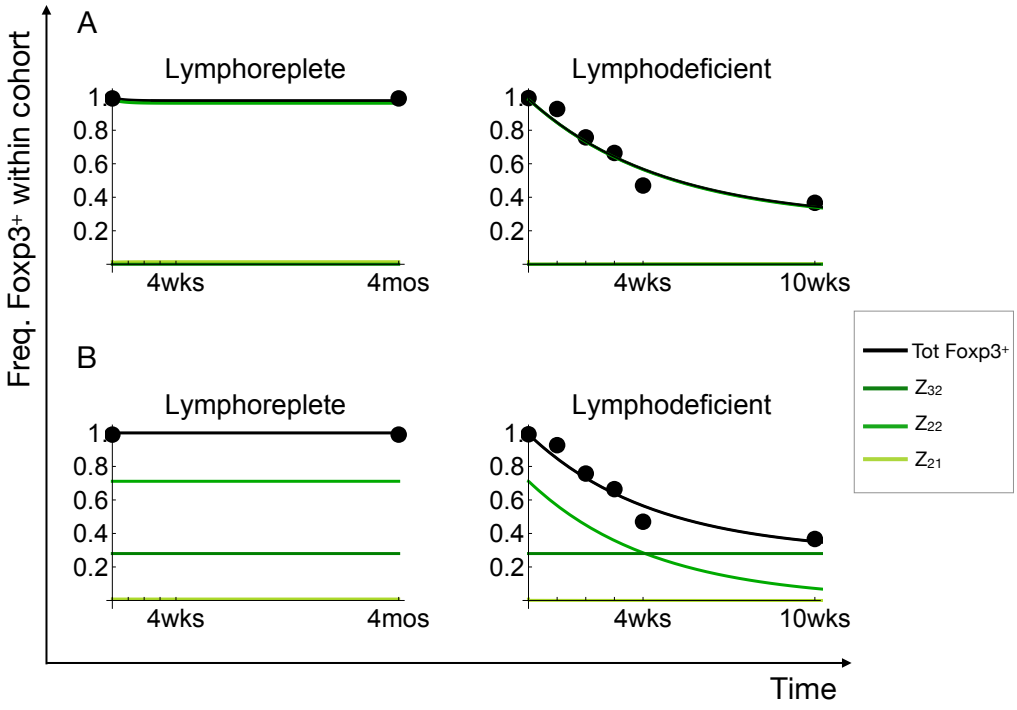

Figure 4.6: **Dynamics of loss of Foxp3 expression observed in lymphodeficiend host is well explained by context-independent epigenetic remodelling of Foxp3 locus over time.** Frequency of Foxp3$^+$ T cells within cohort of CD4 T cells that were 100% Foxp3$^+$ at time zero and where then followed *in vivo*, in two extreme experimental conditions, namely in lymphoreplete mice and in lymphodeficient mice, upon adoptive transfer. Experimental data (dots), model predictions (lines). Model predictions are shown for the frequency of total Foxp3$^+$ cells (black), committed Foxp3$^+$ cells (cells in state Z_{32}), uncommitted Foxp3$^+$ cells with fully active chromatin in Foxp3 locus (cells in Z_{22}), uncommitted Foxp3$^+$ cells with intermediate state of chromatin at the Foxp3 locus (cells in state Z_{21}). Two extreme conditions in parameter space are shown. A: parameter set corresponding to 0.0% of committed cells within Tregs ($\alpha = 0.0\%$): $\varepsilon_{01} = 0.23, \varepsilon_{10} = 0.43, \varepsilon_{12} = 1.52, \varepsilon_{21} = 0.02$, ${}^1\tau_+ = 24.14$, ${}^1\tau_- = 13.03$, ${}^7\tau_+ = 0.57$, ${}^7\tau_- = 36.60$. Also $\beta = 15.36\%$ and $\%98.4$ of context-dependent Tregs are with fully activated state of chromatin.; B: parameter set corresponding to 28% of committed cells within Tregs ($\alpha = 4.2\%$): $\varepsilon_{01} = 1.36, \varepsilon_{10} = 0.94, \varepsilon_{12} = 2.99, \varepsilon_{21} = 0.04$, ${}^1\tau_+ = 29.86$, ${}^1\tau_- = 2.12 * 10^{-5}$, ${}^7\tau_+ = 1.79 * 10^{-12}$, ${}^7\tau_- = 29.86$. Also $\beta = 10.8\%$ and 98.8% of context-dependent Tregs are with fully activated state of chromatin.

iments in our possess, there is no way to distinguish among these possibilities, which one is indeed occuring. In an attempt to overcome this issue, and in particular with the aim of finding predictions for an observable, whose value would strongly vary, depending on the frequency of committed Tregs, allowing us to distinguish between different scenarios, we computed the Foxp3 mean resident time, τ_{foxp3}.

The Foxp3 mean resident time is defined as the time that any uncommitted Treg (no matter whether in state Z_{21} or Z_{22}) would spend in the cluster of states $\{Z_{21}, Z_{22}\}$, before losing Foxp3 expression (see figure 4.7).

This time is computed as the expected value of the density probability distribution $f(t)$, describing the probability that the cell will leave the cluster at time t, given that it is in the cluster at time $t = 0$. The expression is the following:

$$\tau_{foxp3} = \frac{(\varepsilon_{12} + \varepsilon_{21})^2 + \varepsilon_{12}\tau_-}{\varepsilon_{21}(\varepsilon_{12} + \varepsilon_{21})\tau_-}. \tag{4.7}$$

The derivation of the formula is described in detail in supplemental methods section 4.4.3.

In absence of committed Tregs (same parameter regime showed in figure 4.6 A) the model predicts that the average time τ_{foxp3} that any Tregs would express Foxp3, before losing it, is 42 days in lymphodeficient host, and 45 days in physiologic condition (see the dot labelled with b in figure 4.8 A). Then, for increasing values of frequency of committed Tregs, we found that, in lymphodeficient condition, Foxp3 mean residence time decreases (figure 4.8 A, in yellow). Nevertheless, in lymphoreplete condition, the relations was not as clear as compared to lymphopenia, due to an increasing spread in the values found for τ_{foxp3} (figure 4.8 A, in blue), as committed Tregs frequency increases. In fact, as it can be appreciated in figure 4.8 A, for the extreme case of 28% of committed Tregs within Foxp3$^+$ cells, we found the maximum and the minimum value of τ_{foxp3} among all the values (respectively dots c and d in 4.8 A). For the parameter regime showed in figure 4.6 B, the mean residence Foxp3 was found to be infinite, while we found another parameter regime whose corresponding mean residence Foxp3 time was 32.8 days. Nevertheless, in both cases the Foxp3 mean residence time in lymphopenic condition, was 30 days. Results are summarized in table 4.1.

We have just said that, for some values of frequency of committed Tregs,

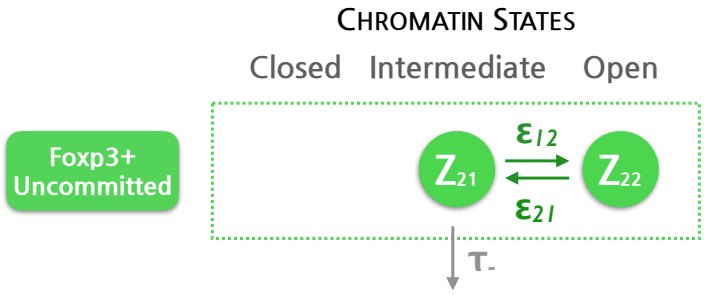

Figure 4.7: **Foxp3 mean residence time.** Cluster of states of uncommitted Tregs, and possible transitions. The Foxp3 mean resident time is defined as the time that any uncommitted Treg (no matter whether in state Z_{21} or Z_{22}) would spend in the cluster of states $\{Z_{21}, Z_{22}\}$, before losing Foxp3 expression.

	no committed Tregs	**committed Tregs (28% of Foxp3⁺ cells)** (I)	(II)
Lymphoreplete	45.5	32.8	forever
Lymphodeficient	42.4	30.4	30.2

Table 4.1: **Foxp3 mean residence time.** Foxp3 mean residence time, in days, of the uncommitted Tregs, in physiologic condition and in lymphopenia, in case of no committed Tregs and for two different parameter regimes (I and II), corresponding to maximum amount of committed Tregs: 28% of Foxp3⁺ CD4 T cells. Regime II corresponds to the solution represented in figure figure 4.6 B.

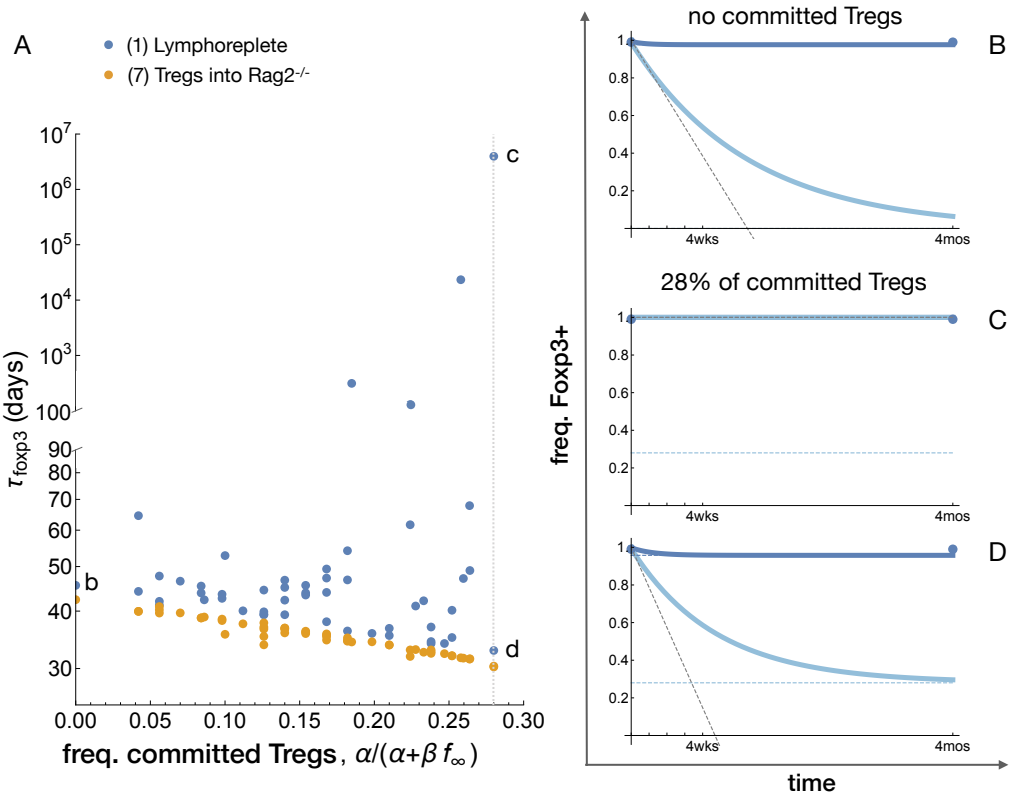

Figure 4.8: **Foxp3 mean residence time *versus* frequency of committed Tregs.** A: Foxp3 mean residence time of the uncommitted Tregs (τ_{foxp3}) plotted against the frequency of committed Tregs within Foxp3⁺ cells ($\alpha/(\alpha+\beta\,\bar{f}_\infty)$), in lymphoreplete conditions (blue) and lymphodeficient condition (yellow). Note the change in scales in the y axis. Letter b in the plot indicates the case of no committed Tregs, letters c and d indicate, respectively, the maximum and the minimum value found for τ_{foxp3} in lymphoreplete condition. They both correspond to the maximum predicted frequency of committed Tregs (28%), as highlighted by the vertical grey dashed line. B, C, D: in dark blue is plotted the frequency of Foxp3⁺ cells, within a cohort of followed Foxp3⁺ CD4 T cells, over time, in lymphoreplete condition. Dots are the experimental data from [Rubtsov et al., 2010], solid lines are the model predictions, dashed lines are the predicted value of equilibrium. In light blue is plotted the frequency over time of cells that, within the same cohort, are Foxp3⁺ and have never lost Foxp3 expression, not even transiently. Solid lines are the model predictions, dashed lines the predicted value of equilibrium, which is the corresponding frequency of committed Tregs. Dashed black line represents the straight line $y(t) = 1 - \tau_{foxp3}^{-1}\,t$. Plot B corresponds to parameter set b in A, C correspond to c and D corresponds to c.

there are several and spread values of τ_{foxp3} compatible with the experimental data in lymphoreplete condition, as can be appreciated by the spread of the blue dots in Figure 4.8 A. Nevertheless, any of this blue dot is associated to a unique function, whose analytical expression is known.

In fact, for any predicted couple of values for the frequency of Foxp3 committed Tregs ($\alpha/(\alpha+\beta\,\bar{f}_{\infty})$) and Foxp3 mean resident time (τ_{foxp3}) of the lymphoreplete condition, there is a unique function that describes the time evolution of the frequency of cells that, within the cohort of Foxp3$^+$ T cells followed in lymphoreplete condition, are Foxp3$^+$ and have never lost Foxp3 expression. Its analytical expression is the following:

$$y(t) = \frac{\alpha + \beta\,\bar{f}_{\infty} e^{-t\,(\tau_{foxp3})^{-1}}}{\alpha + \beta\,\bar{f}_{\infty}}. \tag{4.8}$$

This function, plotted in light blue in figure 4.8 B,C,D, has the tangent in $t = 0$ whose derivative is τ_{foxp3}^{-1} and a plateau whose value is the frequency of predicted committed Tregs, $\alpha/(\alpha+\beta\,\bar{f}_{\infty})$.

4.3 Discussion

In this chapter we proposed an interpretation in terms of cellular mechanism, that quantitatively explains the dynamics observed in vivo in cohorts of Foxp3$^+$ and Foxp3$^-$ cells, in different experimental conditions. We proposed that both stability and lability of Foxp3 expression could be the result of a fast (in terms of hours) end context-dependent transcription translation dynamics combined with a slow (in terms of weeks) and, in principle, context-independent epigenetic dynamics that happen in any peripheral CD4 T cell. We assumed, in particular, that epigenetic dynamics depends on the transcriptional state of the cells and not explicitly on the input the cell receive from the environment.

We used a stochastic model for Foxp3 expression in CD4 T cells, in which both transcription activation and repression and epigenetic remodelling are stochastic process. In our model the model parameters that control the dynamics of either transcription or epigenetic remodeling of the chromatin, can be tuned in a context-dependent or -independent way. We used the model to fit the model to

experimental data.

We found that the different dynamics can be all coherently and quantitatively reproduced, by tuning the ratio between fast Foxp3 transcriptional activation and repression rates across the experiments, while epigenetic rates were kept slow and constant across the experiments.

Also, model predicts that in physiologic condition, where Tregs are stable, the vast majority of Tregs has an active state of chromatin in the Foxp3 locus. This characteristic ensures on the one hand, the stability of Foxp3 expression in lymphoreplete condition, where the context provides stimuli that enhance Foxp3 gene transcription, prevent its repression; on the other hand it explains the slow dynamics of Foxp3 expression loss, observed in those lymphopenic condition where Foxp3 transcription is prevented. Indeed the dynamics of Foxp3 loss, observed in lymphopenia, coincides with the epigenetic remodeling dynamics in Foxp3$^+$ T cell predicted by our model.

4.3.1 Epigenetics as a slow stochastic process

We assumed that the epigenetic state of the Foxp3 locus and its re-modelling over time could be modelled as a step-wise process with stochastic transitions. Before us, others have used this approach [Christogianni et al., 2017, Calado et al., 2006, Josefowicz et al., 2012, Berry et al., 2017, Bintu et al., 2016].

We also assumed that the transitions between epigenetic states could be linked with the transcriptional state of the gene. In fact, on the one hand, we assumed that the epigenetic rates would not depend on the particular context. Therefore, while fitting the model to the data, we looked for the optimum value for epigenetic rates that are conserved across all the experimental environment. Nevertheless, from the other hand, we left the possibility for any epigenetic rate to assume a different and characteristic value. This would tell us whether or not chromatin remodelling depends on the transcriptional state of the cells. Indeed, we found different distributions for the different epigenetic rates. In particular we found that, when Foxp3 is being transcribed, it is much more likely for the chromatin to become more active than the opposite transition.

Also we found that remodelling of the locus that makes it less accessible takes weeks, in case the gene is transcribed, and that this dynamics is the one

that explains the kinetic observed in lymphodeficient host [Duarte et al., 2009].

4.3.2 Peripheral Foxp3 induction

We have observed that, differently from the distributions of the epigenetic remodelling rates in Foxp3$^+$ cells, which were narrowly distributed around the respective expected value, the rates of epigenetic remodelling in Foxp3$^-$ cells were spread (see figure 4.2). This spread is probably due to characteristic of the data concerning Foxp3$^-$ T cell cohort dynamics *in vivo*, that we included in our analysis. These data, differently from the ones on Foxp3$^+$ T cell cohort, do not display variety of behaviors, which indicates much less sensibility to changes of experimental context. Also, none of them show kinetics. In all the cohorts of naive CD4$^+$Foxp3$^-$ T taken in consideration, very small percentage, if not zero, up-regulate Foxp3 in the periphery, no matter whether they are followed in lymphodeficient condition, which is poor in pro-Foxp3 stimuli, or in conditions that provide plenty of pro-Foxp3 stimuli (like natural or "reconstituted" lymphoreplete conditions).

It is known that within CD4SP Foxp3$^-$ thymocytes that have been adoptively transferred into TCR$\beta^{-/-}$ mice, 5 to 10 $\%$ up-regulate Foxp3 in the periphery within 4 weeks [Paiva et al., 2013]. Furthermore, within peripheral CD4$^+$Foxp3$^-$ T cells enriched for Qa-2^{lo} recent thymic emigrants, Foxp3 conversion is observed in around 5$\%$ of cells within 4 weeks upon adoptive transferred into TCR$\beta^{-/-}$ mice [Paiva et al., 2013]. We chose not to include these experiments in our analysis, because we consider thymocytes and recent thymic emigrants as different from mature naive T cells in circulation, mainly due to their developmental stage. All the cohorts of cells included in our meta-analysis are in fact constituted by total peripheral naive CD4 T cells, which are mostly mature cells. Nevertheless, Qa-2^{lo} recent thymic emigrants constitute around 5$\%$ of the CD4$^+$ Foxp3$^-$ peripheral T cells [Paiva et al., 2013]. Also, within their complementary population of CD4$^+$ Foxp3$^-$ Qa-2^{hi} no up-regulation of Foxp3 expression is observed within 4 weeks upon adoptive transferred into TCR$\beta^{-/-}$ mice [Paiva et al., 2013]. Therefore the observation on the recent thymic emigrant as preferential precursors of Tregs differentiated in the periphery, performed in lymphodeficient host, is quantitatively compatible with the observations on total peripheral cells included in our analysis, where less than 2$\%$ of total peripheral CD4$^+$ Foxp3$^-$ up-regulate Foxp3, upon adoptive transfer into lymphodeficient host [Komatsu et al., 2009].

Acquisition in Foxp3 expression in the periphery by naive $CD4^{+}Foxp3^{-}$ has been observed in experiments conduced in DEREG mice. The DEREG mice (DEpletion of REGulatory T cells), are bacterial artificial chromosome (BAC) transgenic mice, whose Foxp3 expressing cells also express the diphtheria toxin (DT) receptors on their surface, allowing for specific depletion of Tregs, upon DT treatment [Lahl and Sparwasser, 2011]. In these mice, it is observed a rebound of Tregs within 6 days: following DT treatment, there is a transient depletion of Tregs, after which the frequency of Foxp3+ Tregs is restored to the level of WT mice. Nevertheless if, shortly after depleting Tregs, $CD4^{+}Foxp3^{-}$ naive T cells are adoptively transferred, then 30 to 40% within the transferred cells, up-regulate Foxp3 [Pratama et al., 2020, Almeida-Santos et al., 2020]. Nevertheless, our current model is not able to reproduce this dynamics. In order to do this, in fact, together with the context dependency of Foxp3 expression, a model is needed that accounts for the population dynamics.

The cross regulation model has already predicted that Tregs mainly compete with Tregs and Teffs mainly compete with Teffs [Carneiro et al., 2007]. Indeed, when Tregs are transferred into lymphoreplete mice, most of them die. Yet, in this case, the ones that to not die remain Tregs [Komatsu et al., 2009]. We incorporated this experiment in our analysis, which lacks the population dynamics, and we interpreted the stability observed as the result of the context which provides enough pro-Foxp3 stimuli. In the same line, when Tregs are simply followed in lymphoreplete environment, Foxp3 expression is stable [Rubtsov et al., 2010]. We also included this experiment in our analysis. Also in this case population dynamics can be neglected, because we expect that in physiologic condition the system is at equilibrium.

When lymphodeficient animals are reconstituted by co-transferring Tregs and Teffs, in proportions that resemble physiologic conditions, Tregs are stable, as a consequence of the richness of pro-Foxp3 stimuli characteristic of the context, yet the up-regulation of Foxp3 in the periphery is almost negligible. The transfer experiment in DEREG mice, seems to suggest that, beside an environment that provides inputs for stable maintenance of Foxp3 expression, it is necessary to ”create space for Tregs” to observe Foxp3 up-regulation in naive CD4 T cells. In fact, when $CD4^{+}Foxp3^{-}$ cells are transferred after Tregs depletion, 30 to 40% up-regulate Foxp3. While donor Teffs would be outcompeted by endogenous Teffs,

not only the environment favours Foxp3 up-regulation (the host is replete with endogenous Teff cells) but also the newly converted Tregs do not have endogenous Tregs to compete with, and can freely proliferate.

We did not include this experiment in this chapter, given that our current model do not account for cells proliferation. Yet it would be interesting to further investigate on this, using a model that integrates population dynamics and cellular dynamics.

4.3.3 Preferential stability of Foxp3 expression in subpopulation of peripheral Foxp3$^+$ is compatible with our analysis

When we assembled the data from literature to perform the meta-analysis, we adopted a criteria of homogeneity among the cell cohorts in which Foxp3 expression is tracked over time. The aim was to ensure a fair comparison among experimental data in different contexts. For this reason, in all the experiment chosen, the cohorts are constituted by total peripheral CD4 T cells, either Foxp3$^+$ or Foxp3$^-$, belonging to adult lymphoreplete mice at steady state. Therefore, we could not include in our analysis experimental data in which the frequency of Foxp3$^+$ cells was assessed in cell cohorts sorted following different criteria, for instance sorting subpopulation within the total peripheral Foxp3$^+$ or Foxp3$^-$ CD4 T cells.

Yet, the tracking of Foxp3 expression in specific CD4 T cell subpopulations has shown the existence of subpopulations of Foxp3$^-$ CD4 T cells which preferentially up-regulate Foxp3 in the periphery, such as the already mentioned case of the recent thymic emigrants [Paiva et al., 2013]. Similarly, among peripheral Foxp3$^+$ T cells, there are subpopulations that exhibit more stable Foxp3 expression, if compared with others. For instance, using genetic fate mapping, which allows to sort Foxp3$^+$ T cells that have recently initiated Foxp3 transcription and to distinguish them from the ones that have expressed Foxp3 for some time, it has been shown that these two subpopulations are different in terms of stability of Foxp3 expression [Miyao et al., 2012]. When peripheral newly developed Foxp3$^+$, sorted as GFP$^+$RFP$^{-/lo}$ from Foxp3$^{GFP\text{-}Cre}\times$ROSA26RFP mice, are adoptively transferred into Rag1$^{-/-}$ mice, together with naive Foxp3$^-$ CD4 T cells (in proportion of 1:4), half of them lose Foxp3 expression within 5 weeks [Miyao et al., 2012]. Yet, if the same experiment is performed with their

complementary subpopulation of peripheral resident Foxp3$^+$ cells, sorted as GFP$^+$RFP^{hi}, 97% of them keep Foxp3 expression.

We used this data to cross-validate our analysis. When total peripheral CD4$^+$ cells are sorted from steady state Foxp3$^{GFP\text{-}Cre}\times$ROSA26RFP mice, 90% of them are GFP$^+$RFP^{hi} and 10% of them are GFP$^+$RFP$^{-/lo}$ [Miyao et al., 2012]. So in a hypothetical experiment in which total peripheral Foxp3$^+$ and Foxp3$^-$ CD4 T cells are co-transferred in lymphodeficient host in proportion of 1:4, we can imagine the Foxp3$^+$ T cell cohort being constituted by 90% of resident Foxp3$^+$ cells and the remaining by newly generated Foxp3$^+$ cells. In this case, the frequency of cells maintaining Foxp3 expression within the total Foxp3$^+$ cohort is expected to be 93%. This frequency is compatible with the correspondent frequencies found in analogous co-transfer experiment and with the model predictions. In fact, although the data set used in our analysis does not include co-transfer in proportion 1:4, it includes co-transfer performed with proportions 1:10 and 1:1. We expect, in accordance with our model prediction, the case 1:4 to be an intermediate case between the case 1:10 and 1:1 in which the frequency of cells maintaining Foxp3 expression is 97% at 4 weeks with a plateau of 97% and 92% at 4 weeks with a plateau of 91%, respectively. Finally, the loss of Foxp3 observed along 5 weeks within the subpopulation of newly generated Foxp3$^+$ is compatible with the prediction of Foxp3 mean residence time.

4.3.4 Foxp3 mean residence time

The Foxp3 mean residence time describes the average time during which any uncommitted Tregs would maintain Foxp3 expression, before losing it. As the definition suggests and the formula in equation 4.7 confirms, its value is function of the epigenetic remodelling rates of a Foxp3 transcribing/translating cell (in our model, rates $\varepsilon_{12}, \varepsilon_{21}$), together with the rate of transcription/ translation repression (τ_-).

We expected its value also to vary together with the frequency of committed Tregs, which in no case can lose Foxp3 expression. In fact, the bigger the fraction of committed Tregs within a cohort of Foxp3$^+$ T cells, the smaller the fraction of cells than, within the same cohort, can lose Foxp3 expression over time. Consequently, the smaller the fraction of cells able to loose Foxp3 expression, the faster the dynamics of these cells, at least in lymphodeficient conditions where

loss of Foxp3 expression is observed over time. When looking at the Foxp3 resident time in lymphopoenia (figure 4.8), we actually found a negative correlation between τ_{foxp3} and the frequency of committed Tregs.

In physiologic condition, we found something slightly different. As expected, the Foxp3 mean residence time in lymphoreplete condition, is always longer than the one in case Tregs are transferred into lymphodeficient host. Apart from that, first of all, for any given frequency of committed Tregs, we found more than one possible value, for τ_{foxp3}, compatible with Foxp3 stability. Moreover, we found that the spread of these values increases as the frequency of committed Tregs increases (see figure 4.8). Finally, while we found some parameter regimes characterized by values of τ_{foxp3} very different between physiology and lymphopenia, in other parameter regimes, the Foxp3 residence time was very similar in the two different and extreme contexts.

Let us remember that, because we assumed conserved epigenetic remodelling rates across experimental contexts, what determines the different values of τ_{foxp3} between physiology and lymphopenia, is the rate τ_{-} of transcription/ translation repression. This implies that in those parameter regimes that share similar Foxp3 residence time in both physiology and lymphopenia, uncommitted Tregs lose Foxp3 expression over time, even in lymphoreplete hosts. In those cases, values of τ_{foxp3} range from 32 to 45 days. The model therefore predicts that, due the context, the loss is transient. In particular, the reacquisition of Foxp3 expression by those uncommitted exTregs is so fast that, within a cohort of Foxp3$^+$ T cells followed over time, as in the experiment (1) in lymphoreplete condition [Rubtsov et al., 2010], at any moment almost all the cells are found Foxp3$^+$.

On the other hand we have also found parameter regimes in which the mean resident time of Foxp3 is very different between physiologic and lymphopenic conditions. If this was the case, it would mean that, in lymphoreplete condition there are no Treg that lose Foxp3 expression, not even transiently. Therefore, in case we were able to label and quantify *in vivo* transient Foxp3 expression within cohort of originally Foxp3$^+$ T cells, we would not find any of them.

4.3.5 Distinguishing between scenarios

Our analysis suggests that data support the possibility of Tregs being a heterogeneous population of cells.

An heterogeneity model has already been proposed, according to which Foxp3$^+$CD4$^+$ T cells are constituted by committed Tregs and by a minor population of plastic cells with the potentiality to convert into Teffs [Komatsu et al., 2009, Miyao et al., 2012]. According to the heterogeneity model, the distinction between committed Tregs and the cells that exhibit promiscuous Foxp3 expression, without being Tregs, resides in the demethylated status of the TSDR which ensures the commitment, irrespective of Foxp3 expression.

In a slightly different way, our analysis focuses on Foxp3 expression. When we talk about heterogeneity within cells, we refer to the property of the cell to committed to Foxp3 expression. In fact, our model account for cells that are committed to Foxp3 expression and others in which Foxp3 expression is context-dependent. While the first subpopulation of cells, committed to Foxp3 expression, are characterized by fully active Foxp3 locus, the cells that belong to the second population can be found in different phenotypic state. More specifically, each cell can switch stochastically among the phenotypic states in response to external cues, by remodelling the Foxp3 locus and by changing its transcriptional activity.

The confrontation of the model with experimental data, suggests the compatibility of the data with Tregs heterogeneity. Nevertheless, in this case, the analysis suggests that only a minor fraction of Tregs would be committed (up to 28$\%$ of them), while the majority would express Foxp3 either stably or transiently, in a context-dependent way. Also, the model prediction accounts for the possibility of Tregs being a homogeneous pool of CD4 T cells in which Foxp3 expression is stable or labile depending on the context. Between these two extreme scenario, there are all the intermediate case in which cells committed to Foxp3 expression represent anything between 0 and 28$\%$ of Tregs. All these scenarios are compatible with data.

As already mentioned, we do not have experimental evidences that could allow us to distinguish among these scenarios. Nevertheless, the predictions on Foxp3 residence time can shed some light in this regard. In case we were able to asses the frequency of cells within Foxp3$^+$ T cell cohort that, at any time, have stopped expressing Foxp3, even transiently, we could compare its dynamics with the model prediction (cfr. Figure 4.8B). This would allow to quantify the Foxp3 mean residence time whose value characterizes each different scenario.

4.4 Methods

4.4.1 Stochastic model for the uncommitted CD4 T cells.

The model for the compartment of uncommitted CD4 T cells, introduced in section 4.2.1, is described by a continuous time Markov chain with four states: Z_{10}, Z_{11}, Z_{21}, Z_{22}. Its solution gives the probability over time for a cell in this compartment to be in any of the four states. Let us define the probability vector:

$$\underline{z}(t) = (z_{10}, z_{11}, z_{21}, z_{22})(t) = (z_{10}(t), z_{11}(t), z_{21}(t), z_{22}(t)), \tag{4.9}$$

where $z_{ij}(t)$, for $i \in \{1, 2\}, j \in \{0, 1, 2\}$, is the probability for a cell to be in state Z_{ij} at time t. Note that we used roman capital letters to indicate the states, whereas the probabilities of being in the respective states are indicated by small italic letters. The model is described by the following system of ordinary differential equation:

$$\dot{\underline{z}} = \underline{z}\,\underline{\Lambda}, \tag{4.10}$$

where $\dot{\underline{z}}$ indicates the time derivative of the vector $\underline{z}(t)$ and $\underline{\Lambda}$ is the infinitesimal generator matrix for the model. $\underline{\Lambda}$ is a 4×4 matrix defined on $\mathbb{R}^+$ as following:

$$\underline{\Lambda} = \begin{pmatrix} -\varepsilon_{01} & \varepsilon_{01} & 0 & 0 \\ \varepsilon_{10} & -(\varepsilon_{10} + \tau_+) & \tau_+ & 0 \\ 0 & \tau_- & -(\tau_- + \varepsilon_{12}) & \varepsilon_{12} \\ 0 & 0 & \varepsilon_{21} & -\varepsilon_{21} \end{pmatrix}. \tag{4.11}$$

Given the rates of state transitions and the initial probability distributions, defined as $\underline{z}(0) = (z^0_{01}, z^0_{10}, z^0_{12}, z^0_{21})$, the solution of the Markov chain model is unique and it is the following:

$$\underline{z}(t) = \underline{z}(0) \cdot e^{\underline{\Lambda} t}, \tag{4.12}$$

where $e^{\underline{\Lambda} t}$ is the matrix exponential and $\cdot$ indicates the scalar product.

Finally, the probability of an uncommitted CD4 T cell to be Foxp3 $^+$ is defined

as:

$$f(t) = z_{21}(t) + z_{22}(t). \tag{4.13}$$

The complete expression for $f(t)$ can be written as:

$$f(t) = x_0 + \sum_{j=1}^{3} x_j e^{r_j t}, \tag{4.14}$$

where r_k, for $k = 1, 2, 3$ are the three root of the following polynomial of degree three:

$$\begin{aligned} q(x) \quad = \quad & \varepsilon_{10}\varepsilon_{21}\vartheta + \varepsilon_{01}\varepsilon_{21}\vartheta - \varepsilon_{10}\varepsilon_{21}\vartheta\rho + \varepsilon_{01}\varepsilon_{12}\vartheta\rho + ... \\ & (\varepsilon_{10}\varepsilon_{21} + \varepsilon_{01}\varepsilon_{21} + \varepsilon_{10}\varepsilon_{12} + \varepsilon_{01}\varepsilon_{12} + \varepsilon_{10}\vartheta + \varepsilon_{01}\vartheta + ... \\ & \varepsilon_{21}\vartheta - \varepsilon_{10}\vartheta\rho + \varepsilon_{12}\vartheta\rho)x + (\varepsilon_{10} + \varepsilon_{01} + \varepsilon_{21} + \varepsilon_{12} + \vartheta)x^2 + x^3. \end{aligned} \tag{4.15}$$

In case the initial conditions are $(0, 0, 1 - z_{21}^0, z_{21}^0)$ the expression for the constants x_j, $j = 0, 1, 2, 3$, are:

$$\begin{aligned} x_0 \quad = \quad & \frac{-r_1 r_2 r_3 - r_1 r_2 \tau_- - r_1 r_3 \tau_- - r_2 r_3 \tau_- - r_1 \tau_-^2 - r_2 \tau_-^2 - r_3 \tau_-^2 - \tau_-^3}{D_0} + ... \\ & \frac{-r_1 \tau_- \tau_+ - r_2 \tau_- \tau_+ - r_3 \tau_- \tau_+ - 2\tau_-^2 \tau_+ - \tau_- \tau_+^2 - \tau_- \tau_+ \varepsilon_{10} - r_1 \tau_- \varepsilon_{12}}{D_0} + ... \\ & \frac{-r_2 \tau_- \varepsilon_{12} - r_3 \tau_- \varepsilon_{12} - 2\tau_-^2 \varepsilon_{12} - \tau_- \tau_+ \varepsilon_{12} - \tau_- \varepsilon_{12}^2 - \tau_- \varepsilon_{12} \varepsilon_{21} + r_1 r_2 \tau_- z_{22}}{D_0} + ... \\ & \frac{+r_1 r_3 \tau_- z_{22} + r_2 r_3 \tau_- z_{22} + r_1 \tau_-^2 z_{22} + r_2 \tau_-^2 z_{22} + r_3 \tau_-^2 z_{22} + \tau_-^3 z_{22}}{D_0} + ... \\ & \frac{+r_1 \tau_- \tau_+ z_{22} + r_2 \tau_- \tau_+ z_{22} + r_3 \tau_- \tau_+ z_{22} + 2\tau_-^2 \tau_+ z_{22} + \tau_- \tau_+^2 z_{22} + \tau_- \tau_+ \varepsilon_{10} z_{22}}{D_0} + ... \\ & \frac{+r_1 \tau_- \varepsilon_{12} z_{22} + r_2 \tau_- \varepsilon_{12} z_{22} + r_3 \tau_- \varepsilon_{12} z_{22} + 2\tau_-^2 \varepsilon_{12} z_{22} + \tau_- \tau_+ \varepsilon_{12} z_{22} + \tau_- \varepsilon_{12}^2 z_{22}}{D_0} + ... \\ & \frac{+r_1 \tau_- \varepsilon_{21} z_{22} + r_2 \tau_- \varepsilon_{21} z_{22} + r_3 \tau_- \varepsilon_{21} z_{22} + \tau_-^2 \varepsilon_{21} z_{22} + \tau_- \tau_+ \varepsilon_{21} z_{22}}{D_0} + ... \\ & \frac{+2\tau_- \varepsilon_{12} \varepsilon_{21} z_{22} + \tau_- \varepsilon_{21}^2 z_{22}}{D_0}, \end{aligned}$$

$$
\begin{aligned}
x_1 \;=\; & \frac{-r_2 r_3 \tau_- - r_2 \tau_-^2 - r_3 \tau_-^2 - \tau_-^3 - r_2 \tau_- \tau_+ - r_3 \tau_- \tau_+ - 2\tau_-^2 \tau_+ - \tau_- \tau_+^2 - \tau_- \tau_+ \varepsilon_{10}}{D_1} + \ldots \\
& \frac{-r_2 \tau_- \varepsilon_{12} - r_3 \tau_- \varepsilon_{12} - 2\tau_-^2 \varepsilon_{12} - \tau_- \tau_+ \varepsilon_{12} - \tau_- \varepsilon_{12}^2 - \tau_- \varepsilon_{12} \varepsilon_{21} + r_2 r_3 \tau_- z_{22}}{D_1} + \ldots \\
& \frac{+r_2 \tau_-^2 z_{22} + r_3 \tau_-^2 z_{22} + \tau_-^3 z_{22} + r_2 \tau_- \tau_+ z_{22} + r_3 \tau_- \tau_+ z_{22} + 2\tau_-^2 \tau_+ z_{22} + \tau_- \tau_+^2 z_{22}}{D_1} + \ldots \\
& \frac{+\tau_- \tau_+ \varepsilon_{10} z_{22} + r_2 \tau_- \varepsilon_{12} z_{22} + r_3 \tau_- \varepsilon_{12} z_{22} + 2\tau_-^2 \varepsilon_{12} z_{22} + \tau_- \tau_+ \varepsilon_{12} z_{22} + \tau_- \varepsilon_{12}^2 z_{22}}{D_1} + \ldots \\
& \frac{+r_2 \tau_- \varepsilon_{21} z_{22} + r_3 \tau_- \varepsilon_{21} z_{22} + \tau_-^2 \varepsilon_{21} z_{22} + \tau_- \tau_+ \varepsilon_{21} z_{22} + 2\tau_- \varepsilon_{12} \varepsilon_{21} z_{22} + \tau_- \varepsilon_{21}^2 z_{22}}{D_1},
\end{aligned}
$$

$$
\begin{aligned}
x_2 \;=\; & \frac{-r_1 r_3 \tau_- - r_1 \tau_-^2 - r_3 \tau_-^2 - \tau_-^3 - r_1 \tau_- \tau_+ - r_3 \tau_- \tau_+ - 2\tau_-^2 \tau_+ - \tau_- \tau_+^2 - \tau_- \tau_+ \varepsilon_{10}}{D_2} + \ldots \\
& \frac{-r_1 \tau_- \varepsilon_{12} - r_3 \tau_- \varepsilon_{12} - 2\tau_-^2 \varepsilon_{12} - \tau_- \tau_+ \varepsilon_{12} - \tau_- \varepsilon_{12}^2 - \tau_- \varepsilon_{12} \varepsilon_{21} + r_1 r_3 \tau_- z_{22}}{D_2} + \ldots \\
& \frac{+r_1 \tau_-^2 z_{22} + r_3 \tau_-^2 z_{22} + \tau_-^3 z_{22} + r_1 \tau_- \tau_+ z_{22} + r_3 \tau_- \tau_+ z_{22} + 2\tau_-^2 \tau_+ z_{22} + \tau_- \tau_+^2 z_{22}}{D_2} + \ldots \\
& \frac{+\tau_- \tau_+ \varepsilon_{10} z_{22} + r_1 \tau_- \varepsilon_{12} z_{22} + r_3 \tau_- \varepsilon_{12} z_{22} + 2\tau_-^2 \varepsilon_{12} z_{22} + \tau_- \tau_+ \varepsilon_{12} z_{22} + \tau_- \varepsilon_{12}^2 z_{22}}{D_2} + \ldots \\
& \frac{+r_1 \tau_- \varepsilon_{21} z_{22} + r_3 \tau_- \varepsilon_{21} z_{22} + \tau_-^2 \varepsilon_{21} z_{22} + \tau_- \tau_+ \varepsilon_{21} z_{22} + 2\tau_- \varepsilon_{12} \varepsilon_{21} z_{22} + \tau_- \varepsilon_{21}^2 z_{22}}{D_2},
\end{aligned}
$$

$$
\begin{aligned}
x_3 \;=\; & \frac{-r_1 r_2 \tau_- - r_1 \tau_-^2 - r_2 \tau_-^2 - \tau_-^3 - r_1 \tau_- \tau_+ - r_2 \tau_- \tau_+ - 2\tau_-^2 \tau_+ - \tau_- \tau_+^2 - \tau_- \tau_+ \varepsilon_{10}}{D_3} + \ldots \\
& \frac{-r_1 \tau_- \varepsilon_{12} - r_2 \tau_- \varepsilon_{12} - 2\tau_-^2 \varepsilon_{12} - \tau_- \tau_+ \varepsilon_{12} - \tau_- \varepsilon_{12}^2 - \tau_- \varepsilon_{12} \varepsilon_{21} + r_1 r_2 \tau_- z_{22}}{D_3} + \ldots \\
& \frac{+r_1 \tau_-^2 z_{22} + r_2 \tau_-^2 z_{22} + \tau_-^3 z_{22} + r_1 \tau_- \tau_+ z_{22} + r_2 \tau_- \tau_+ z_{22} + 2\tau_-^2 \tau_+ z_{22} + \tau_- \tau_+^2 z_{22}}{D_3} + \ldots \\
& \frac{+\tau_- \tau_+ \varepsilon_{10} z_{22} + r_1 \tau_- \varepsilon_{12} z_{22} + r_2 \tau_- \varepsilon_{12} z_{22} + 2\tau_-^2 \varepsilon_{12} z_{22} + \tau_- \tau_+ \varepsilon_{12} z_{22} + \tau_- \varepsilon_{12}^2 z_{22}}{D_3} + \ldots \\
& \frac{+r_1 \tau_- \varepsilon_{21} z_{22} + r_2 \tau_- \varepsilon_{21} z_{22} + \tau_-^2 \varepsilon_{21} z_{22} + \tau_- \tau_+ \varepsilon_{21} z_{22} + 2\tau_- \varepsilon_{12} \varepsilon_{21} z_{22} + \tau_- \varepsilon_{21}^2 z_{22}}{D_3},
\end{aligned}
$$

where:

$$
\begin{aligned}
D_0 &= \tau_+\varepsilon_{01}\varepsilon_{12} + \tau_-\varepsilon_{01}\varepsilon_{21} + \tau_+\varepsilon_{01}\varepsilon_{21} + \tau_-\varepsilon_{10}\varepsilon_{21},\\
D_1 &= 4r_1^3 + 3r_1^2\tau_- + 3r_1^2\tau_+ + 3r_1^2\varepsilon_{01} + 2r_1\tau_-\varepsilon_{01} + 2r_1\tau_+\varepsilon_{01} + 3r_1^2\varepsilon_{10} + 2r_1\tau_-\varepsilon_{10} + ...\\
&\quad +3r_1^2\varepsilon_{12} + 2r_1\tau_+\varepsilon_{12} + 2r_1\varepsilon_{01}\varepsilon_{12} + \tau_+\varepsilon_{01}\varepsilon_{12} + 2r_1\varepsilon_{10}\varepsilon_{12} + 3r_1^2\varepsilon_{21} + 2r_1\tau_-\varepsilon_{21} + ...\\
&\quad +2r_1\tau_+\varepsilon_{21} + 2r_1\varepsilon_{01}\varepsilon_{21} + \tau_-\varepsilon_{01}\varepsilon_{21} + \tau_+\varepsilon_{01}\varepsilon_{21} + 2r_1\varepsilon_{10}\varepsilon_{21} + \tau_-\varepsilon_{10}\varepsilon_{21},\\
D_2 &= 4r_2^3 + 3r_2^2\tau_- + 3r_2^2\tau_+ + 3r_2^2\varepsilon_{01} + 2r_2\tau_-\varepsilon_{01} + 2r_2\tau_+\varepsilon_{01} + 3r_2^2\varepsilon_{10} + 2r_2\tau_-\varepsilon_{10} + ...\\
&\quad +3r_2^2\varepsilon_{12} + 2r_2\tau_+\varepsilon_{12} + 2r_2\varepsilon_{01}\varepsilon_{12} + \tau_+\varepsilon_{01}\varepsilon_{12} + 2r_2\varepsilon_{10}\varepsilon_{12} + 3r_2^2\varepsilon_{21} + 2r_2\tau_-\varepsilon_{21} + ...\\
&\quad +2r_2\tau_+\varepsilon_{21} + 2r_2\varepsilon_{01}\varepsilon_{21} + \tau_-\varepsilon_{01}\varepsilon_{21} + \tau_+\varepsilon_{01}\varepsilon_{21} + 2r_2\varepsilon_{10}\varepsilon_{21} + \tau_-\varepsilon_{10}\varepsilon_{21},\\
D_3 &= 4r_3^3 + 3r_3^2\tau_- + 3r_3^2\tau_+ + 3r_3^2\varepsilon_{01} + 2r_3\tau_-\varepsilon_{01} + 2r_3\tau_+\varepsilon_{01} + 3r_3^2\varepsilon_{10} + 2r_3\tau_-\varepsilon_{10} + ...\\
&\quad +3r_3^2\varepsilon_{12} + 2r_3\tau_+\varepsilon_{12} + 2r_3\varepsilon_{01}\varepsilon_{12} + \tau_+\varepsilon_{01}\varepsilon_{12} + 2r_3\varepsilon_{10}\varepsilon_{12} + 3r_3^2\varepsilon_{21} + 2r_3\tau_-\varepsilon_{21} + ...\\
&\quad +2r_3\tau_+\varepsilon_{21} + 2r_3\varepsilon_{01}\varepsilon_{21} + \tau_-\varepsilon_{01}\varepsilon_{21} + \tau_+\varepsilon_{01}\varepsilon_{21} + 2r_3\varepsilon_{10}\varepsilon_{21} + \tau_-\varepsilon_{10}\varepsilon_{21}.
\end{aligned}
$$

4.4.2 Fitting the model to the experimental data.

To fit the model solution defined in section 4.2.1 to the experimental data we minimized the score function S which is given by the residuals between the model prediction and the experimental data set across ten different experimental conditions.

Under the assumption that the epigenetic remodeling rates $\varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}$ are context-independent, while the transcription activation and repression rates, τ_+ and τ_- depend on the experimental context, we defined the following context-dependent rates:

$$
{}^i\tau_+ \quad {}^i\tau_- \qquad i = 1, 2, ..., 10, \tag{4.16}
$$

where i refers to the i-th experimental setting. Accordingly, we defined the ratio ${}^i\rho$, as a generalization of the one in equation 4.4:

$$
{}^i\rho = \frac{{}^i\tau_+}{{}^i\tau_+ + {}^i\tau_-} \qquad i = 1, 2, ..., 10. \tag{4.17}
$$

Finally, for convenience, we defined also the following context-independent

variable:

$$\vartheta = {}^{i}\tau_{+} + {}^{i}\tau_{-} \qquad i = 1, 2, ..., 10. \tag{4.18}$$

Defining with:

$${}^{i}f(t) = {}^{i}f(t, \alpha, \beta, \varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}, \vartheta, {}^{i}\varrho), \qquad i = 1, 2, ..., 10. \tag{4.19}$$

the model prediction for the frequency over time of Foxp3^{+} cells within the cell cohort followed in the i-th experimental setting, then the score function was defined as following:

$$S = \sum_{i=1}^{10} \sum_{\tau \in T_i} \left({}^{i}f(\tau) - {}^{i}\hat{f}(\tau) \right)^2, \tag{4.20}$$

with ${}^{i}\hat{f}(\tau)$ being the i-th experimental data set, where T_i for $i = 1, ..., 10$, is the set of the discrete time points τ of the i-th experiment.

We the fixed values for the parameter α, uniformly distributed within the interval

$$I_\alpha = [0, 0.15], \tag{4.21}$$

and for each fixed value, we minimized the score function S with respect to the parameters $\beta, \varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}, \vartheta, {}^{1}\varrho, {}^{2}\varrho, ..., {}^{10}\varrho$.

The minimization was subjected to the following constraints:

1. $0 \leq \alpha + \beta \leq 1$;
2. $0 \leq \alpha + \beta\, f_\infty \leq 0.15$;
3. $\max_{\{i=1,...,10\}}\{\theta\ {}^{i}\rho\} > 8 \max\{\varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}\}$;
4. $\max_{\{i=1,...,10\}}\{\theta\,(1 - {}^{i}\rho)\} > 8 \max\{\varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{12}\}$.

The symbol f_∞ in constraint 2 represents the frequency of equilibrium in physiologic condition, of the uncommitted Foxp3^{+}. The constraints, therefore, translates the well known result that in physiologic condition 10-15$\%$ of CD4 T cells are found to be Foxp3^{+}. Constraints 2 and 3 ensure the transcription We explored the parameter space choosing random initial values for the parameters

ranging in specific intervals:

$$\begin{aligned} \beta \in I_\beta &= [0,1], \\ \varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{21} \in I_\varepsilon &= [0,3], \\ \vartheta \in I_\vartheta = [0,100], \quad {}^i\varrho \in I_\varrho &= [0,1], \quad i = 1,2,...,10; \end{aligned} \tag{4.22}$$

We used the "Principal Axis" algorithm [Brent, 2013] to minimize the score function S. To avoid local minima, we fixed a threshold for the maximum score value allowed, which was equal to $th = 0.01878$.

4.4.3 Foxp3 mean residence time, derivation of the formula

In this section we derive the formula of the Foxp3 mean residence time. Let us recall that, because according to our model a cell is Foxp3$^+$ provided it is either is state Z_{21} or in state Z_{22}, the Foxp3 mean residence time, τ_{foxp3}, is the expected time the cell remains in the cluster of states:

$$F^+ = \{Z_{21}, Z_{22}\}, \tag{4.23}$$

once it gets there. We will here derive a general formula, assuming that the cell can leave the cluster (therefore becoming Foxp3$^-$) through any of the two states of the cluster, respectively with rate τ_{-1} (if it leaves through the state Z_{21}), or τ_{-2} (in case it leaves through the state Z_{22}). The formula used in the main text can then be derived by setting $\tau_{-1} = \tau_-$ and $\tau_{-2} = 0$.

From its definition, it follows that the Foxp3 mean residence time is computed as:

$$\mathbb{E}[X_+] = \int_0^\infty t\, f_+(t)\, dt \tag{4.24}$$

where $f(t)$ is the density probability function, of the continuous time random variable $X_+ = X_+(t)$, describing the probability the cell leaves the cluster F^+ at time t, given that the cell was in the cluster at time $t = 0$, no matter in which of the two states.

At any given time t, the cell can leave the cluster through one out of an infinite number of paths. Each path has an associated probability, that decreases together with the number of transitions involved. We can group those density

probability functions into four groups, defining the following four density probability functions,

$$f_{ij}(t) \qquad i,j \in \{1,2\} \tag{4.25}$$

each of which defined as the density probability that the cell leaves the cluster at time t from the state Z_{2j}, given that at time $t = 0$ the cell was in state Z_{2i}, times the probability p_i that the cell was in the state Z_{2i} at time $t = 0$. Now, each $f_{ij}(t)$ is the sum of a serie of function, given that there are infinite paths that start from state Z_{2i} and leave the cluster from state Z_{2j}. Then the density probability function $f_+(t)$ of interest is defined as:

$$f_+(t) = \sum_{i,j \in 1,2} f_{ij}(t). \tag{4.26}$$

In order to define the $f_{ij}(t)$, for $i,j \in \{1,2\}$ let us first define λ_i (for $i = 1,2$) as the sum of the rates that go out from the state Z_{2i} :

$$\lambda_1 = \tau_{1-} + \varepsilon_{12} \tag{4.27}$$

$$\lambda_2 = \tau_{2-} + \varepsilon_{21}. \tag{4.28}$$

Then we can define the density function $f_i(t)$ (for $i = 1,2$) describing the probability of leaving the state Z_{2i} at time t as following:

$$f_i(t) = \lambda_i \, e^{-\lambda_i t}. \tag{4.29}$$

Also we define the operator $\mathscr{F}$ as following:

$$\begin{aligned} \mathscr{F} : C[0,\infty) &\to C[0,\infty) \\ g &\mapsto \mathscr{F}(g) = (f_1 * f_2) * g \end{aligned} \tag{4.30}$$

where $*$ is the product of convolution.

The operator $\mathscr{F}$ can be applied to the identity function i. In that case we write:

$$\mathscr{F}(i) = \mathscr{F} = f_1 * f_2. \tag{4.31}$$

Also, the operator $\mathscr{F}$ can be repeatedly applied, in the sense that it can be com-

posed with itself:

$$\mathscr{F}^{\mathsf{n}}(g) = (\underbrace{\mathscr{F} \circ \cdots \circ \mathscr{F}}_{\text{n times}})(g) = \underbrace{\mathscr{F}(\ldots(\mathscr{F}))}_{\text{n times}}(g) \qquad \text{n=0,1,2,...} \tag{4.32}$$

where

$$\mathscr{F}^{0}(g) = I(g) = g. \tag{4.33}$$

This said, we define the four aforementioned density probability functions f_{ij} for $i, j \in \{1, 2\}$ as following:

$$\begin{aligned}
f_{11} &= p_1 \frac{\tau_{1-}}{\lambda_1} \sum_{n=0}^{\infty} \left(\frac{\varepsilon_{12}\varepsilon_{21}}{\lambda_1\lambda_2} \right)^n \mathscr{F}^n(f_1)(t); \\
f_{12} &= p_1 \frac{\varepsilon_{12}\tau_{2-}}{\lambda_1\lambda_2} \sum_{n=0}^{\infty} \left(\frac{\varepsilon_{12}\varepsilon_{21}}{\lambda_1\lambda_2} \right)^n \mathscr{F}^{n+1}(t); \\
f_{21} &= p_2 \frac{\tau_{1-}\varepsilon_{21}}{\lambda_1\lambda_2} \sum_{n=0}^{\infty} \left(\frac{\varepsilon_{12}\varepsilon_{21}}{\lambda_1\lambda_2} \right)^n \mathscr{F}^{n+1}(t); \\
f_{22} &= p_2 \frac{\tau_{2-}}{\lambda_2} \sum_{n=0}^{\infty} \left(\frac{\varepsilon_{12}\varepsilon_{21}}{\lambda_1\lambda_2} \right)^n \mathscr{F}^n(f_2)(t).
\end{aligned} \tag{4.34}$$

4.4.3.1 Sum of the serie in the Laplace s-Space

To compute the sum of the four series of functions that define f_{ij}, we take advantage of the following properties of the Laplace transform $\mathcal{L}_s(\cdot)$:

- $\mathcal{L}_s$ is a linear operator;
- $\mathcal{L}_s(f * g) = \mathcal{L}_s(f)\,\mathcal{L}_s(g)$;
- $\mathcal{L}_s(\lambda e^{-\lambda t}) = \frac{\lambda}{s+\lambda}$.

In the Laplace space we get:

$$\begin{aligned}
\mathcal{L}_s(f_{11}) &= p_1 \frac{\tau_{1-}}{s+\lambda_1} \sum_{n=0}^{\infty} \left(\frac{\varepsilon_{12}\varepsilon_{21}}{(s+\lambda_1)(s+\lambda_2)} \right)^n \overset{*}{=} \\
&= p_1 \tau_{1-} \frac{s+\lambda_2}{(s+\lambda_1)(s+\lambda_2) - \varepsilon_{12}\varepsilon_{21}}; \\
\mathcal{L}_s(f_{12}) &= p_1 \varepsilon_{12} \tau_{2-} \frac{1}{(s+\lambda_1)(s+\lambda_2) - \varepsilon_{12}\varepsilon_{21}}; \\
\mathcal{L}_s(f_{21}) &= p_2 \tau_{1-} \varepsilon_{21} \frac{1}{(s+\lambda_1)(s+\lambda_2) - \varepsilon_{12}\varepsilon_{21}}; \\
\mathcal{L}_s(f_{22}) &= p_2 \tau_{2-} \frac{s+\lambda_1}{(s+\lambda_1)(s+\lambda_2) - \varepsilon_{12}\varepsilon_{21}};
\end{aligned} \tag{4.35}$$

Where the equality (*) holds because the serie is a geometric one, with ratio $0 < q < 1$.

The transform of the density probability distribution $f_+(t)$ in the Laplace space is therefore:

$$\mathcal{L}_s(f_+) = \frac{p_1(\lambda_1\tau_{1-} + \varepsilon_{12}\tau_{2-}) + p_2(\lambda_2\tau_{2-} + \tau_{1-}\varepsilon_{21})}{(s+\lambda_1)(s+\lambda_2) - \varepsilon_{12}\varepsilon_{21}}. \tag{4.36}$$

4.4.3.2 Back to the real t-Space

Applying the anti-transform to (4.36) we finally get the expression for the desired density probability distribution:

$$\begin{aligned}
f_+(t) = \frac{e^{-\frac{1}{2}t(\Lambda+\lambda)}}{2\Lambda} \left[e^{\Lambda t}(\Lambda - \lambda) + \Lambda + \lambda \right] (p_1\tau_{1-} + p_2\tau_{2-}) + \\
+ \frac{e^{-\frac{1}{2}t(\Lambda+\lambda)}}{\Lambda} (e^{\Lambda t} - 1)(\tau_{1-}\lambda_2 + \varepsilon_{12}\tau_{2-})(p_1 + p_2)
\end{aligned} \tag{4.37}$$

where

$$\begin{aligned}
\lambda &= \tau_{1-} + \varepsilon_{12} + \tau_{2-} + \varepsilon_{21}; \\
\Lambda &= \sqrt{(\lambda_1 - \lambda_2)^2 + 4\varepsilon_{12}\varepsilon_{21}}.
\end{aligned} \tag{4.38}$$

Let's stress the fact that:

$$\int_0^\infty f_+(t)\, dt = p_1 + p_2 \tag{4.39}$$

which, under the hypothesis of starting in the F^+ cluster, is equal to 1.

The mean residence time of the Foxp3^+ cluster, computed using the (4.24) is therefore the following:

$$\tau_+ = \frac{p_1\tau_{2-} + p_2\tau_{1-} + (\varepsilon_{12} + \varepsilon_{21})}{\tau_{1-}\tau_{2-} + \tau_{1-}\varepsilon_{21} + \tau_{2-}\varepsilon_{12}}. \tag{4.40}$$

Finally, to get the formula 4.7 used in the main text for the Foxp3 mean residence time, we substitute $\tau_{-1} = \tau_-$, $\tau_{-2} = 0$ and $p_2 = \varepsilon_{12}/(\varepsilon_{12} + \varepsilon_{21})$ in equation 4.40 and rearrange the terms:

$$\tau_{foxp3} = \frac{(\varepsilon_{12} + \varepsilon_{21})^2 + \varepsilon_{12}\tau_-}{\varepsilon_{21}(\varepsilon_{12} + \varepsilon_{21})\tau_-}. \tag{4.41}$$

4.5 Supplemental material

4.5.1 Model fitting of the experimental data on Foxp3 *in vivo* dynamic

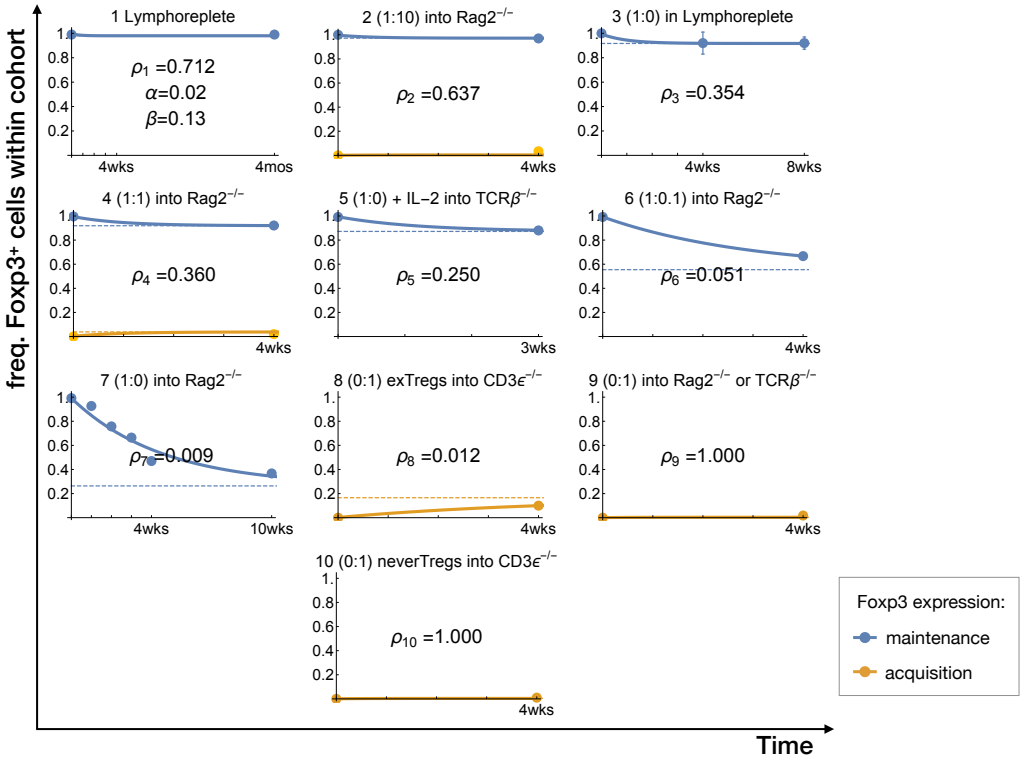

Figure 4.9: **Model predictions reproducing the experimental data on Foxp3 in vivo dynamics.** Frequency of Foxp3$^+$ cells within cohort of cells followed *in vivo* over time, in different experimental conditions. Dots represent experimental data (experiments have been described in details, in materials and methods section of the previous chapter), lines are the model predictions from one representative parameter set out of the equally good found sets. Solid lines are the temporal solutions of the stochastic model, dashed lines represent the predicted value of equilibrium. In blue, the experimental data in which maintenance of Foxp3 expression over time, is assessed, in yellow, the experimental data in which acquisition of Foxp3 expression assessed over time. In each experiment is reported the value of the ratio ${}^i\rho$ obtained through the fitting. Other parameter values are: $\alpha = 0.02, \beta = 0.13, \vartheta = 28.56, \varepsilon_{01} = 0.28, \varepsilon_{10} = 1.28, \varepsilon_{12} = 2.83, \varepsilon_{21} = 0.03$.

4.5.2 Distribution of rates without constraints

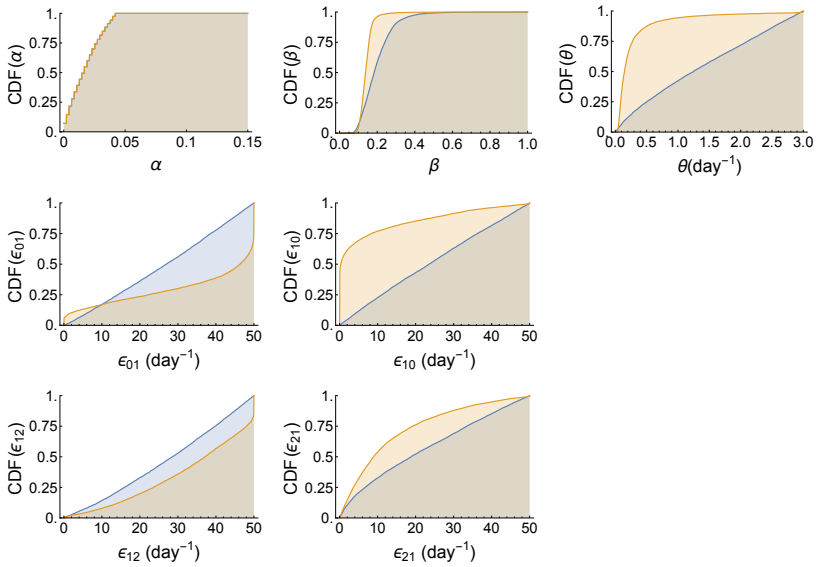

Figure 4.10: **Convergence of optimization algorithm (I).** Cumulative distribution function (CDF) of the model parameters $\alpha, \beta, \vartheta, \varepsilon_{01}, \varepsilon_{10}, \varepsilon_{12}, \varepsilon_{21}$ optimized to fit the experimental data under constraints 1 and 2 (see materials and method section 4.4.2). In blue the distribution of the initial values provided as initial values to the algorithm, in order to minimize sum of residuals between experimental data and model solution. In yellow the distribution of the optimum values.

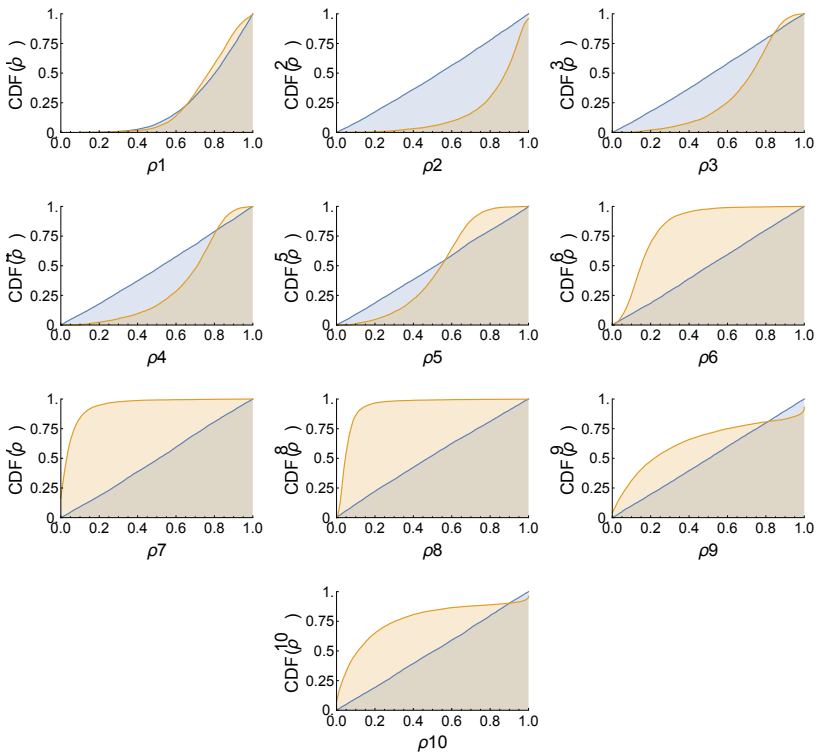

Figure 4.11: **Convergence of optimization algorithm (II).** Cumulative distribution function (CDF) of the model parameters ${}^{i}\rho, i = 1, ..., 10$ optimized to fit the experimental data under constraints 1 and 2 (see materials and method section 4.4.2). In blue the distribution of the initial values provided as initial values to the algorithm, in order to minimize sum of residuals between experimental data and model solution. In yellow the distribution of the optimum values.

4.5.3 Foxp3 mean residence time *versus* frequency of committed Tregs.

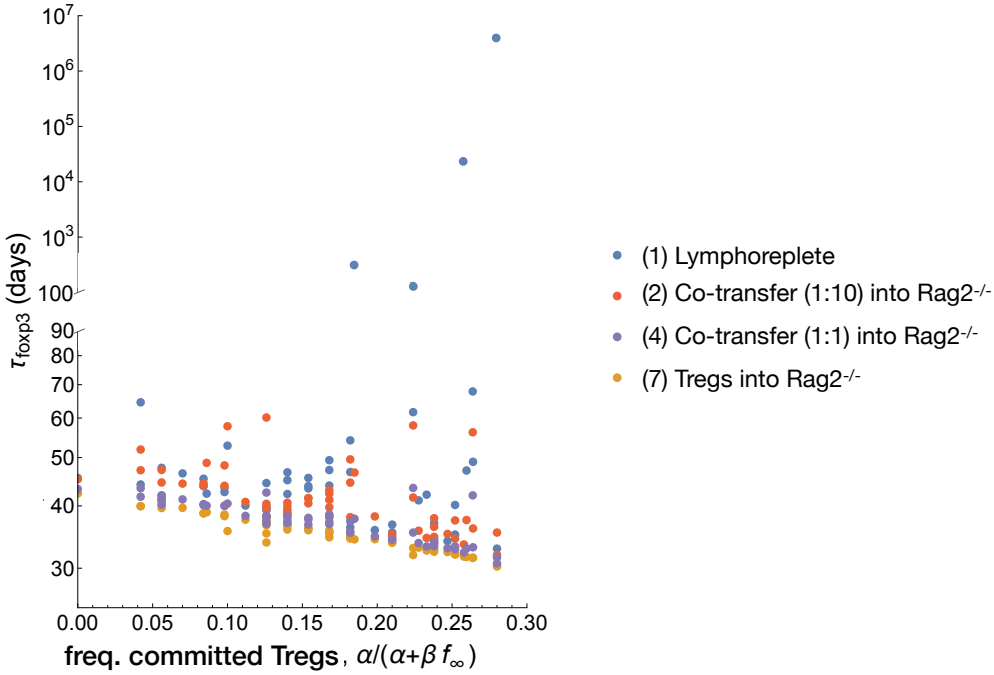

Figure 4.12: **Foxp3 mean residence time *versus* frequency of committed Tregs in different experimental conditions.** Foxp3 mean residence time of the uncommitted Tregs (τ_{foxp3}) plotted against the frequency of committed Tregs within Foxp3$^+$ cells ($\alpha/(\alpha+\beta\,\bar{f}_\infty)$), in four experimental conditions: lymphoreplete conditions, experiment 1 from [Rubtsov et al., 2010] (blue); co-transfer of Tregs and CD4$^+$Foxp3$^-$ T cells in proportion (1:10) into Rag2$^{-/-}$, experiment 2 from [Komatsu et al., 2009] (red); co-transfer of Tregs and CD4$^+$Foxp3$^-$ T cells in proportion (1:1) into Rag2$^{-/-}$, experiment 4 from [Komatsu et al., 2009, Duarte et al., 2009] (violet); transfer of Tregs into Rag2$^{-/-}$, experiment 7 from [Duarte et al., 2009] (yellow). Note the change in scales in the y axis.

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5

General discussion

5.1 Overview

Immunological tolerance is a robust process that relies on the development and maintenance of population of functional Tregs, able to prevent the clonal expansion of auto-reactive Teffs that circulate in the periphery. Maintenance of a functional population of Tregs, in balance with the population of Teffs they control, requires sustained Foxp3 expression at the cellular level and the stable coexistence of Teff and Treg populations with shared specificities.

This thesis aimed at theoretically investigating the sub-cellular and super-cellular mechanisms that preside over tolerance and its robustness.

We started with the very fundamental and general question of whether it is possible to maintain Treg and Teff populations in dynamic balance under the assumption of Foxp3 lability. To do that, in chapter 2, we built upon the cross regulation model for APCs-mediated CD4 T cells interactions [León et al., 2000, Carneiro et al., 2007], introducing context-dependent Foxp3 loss. We considered the worst case scenario of no peripheral Foxp3 up-regulation that could counterbalance Foxp3 loss. In this extended model, tolerance is maintained provided that Foxp3 loss is slower than a critical value. Furthermore, the introduction of Foxp3 lability determines a qualitative change in the so called tolerance equilibrium, particularly in the trajectories of the system when subjected to a perturbation. As a result, robustness of tolerance increases. It was shown that robust tolerance can be guaranteed by the non-linear and density-dependent

population dynamics of a Foxp3 committed Tregs population, together with cognate Teffs and APCs populations [Carneiro et al., 2007]. Our result implies that a population of uncommitted Tregs, in which Foxp3 expression is determined by the context, could better cope with perturbations.

The requirement for a critical slow Foxp3 loss was a non trivial theoretical result that raised the question of estimating the characteristic time of Foxp3 loss *in vivo*. To this end, in chapters 3 and 4 we moved to the cellular level. We focused on Foxp3 expression dynamics in CD4 T cells, by the implementation and analysis of stochastic models of gene expression. These models allowed us to investigate, first, the characteristic time scale of Foxp3 loss *in vivo* and, secondly, the commitment and plasticity of Tregs.

Specifically, in chapter 3 we investigated whether postulating commitment to Foxp3 expression is necessary for the observed Foxp3 stability and what can control stability and lability of Foxp3 expression. We investigated also whether we could make quantitative predictions on heterogeneity of Tregs, regarding their ability to maintain stable Foxp3 expression. We fitted the stochastic model of Foxp3 expression to the experimental data on Foxp3 stability *in vivo* in different experimental contexts. Our analysis showed that experimental data are compatible with Tregs being a homogeneous pool of CD4 T cells in which Foxp3 expression is stable or labile depending on the context. We found positive correlation between stability of Foxp3 expression and number and frequency of Teffs in the host. We quantified the average time needed for the loss of Foxp3 expression in peripheral Tregs in absence of pro-Foxp3 stimuli as four weeks. Data also suggest that the majority of CD4 T cells cannot be induced *in vivo* to express Foxp3 regardless of the context.

In chapter 4 we asked whether the Foxp3 loss rate, estimated in chapter 3, can be the consequence of slow epigenetic dynamics. Fitting of an extended model, combining fast transcriptional activation with slow epigenetic dynamics, suggested that this could be the case.

In each chapter, we have discussed the respective results. Here, we would like to make some considerations of a more general character, integrating cross-cutting aspects of our results. Also, we try to propose some experimental approaches that could be useful to validate our predictions.

5.2 Three subpopulations of CD4 T cell

Navigating through the Treg literature can be challenging. As more experimental evidences have been collected regarding Foxp3 expression in CD4 T cells, more definitions for Tregs have been introduced [Abbas et al., 2013, Shevach and Thornton, 2014, Koizumi and Ishikawa, 2019]. These definitions reflect the idea that many subpopulations of Foxp3$^+$ Tregs with distinct identities exist. Accordingly, several interpretations and models for these populations have been proposed.

Some Foxp3$^+$ cell subpopulations were defined according to the location of their differentiation: tTreg differentiate in the thymus [Fontenot et al., 2005a, Hsieh et al., 2012, Klein et al., 2019], pTreg in the periphery [Chen et al., 2003], iTreg are generated *in vitro* [Curotto de Lafaille and Lafaille, 2009]. Helios and Neuropilin-1 (Nrp1) have been used to distinguish between tTreg (Helios$^+$ Nrp1$^+$) and pTreg (Helios$^-$ Nrp1$^-$) [Thornton et al., 2010, Yadav et al., 2012, Weiss et al., 2012]. However the ambiguity between tTregs and pTregs arises since high versus low expression of Nrp1 and Helios have been shown not to unequivocally identify tTreg and pTregs [Szurek et al., 2015]. Furthermore, by using the Foxp3-Tocky (Timer of cell kinetics and activity) reporter mice, it has been shown that both Nrp1 and Helios are dynamically regulated *in vivo*, according to Foxp3 transcription dynamics [Bending et al., 2018b]. These observations have led to question the significance of these two markers [Bending and Ono, 2019].

Other Foxp3$^+$CD4$^+$ T cell subpopulations were defined depending on the activation status of the cells. In particular, tTregs have been divided into two distinct subpopulations. Naive tTregs are defined as central Treg (cTreg) cells, also called resting Tregs; while tTregs that have been activated upon TCR stimulation are called effector Treg (eTreg) cells, or activated Tregs or effector memory Tregs [Levine et al., 2014, Liston and Gray, 2014, Li and Rudensky, 2016]. Markers for cTregs are generally CD62L^{hi}CD44^{lo} while eTregs are characterised as CD62L^{lo}CD44^{hi}.

Many other definitions reflect rather the interpretations of the dynamics of Foxp3 expression in these cells. Often some of these definitions for CD4 T cells different populations are not associated with distinctive markers. The debate has been wide [Bailey-Bucktrout and Bluestone, 2011, Hori, 2011a, Sakaguchi et al., 2013, Hori, 2014, Qiu et al., 2020]. Some authors mention

Treg lineage stability when Tregs differentiation appears to be stable, regardless of the different perturbations in the extracellular environment they are subjected to [Hori, 2011b, Josefowicz et al., 2012]. Phenotypic plasticity of Tregs, on the other hand, refers to Tregs ability to change their gene expression in response to external inputs [Josefowicz et al., 2012, Campbell and Koch, 2011, Burzyn et al., 2013]. Finally, the context-dependent change in gene expression and the ability of Tregs to acquire gene signatures and functions characteristics of various Teff cell types has also been interpreted as reprogramming. According to this interpretation, these cells are susceptible to lineage or developmental plasticity being referred to as reprogrammed exTregs [Zhou et al., 2009, Mellor and Munn, 2011, Liston and Piccirillo, 2013]. However, the cellular markers of reprogramming are unclear. Observing loss of Foxp3 expression is not a sufficient indication for reprogramming to functional Teffs because these exTregs may re-express Foxp3 stably and display suppressive function [Miyao et al., 2012]. Also, the expression of pro-inflammatory cytokines by T cells does not allow to conclude that one is in the presence of functional Teffs. For instance, in the contest of bone marrow adoptive transfer, donor Foxp3$^+$ T cells protect the host from graft-versus-host disease [Hoffmann et al., 2002]. However, in this highly inflammatory model, more than 50% of donor Foxp3$^+$ T cells produce INF-γ while displaying fully demethylated TSDR and stably expressing Foxp3. Also, the protection is INF-γ-dependent, given that blocking INF-γ with specific mAb abolishes the beneficial effect of donor Foxp3$^+$ T cells [Koenecke et al., 2012]. Also, Foxp3$^+$ cells isolated from INF-γ deficient mice fail to protect the host from graft-versus-host disease [Sawitzki et al., 2005]. Finally, Foxp3$^+$ T cells have been reported to acquire Teffs-like features without losing Foxp3 expression *in vivo* and *in vitro*. Foxp3$^+$ cells expressing ROR-γt and IL-17 [Zhou et al., 2008] or T-bet and INF-γ [Oldenhove et al., 2009] have been found in normal or *Toxoplasma gondii* infected mice, respectively. The latter phenotype was also induced in Foxp3$^+$ cells stimulated *in vitro* in Th1-promoting conditions [Wei et al., 2009, Koch et al., 2012, Zhao et al., 2012].

Genetic fate mapping allowed to sort Foxp3$^+$ T cells that have recently initiated Foxp3 transcription and to distinguish them from the ones that have expressed Foxp3 for some time [Miyao et al., 2012, Zhou et al., 2009]. Newly developed Foxp3$^+$ T cells exhibit transient Foxp3 expression [Miyao et al., 2012].

This observation have led to wonder whether these cells are developmental intermediates on the way to become stable Tregs [Hori, 2010] or activated Teffs that transiently and promiscuously express Foxp3, regardless of Treg differentiation [Miyao et al., 2012, Hori, 2014]. In this line it has also been proposed that Tregs represent a ”meta-stable” activation state, rather than a distinct lineage of suppressive cells [Hori, 2014, Bending and Ono, 2019].

A heterogeneity model was proposed [Komatsu et al., 2009, Hori, 2010, Miyao et al., 2012] that overcomes the reprogramming of committed Tregs definition by calling into play conversion and selection. These authors proposed that there are two distinct populations of Foxp3^{+} CD4 T cells: the majority are committed Tregs, whereas a minor subpopulation are uncommitted, plastic and have the potentiality to convert into Teffs. This minor population does not proliferate in lymphoreplete conditions while it preferentially proliferates in lymphopenia and under inflammation.

The heterogeneity model was extended to the population of former Foxp3^{+} [Miyao et al., 2012, Hori, 2014]. This population was portrayed as a mixture of a population of Teffs that transiently expressed Foxp3 after activation, and a population of Tregs that have lost Foxp3 expression but retain epigenetic memory of Foxp3 expression and suppressive function. The latter population was defined as latent Tregs. This model was introduced to accommodate the observations that the population of former Foxp3^{+} cells displayed partially demethylated TSDR. Upon transfer into Rag1$^{-/-}$, some cells reacquired Foxp3 expression, becoming fully suppressive and showing fully demethylated TSDR. Other cells remained negative to Foxp3 expression, were not suppressive and had fully methylated TSDR [Komatsu et al., 2009, Miyao et al., 2012, Hori, 2014].

It was proposed that the level of CD25 expression could be a distinctive marker to deal with the heterogeneity. Committed or latent Tregs would be CD25^{high} whereas non-Tregs would be CD25^{low}, regardless of Foxp3 expression. Yet, within CD25^{low}Foxp3^{+} cells there are some *bona fide* Tregs with stable Foxp3 expression, and CD25^{high}Foxp3^{+} cells contain some cells that exhibit unstable Foxp3 expression [Komatsu et al., 2009]. In addition, CD25 expression in Foxp3^{+} cells is dynamically regulated, depending on IL-2 availability and proliferation status [Fontenot et al., 2005b, Zelenay et al., 2005, Almeida et al., 2006a]. Finally it was proposed that the distinction between committed Tregs and cells

that exhibit promiscuous Foxp3 expression, without being Tregs, is based on the differential methylation status of the Foxp3 locus [Miyao et al., 2012]. In fact, the demethylated status of TSDR, identifies both Tregs expressing Foxp3 and the Foxp3$^-$ latent Tregs that retain epigenetic memory. Therefore, according to the heterogeneity model, Tregs are a stable cell lineage, whose commitment is provided by the demethylation of the Foxp3 locus, regardless of ongoing Foxp3 expression [Miyao et al., 2012].

In the face of such multiplicity of definitions, we proposed here a model that divides the pool of CD4 T cells into three populations: committed Foxp3$^-$ cells, committed Foxp3$^+$ cells, and cells with context-dependent Foxp3 expression. The confrontation of this model with the data indicated that there is no absolute requirement to postulate the existence of the committed Foxp3$^+$ cell population. In this simplified view, the majority of Treg populations defined in literature, become a single population of context-dependent cells. More specifically, instead of distinguishing subpopulations of CD4 T cells, we consider different phenotypic states that some CD4 T cells can assume. Each cell can switch stochastically among these phenotypic states in response to external cues, by remodelling of the Foxp3 locus and by changing its transcriptional activity. For a cell of this context-dependent population, being regarded as exTreg, latent Treg, committed Treg or newly developed Treg, depends on the recent history of the cell before the sorting, but it does not necessarily mirror its function nor predicts its fate.

As we have discussed in chapters 3 and 4, our stochastic model can reconcile the experimental observations on stability and lability of Foxp3 expression *in vivo* [Rubtsov et al., 2010, Duarte et al., 2009, Komatsu et al., 2009, Paiva et al., 2013]. Moreover, the model predictions are compatible with observations that were not included in the analysis. For instance, the apparent heterogeneity of former Foxp3$^+$ cells mentioned above [Komatsu et al., 2009, Miyao et al., 2012, Hori, 2014] can be alternatively explained by the slow and stochastic epigenetic remodelling of the Foxp3 locus over time, which depends on the transcriptional state of the cell. According to our stochastic model, former Foxp3$^+$ cells that are found with partially demethylated TSDR are cells in state Z_{11}. Also, the model explains how only a fraction of these cells re-acquire Foxp3 expression and become fully demethylated at the TSDR. This fraction is interpreted as those cells that make the transition from state Z_{11} to Z_{21} and sub-

sequently to Z_{22}. Likewise, the model explains the fraction of exTregs that are pathogenic [Miyao et al., 2012, Duarte et al., 2009], as those that happen not to require Foxp3 remaining in states Z_{11} or Z_{10}.

Although many heterogeneous behaviours and promiscuous profiles of Tregs can be quantitatively interpreted through our model, some cannot. For instance, given that our model does not account for CD25 expression, it cannot explain CD25 dynamics. Nevertheless, based on the result (chapter 4) that the vast majority of stable Tregs are in state Z_{22}, we expect cells in state Z_{21} to be enriched in $CD25^{low}$, while cells in state Z_{22} to be enriched in $CD25^{high}$. For simplicity, we made the choice of introducing only three epigenetic states and we assumed that cells with fully active chromatin do not lose Foxp3 expression, unless they first undergo chromatin remodelling. The last assumption corresponds to saying that Foxp3 loss is negligible in state Z_{22}. Observed $CD25^{high}Foxp3^{+}$ T cells with partial demethyletad TSDR but exhibiting stable Foxp3 expression and suppressive function [Hori, 2014] could be perhaps better explained in case we introduced some more intermediate epigenetic states.

It is not straightforward, with our model, to reproduce the observation that most immature $CD25^{high}Foxp3^{+}$ thymocytes stably express Foxp3, while having largely methylated TSDR [Toker et al., 2013]. This observation has been interpreted as CpG demethylation in TSDR occurs after Foxp3 stable expression has been achieved, in the sense that TSDR demethylation maintains Foxp3 stability, rather than inducing it. Consistently, demethylation at the TSDR is not required for initiation of Foxp3 expression, but it is needed for its long-term maintenance according to [Polansky et al., 2008, Huehn et al., 2009, Zheng et al., 2010]. More recently, Bending *et al.* have investigated the *in vivo* dynamics of demethylation of the Foxp3 gene, by using the Foxp3-Tocky reporter mice [Bending et al., 2018a]. In this mice the transcriptional activity of the Foxp3 gene is reported by Fluorescent Timer protein, which spontaneously shifts its emission spectrum from blue to red after translation. Using the Foxp3-Tocky reporter mice, Bending *et al.* have shown that in $Foxp3^{+}$ thymocytes, Foxp3 expression occurs before TSDR demethylation, while the most active demethylation process happens when Foxp3 expression is already sustained [Bending et al., 2018a]. Also for these cases, the data could be potentially better explained by a model featuring more epigenetic states, particularly if the balance

between of transcriptional activation and repression increases as the locus become more accessible. This kind of stochastic model of gene expression has been described for the IL-10 gene [Paixão et al., 2007].

Our modelling results suggest that exTregs and newly generated Tregs can be observed in lymphoreplete conditions, thus reconciling the apparent contrasting observations made by fate mapping strategies [Miyao et al., 2012, Zhou et al., 2009, Rubtsov et al., 2010], as further discussed in section 5.5.

Several experimental observations indicate that Foxp3 expression, per se, is not sufficient to ensure its continued and stable expression. Therefore, Foxp3 transcription and translation does not work as a molecular switch, as it was originally described for the transcription of other master transcription factors of CD4 T cell differentiation such as T-bet or GATA-3 for Th1 and Th2 lineages, respectively [Szabo et al., 2000, Zheng and Flavell, 1997]. Already for Th1 and Th2 differentiation, it appeared soon clear that lineage specification is more plastic than it was originally thought [Evans and Jenner, 2013]. That said, our analysis suggests that what makes a CD4 T cell be a Treg, expressing Foxp3 in a sustained and prolonged manner, are sustained environmental cues. Likewise, inhibition of suppressive activity and ”reprogramming” toward Teff function occur in scenarios in which the environmental context ensures that Foxp3 expression is prevented for a sufficiently long period.

We found a positive correlation between the number of Foxp3$^-$ CD4 T cells in the host and stability of Foxp3 expression. This result, reported in chapter 3, holds also in the analysis described in chapter 4. This correlation is in agreement with the indexation of Treg cells to the number of activated IL-2-producing cells [Almeida et al., 2006b], which was posited to be a mechanism for maintaining homeostasis and preventing autoimmune or lymphoproliferative diseases. This correlation is also a straightforward prediction of the original cross regulation model [Carneiro et al., 2007] which still holds in the presence of context-dependent Foxp3 expression, as for the model presented and studied in this work in chapter 2. Finally, it is in agreement with the result that the number of Teffs, producing IL-2, is controlled via a quorum sensing?like feedback loop where the IL-2 is sensed by both the activated Teffs and by Tregs [Amado et al., 2013, Reynolds et al., 2014]

5.3 Consequences of neglecting population dynamics while addressing Foxp3 cellular expression

In chapters 3 and 4, that focus on Foxp3 expression at cellular level, we put aside the population dynamics. In fact, while focusing on the frequency of Foxp3$^+$ cells within cell cohorts, we did not consider cell proliferation nor death.

To clarify the implications of this choice, let us recall that we assumed that the temporal evolution of the observed frequency depended on the context in which the cohort of cells was followed. Beyond antigens presentation, that we assume not to vary across the different experimental contexts examined, in some experiments the context is mainly constituted by the followed cohort itself. This is the case for the experiments in which Foxp3$^+$ cells are transferred alone into lymphodeficient host mice. In the remaining experiments the context is constituted by the followed cohort together with other lymphocytes populations, either co-transferred into, or endogenous to, the host. In each experiment considered in our analysis, there were portions of cells, within the cohort, whose Foxp3 expression state would change over time: they either up-regulated or down-regulated the gene. Neglecting cell proliferation and death mainly implied that, within each followed cohort, we neglected any preferential proliferation of Foxp3$^-$ cells over Foxp3$^+$ cells or vice-versa, regardless of the context.

When transferring 2.5×10^5 Foxp3$^+$ T cells alone into lymphodeficient host, Duarte *et al.* "spiked" the Treg cells preparation with 2% of CD4$^+$Foxp3$^-$ Teffs cells (as few as 5×10^3 cells). These contaminants could be distinguished by the others T cells thanks to genetic marker. After 4 weeks the contaminants accounted for 15% of the total CD4$^+$Foxp3$^-$ recovered.

Cells that have up-regulated Foxp3 expression in the periphery, lose Foxp3 expression in lymphodeficient condition [Miyao et al., 2012]. Also, they preferentially proliferate in lymphodeficient condition, if compared with the total pool of ex-Foxp3 cells that have lost Foxp3 expression in lymphodeficient condition. While the latter cohort of cells do not undergo major proliferation, the former cohort of cells exhibits a variety of behaviors: within 4 weeks they can either increase up to 50 folds or be halved [Miyao et al., 2012]. Starting from a small amount of cells as 2×10^4, which constitute the 0.5% of the total cohort of the Foxp3$^+$ cells transferred into lymphopenic host, after 4 weeks they can constitute, on average, from

less than 1% up to 20% of the Foxp3$^-$ cells accumulated. Based on this observation, researchers have been proposed that the accumulation of cells that have lost Foxp3 expression, observed in lymphopenic condition and under inflammation, is mainly the result of preferential proliferation of a minor population of Teffs that transiently express Foxp3, rather than the pure conversion of Foxp3$^+$ cells into Foxp3$^-$ [Miyao et al., 2012]. Although this might be the case, the link between the proliferation and the change in frequency was not quantitatively established, leaving the door open to alternative interpretation. Also, the high non linearity that affects Tregs and Teffs dynamics when out of their equilibrium, makes the interpretation of these observations anything but trivial.

Conventional CD4$^+$ T cells poorly expand when adoptively transferred into lymphoreplete, presumably being outcompeted by endogenous cells. In contrast, when conventional CD4$^+$ T cells are adoptively transferred into lymphodeficient hosts, they undergo massive proliferation [Annacker et al., 2000, Annacker et al., 2001, Almeida et al., 2002]. Likewise, in case of adoptive transfer of Tregs into lymphodeficient mice, we expect that the cells that first lose Foxp3 expression will, at least transiently, proliferate more than the cells that remain Foxp3$^+$. These expectations are in line with the original cross regulation model predictions [León et al., 2001, Carneiro et al., 2007] and still hold in the model presented in chapter 2. According to the cross regulation model, the T cell per capita net growth rate, $\frac{dT}{Tdt}$, increases as the cell density decreases, thus being larger in lymphodeficient as compared to lymphoreplete hosts. This rate is zero in lymphoreplete condition which corresponds to the equilibrium of the system. Moreover, at low T cells density the co-conjugation of Tregs and Teffs on APCs is diluted by the excess of APCs, since T cells likely conjugate alone with APCs. Under these conditions, Teffs are activated and proliferate, whereas Tregs do not. The preferential Teffs proliferation is expected to be transient. As soon as Teffs density grows, the chance for Tregs to form a multicellular conjugate with Teffs increases, and Teffs start ”fuelling” Tregs proliferation, while they are inhibited by activated Tregs. This dynamics is evident in the trajectories of Teffs and Tregs populations toward the equilibrium (see figures 2.4 and 2.5): any trajectory that starts within the basin of attraction of the tolerance equilibrium with minute Teff densities, displays initially a Teffs population growth, before the equilibrium, dominated by Tregs, is reached.

In our models, introduced in chapters 3 and 4, we focused on cellular frequencies which is analogous to a scenario of equal proliferation and death of Foxp3$^-$ and Foxp3$^+$ CD4 T cells. This was the simplest approach in our first approximation to the problem. We are aware that we are neglecting the transient preferential proliferation of Teffs that occurs at low T cells densities. We expect this process not to happen in the lymphoreplete condition and to be mitigated in co-transfers experiments. Concerning lymphodeficient condition, we are assuming that the preferential proliferation of Teffs during a the initial transient period, can be neglected in comparison with the longer time window of weeks, during which most of the dynamics is observed. Nevertheless, we cannot rule out the possibility that neglecting this preferential proliferation has led us to overestimate the frequency of cells that express Foxp3 in a context dependent way. For this reason, including the population dynamics in this analysis is a desirable path for future research.

In the study of Foxp3 cellular expression (chapters 3 and 4), we assumed that each host provides a particular environment for the CD4 T cells. That is to say, we assumed that any CD4 T cell in a specific host, given the host particular composition of immune cells populations, is subjected to certain interactions with other CD4 T cells, mediated by APCs. This average host-specific context, to which each CD4 T cell is subjected during the time window of any experiment, is reflected in the context-dependent model parameters values. We also assumed that the change in Foxp3 transcriptional state occurring in any CD4 T cell, does not impact the average characteristics of the field of inputs in which all the cell are embedded. It means that the model parameter values do not change over the time of the experiments, but reflect the average characteristics of the experiment. Also, in this sense, the population dynamics is neglected in these two chapters.

With such an approximation, we regarded any mouse as if it was a well-stirred system, in which, each cell being sampled is representative of any CD4 T cell: given that the time window of the experimental observation is long enough, any sampled cell would receive, on average and throughout the experiment, the same input as any other. We are, therefore, using a mean field approximation to analyse the interactions among T cells mediated by APCs. The idea of the mean field approximation, in case of stochastic model involving high-number of interacting particles, is to focus on one average par-

ticle. In our case the ”particles” are cells. The approximation assumes that the effect of the neighbouring cells on any given cell is approximated by a single averaged effect of what is called mean field. This approximation reduces a many-body problem to a one body problem. The approach of mean field approximation in modelling the adaptive immune system is not new. It has been used to study the maintenance of diversity in T cells repertoire [Stirk et al., 2008, Stirk et al., 2010], the mechanism of T cell receptor triggering and down-regulation [Milutinovic et al., 2003, Sousa and Carneiro, 2000] and the dynamics of the TCR expression level distribution [Milutinović et al., 2007].

5.4 Can sub-cellular and super-cellular dynamics be reconciled?

The population dynamics analysis in chapter 2 suggested that loss of Foxp3 expression needs to be a slow process such that tolerance is maintained. The analysis of the cellular dynamics of Foxp3 expression in CD4 T cells, conducted in chapter 3 with the use of experimental data, gave us an estimation for Foxp3 rate loss. Irrespective of the fractions of committed Tregs and plastic Tregs, the rate at which un-stimulated Tregs convert into Teffs is of the order of weeks. Finally, the analysis in chapter 4 showed that the slow rate of Foxp3 loss can be explained by epigenetic remodelling of the Foxp3 locus that controls the accessibility for the transcription machinery. Let us recall that, because epigenetic marks are inherited traits, epigenetic dynamics needs to be, by definition, slow. At least, it cannot be faster than cell proliferation, otherwise epigenetic marks, rather than being inherited, would be stochastic.

In the interaction between Tregs and Teffs that we have studied here, the cellular and population levels are actually intertwined and interdependent. In fact population dynamics, which controls proliferation and death of the two populations, and Foxp3 expression dynamics, which defines the identity of the cells, depend on the densities of Tregs, Teffs and APCs. On the one hand, T cell populations interact through multicellular conjugates and the kind of inhibitory or activatory interactions depends on the conjugate stoichiometry that in turn is determined by the densities of the populations. On the other hand, the cellular dynamics, which determines the change in identity between Tregs and Teffs, de-

pends on what we have called the environment of the cells. In chapter 3 we also showed that the environment can be likely explained by density of Teffs. Cellular dynamics, therefore, depends on the densities of the cell populations.

Therefore, population dynamics and cellular Foxp3 dynamics depend on cells density and eventually determine the densities themselves. While the global equilibrium, at cellular and population level can be studied (and, in a way, is the assumption behind the analysis in chapters 3 and 4), investigating the global dynamics can be tricky. In fact the two levels determine one another. If their respective time scales were different, one could simply address their joint dynamics, by quasi-steady-state approximation, for instance. However this is not the case: in addition to determining one another, the dynamics at sub-cellular and super-cellular level share the same time-scale.

We incorporated the epigenetic dynamics into the population dynamics model and did some preliminary analysis. We introduced two sub-populations of Tregs, distinguished by their epigenetic state of the Foxp3 locus. In the model, cells belonging to the first Tregs subpopulation can lose Foxp3 expression in a context-dependent way, as the Tregs in the model introduced in chapter 2. The other subpopulation of Tregs cannot lose Foxp3 expression, unless they first convert into the other population. Transition between the two populations happens in a context-independent way.

We used this model to reproduce the experiments showing stability of Foxp3 expression in physiologic condition [Rubtsov et al., 2010] and the experiments where Tregs are adoptively transferred into lymphodeficient host [Duarte et al., 2009, Komatsu et al., 2009]. We managed to fit the experimental data, using a rate of Foxp3 loss, for un-stimulated Tregs, equal to the one determined by the analysis in chapter 3. Nevertheless, in the regime we found, the dynamics of loss of Foxp3 in the lymphodeficient condition displayed damped oscillations. This means that, according to the model interpretation, the frequency of Foxp3$^+$ cells would converge to the equilibrium in the new environment through transient oscilllations of progressively low amplitude. For this reason, further investigation should be carried to know the bifurcation diagram of the integrated model and check whether it would be possible to reproduce the experimental data without oscillations.

5.5 Dynamical interpretation of Foxp3 expression and stability

The two stochastic models for cellular Foxp3 expression (chapters 3 and 4) allowed us to interpret the different temporal dynamics of Foxp3 expression, observed *in vivo* in different hosts, under a single framework. Our analysis included experiments performed in lymphoreplete as well as lymphodeficient conditions, encompassing several intermediate cases. Moreover, the data were produced by experiments assessing the frequency of cells that maintained or that acquired Foxp3 expression.

Prior to performing this analysis, we fitted the same data sets, using simpler stochastic models for just down-regulation of Foxp3, in case the experiments addressed maintenance, or for just up-regulation of Foxp3, in case the data addressed acquisition (see Figure 5.1). We used single-step models, which represent the most parsimonious choice, as well as multi-step models, which consider several epigenetic remodelling steps of the locus or, alternatively, account for other molecular mechanisms that influence gene transcription and translation. In short, within several possible linear combinations of exponential functions, we choose the most reasonable model, based on the Akaike information criterion (AIC) [Akaike et al., 1973, Bozdogan, 1987], which makes a trade-off between the likelihood of the fit and the parsimony of the model. This is a way to quantify the characteristic rate of Foxp3 loss within the cohort, in case the cohort was of Foxp3$^+$ cells. More specifically, it is a way to quantify the ”net rate of decay”. Likewise, in case the cohort was of Foxp3$^-$ cells, the ”net rate of acquisition” would be quantified. Obviously this analysis was only performed on time series with three or more points. Each data on Foxp3 maintenance was then associated to a net rate decay (function of the model rates), and each data on Foxp3 acquisition to a net rate of acquisition. The drawback of this approach was that, by construction, the two rates were independent of each other, even in case maintenance and acquisition of Foxp3 expression were assessed in the same host, by simultaneously following two cell cohorts (one positive and one negative for Foxp3 expression, at time zero). This approach regards Foxp3 maintenance and up-regulation as they were independent from each other, rather than the result of the same context that provides the inputs which regulate gene activation and repression.

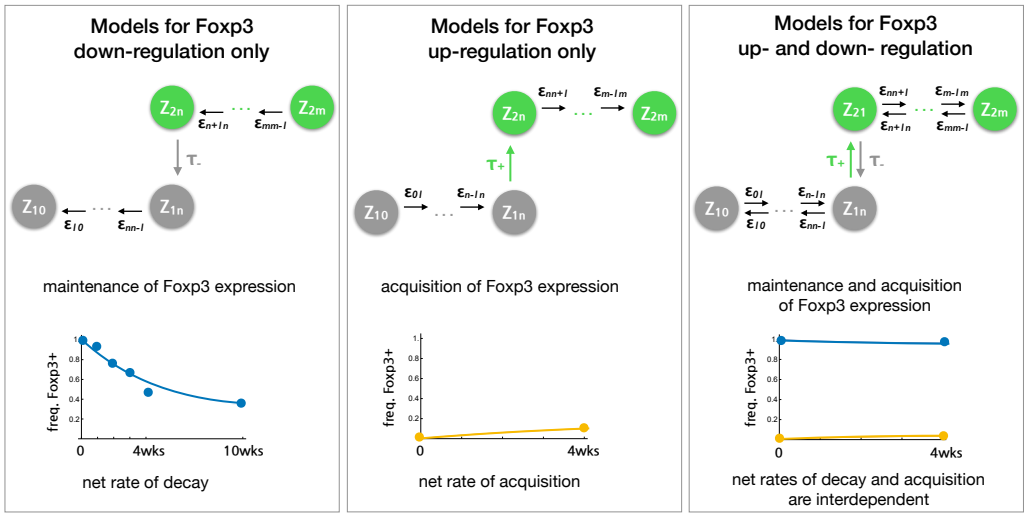

Figure 5.1: **Comparison among different multi-step stochastic models for Foxp3 expression and consequences on dynamical interpretation of Foxp3 expression.** Not any multi-step stochastic model for gene expression allow for dynamical of Foxp3 expression. Left: models for Foxp3 down-regulation only, can be used to perform fitting of experimental data assessing the maintenance of Foxp3 expression over time in cohorts of Foxp3$^+$ cells and to quantify the net rate of Foxp3 expression decay within the cell cohort. Center: models for Foxp3 up-regulation only can be used to perform fitting of experimental data assessing the acquisition of Foxp3 expression over time in cohorts of Foxp3 Foxp3$^-$ cells and to quantify the net rate of Foxp3 expression acquisition within the cohort. Right: models accounting for both Foxp3 up- and down- regulation, as the ones used in chapters 3 and 4, allow for dynamical interpretation of Foxp3 expression and stability. They can be used to fit experimental data assessing maintenance as well as acquisition of Foxp3 expression in cohorts of Foxp3$^+$ and Foxp3$^-$ cells, respectively, even when the assessment is performed in the same host. When using these models to reproduce experimental data, each host is characterised by net rates of Foxp3 decay as well as acquisition and the two rates are interdependent by construction. As a consequence, these models permit to disentangle the net behaviour of the cohort of cells from the dynamics of its individual cells.

On the other hand, models in chapters 3 and 4 assume that, in each host, both up- and down- regulation of Foxp3 are allowed. And yet to any of the activation or repression rates, it can be attributed a value so small as to be practically zero relative to the life of a mouse. With these models, therefore, each host (meaning each context in which CD4 T cells are embedded) is characterised by two rates: the rate at which a CD4 T cell switches from Foxp3$^+$ to Foxp3$^-$ and the rate of reverse transition. This assumption means that the empirically observed frequencies of Foxp3$^+$ cells within any cohort of cells is seen as the net result of both transitions. And in fact, the prediction of a large fraction of Teffs that never up-regulates Foxp3 in lymphoreplete conditions was a result of fitting the models to the data from Tregs and Teffs co-transfers, where both maintenance as well as up-regulation were assessed. In these experiments, the cohort of Foxp3$^+$ cells showed stable Foxp3 expression whereas almost no up-regulation of Foxp3 was observed in the Foxp3$^-$ cohort. The discrepancy observed in the frequency of equilibrium of Foxp3$^+$ cells within the two cohorts together with the fact that those frequencies are the net result of the shared up- and down- regulation rates support the existence of a subpopulation of Teff cells that, no matter the rate of Foxp3 up-regulation, do not make the transition. Also, the constraint on the observed fraction of Foxp3$^+$ cells within CD4 T cells compartment, came into play in the quantification of this subset of CD4 T cells. If we had dissociated up- and down- regulation in the analysis, we would not have obtained this quantitative insight into Teff heterogeneity.

Furthermore, using these models, we could compute the net decay rate associated with the dynamics of the frequency of Foxp3$^+$ cells in a selected cohort of Foxp3-expressing cells but also the rate at which any of these cells lost Foxp3 expression for the first time. In this way the models permitted to disentangle the net behaviour of the cohort of cells from the dynamics of its individual cells. In particular, this allowed to account for possible loss of Foxp3 expression occurring in physiologic conditions that, nevertheless, does not impact on the apparent rate of decay, because it is transient.

The analysis opened the door to a dynamical interpretation of the stability of Foxp3 expression, observed in lymphoreplete condition, that so far has been often overlooked. As already mentioned, our analysis puts forward the possibility that stable Foxp3 expression can be the result of the context, which provides

sustained pro-Foxp3 stimuli to a subset of CD4 T cells, without a necessary commitment to Foxp3 expression. Furthermore, our analysis suggests that stability of Foxp3 expression can be the apparent outcome of a slow loss of Foxp3 expression in Tregs counterbalanced by a fast Foxp3 up-regulation. Also, according to our analysis, the majority of Tregs (at least the 72$\%$) if not all, would be subjected to the slow Foxp3 loss. This results, as we have shown in chapter 4, is compatible with the high level of CpG demethylation in Foxp3 locus, observed in vast majority of stable Tregs [Miyao et al., 2012, Ohkura et al., 2012]. Recently, Bending *et al.* have investigated the Foxp3 transcriptional dynamics, using the Foxp3-tocky reporter mice. These transgenic mice allow to distinguish among cells that have just initiated Foxp3 transcription, cells with sustained transcription and cells that have recently down-regulated Foxp3 expression and are not transcribing the gene any longer [Bending et al., 2018a, Bending et al., 2018b]. They proposed a dynamical perspective on Foxp3, regarded as a dynamically expressed gene [Bending and Ono, 2019]. In their perspective the activation of the Foxp3 transcription is regulated by temporally persistent TCR signalling and enhanced by IL-2, TGF-β and retinoic acid signalling. Furthermore, the maintenance of Foxp3 transcription requires the demethylation of the Foxp3 locus which allows the Foxp3-RUNX1/CBF-β-mediated auto-regulatory transcriptional loop to occur and that can be affected by IL-2 signalling. Finally they suggest a mutual relation between what they called ”the dynamic Foxp3 auto-regulatory loop” and the chromatin remodelling of the Foxp3 gene.This view is fully compatible with our results and interpretations.

Transient Foxp3 expression has been observed in physiologic condition by genetic lineage tracking of Foxp3$^+$ cells [Zhou et al., 2009, Miyao et al., 2012]. In these studies, 10 to 20 $\%$ of the Foxp3$^+$ cells lost the expression of this marker in physiologic conditions. This result was interpreted as a proof of the dynamical regulation of Foxp3 expression. Nevertheless, continuous labelling was criticised as leading to overestimate the amount of Tregs that would lose Foxp3 expression in physiologic conditions. The rationale behind the criticism was that, in presence of continuous labelling, cells that had just transiently expressed Foxp3, without ever having acquired suppressive function would be regarded as exTregs [Rubtsov et al., 2010]. As a matter of fact, in case of inducible labelling upon tamoxifen administration in adult mice, as we discussed at length in this work, no

accumulation of exFoxp3 population is observed [Rubtsov et al., 2010].

Rather than being interested in cells that transiently express Foxp3, we suggest to focus on cells that transiently lose Foxp3 expression. The analytic expressions of the Foxp3 mean residence time, that we derived, allows for quantitative prediction of the frequency of cells that have never lost Foxp3 expression (see equation 4.8) and, therefore, allows for experimental testing. The major issue then becomes the fact that available experimental data, so far, measure only the net rate of Foxp3 loss in a population, which overlooks transient loss.

5.6 An experimental approach to reveal Foxp3 commitment

The analysis carried out in chapters 3 and 4 suggests the possibility of Tregs being heterogeneous and constituted by two populations. A population of cells committed to Foxp3 expression and another population of plastic cells, as previously suggested [Miyao et al., 2012]. Nevertheless, our model predicts that, in case of Tregs heterogeneity, only a minor fraction of Tregs would be committed (at maximum 28%), while the majority would be plastic, expressing Foxp3 stably or transiently depending on the context. The model fitting also supports the possibility that all the Tregs belong to this plastic pool of CD4 T cells and there are no committed Tregs.

It is still unclear whether an animal in normal physiologic conditions possesses a core of CD4 T cells that, once have acquired Foxp3, cannot lose it. This question could be addressed by performing successive transfers of Foxp3$^+$ cells isolated from lymphodeficient recipients. However, these experiments have major technical hurdles. The number of Tregs that are recovered after two consecutive adoptive transfers in lymphodeficient mice (either Rag2$^{-/-}$, TCR$\beta^{-/-}$ or CD3$\varepsilon^{-/-}$) is very small and does not allow for robust assessment (as reported by Demengeot laboratory, unpublished data).

The theoretical results on Foxp3 residence time suggested that the existence of plastic Tregs in adult mice in lymphoreplete condition could be revealed by experiments that do not involve adoptive transfer of cells. If plastic Tregs exist, they should be identified in physiologic conditions and in an experimentally manageable time span (30 and 45 days) (see figure 4.8).

Such an experimental observation would be possible, for instance, in case we could engineer the Foxp3$^{\text{GFP-Cre-ERT2}}$ $\times$ ROSA26YFP transgenic mouse [Rubtsov et al., 2010], to allow for permanent and inducible labelling of cells that lose Foxp3 expression, within a cohort of already labelled Foxp3^{+} cells. This mouse model would allow to quantify a putative fraction of cells that transiently lost and subsequently re-acquired Foxp3 expression, within the cohort of YFP^{+} cells that was found to stably express Foxp3 in the Foxp3$^{\text{GFP-Cre-ERT2}}$ $\times$ ROSA26YFP transgenic mouse [Rubtsov et al., 2010] (see Figure 5.2).

The design of such a transgenic mouse model presents technical hurdles. It would require, along with the tamoxifen-induced Cre-Loxp recombination system, a second system of inducible and permanent labelling, upon a different drug administration. Furthermore, it is not trivial to induce permanent labelling in cells that do not express a gene (in this case the Foxp3 gene). The molecular description of such a mouse model goes beyond the scope of this work. Here we would like to describe the experiment that the model would allow, in case it was viable (Figure 5.2). We assume that in this mouse, Foxp3 expression in CD4 T cells is reported by GFP. Upon drug 1 administration, cells that express Foxp3 at the time of treatment are permanently labelled as YFP^{+}. Drug 1 administration, therefore, establishes the cohort of Foxp3^{+} cells in which Foxp3 dynamics will be assessed. Starting from time $t = 0$ of the experiment, few days after drug 1 administration, mice are provided with food containing drug 2, which induces the RFP permanent labelling of CD4 T cells that, from time $t = 0$ on, do not express Foxp3. At any later time point, the cells belonging to the followed cohort, that have never lost Foxp3 expression since time $t = 0$, are labelled as GFP^{+}YFP^{+}RFP^{-}, the cells that have transiently lost Foxp3 expression are labelled as GFP^{+}YFP^{+}RFP^{+}. Finally, the cells that have lost Foxp3 expression without re-acquiring it are labelled as GFP^{-}YFP^{+}RFP^{+}. The frequency of GFP^{+}YFP^{+}RFP^{-} cells within the YFP^{+} cell cohort over time can be used to infer the Foxp3 residence time and the frequency of committed Tregs, as described in section 4.2.4 and Figure 4.8.

This transgenic mouse model would uncover putative transient loss and reacquisition of Foxp3 expression. Nevertheless, as predicted by our analysis, it would not be resolutive in case of permanent yet context-dependent Foxp3 expression. Let us explain it in more details.

Our model fitting analysis showed that experimental data are compatible with

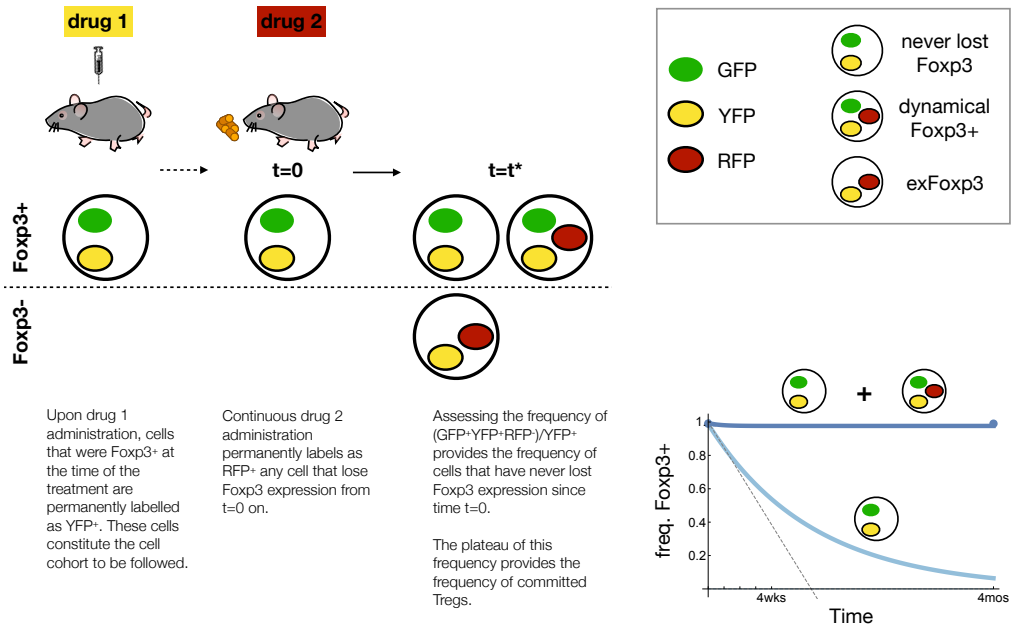

Figure 5.2: **Experimental design to assess dynamical Foxp3 expression in physiologic condition.** Mice are the transgenic mice model proposed in the main text. In them, Foxp3 expression is reported by GFP. Upon drug 1 administration Foxp3$^+$ cells are permanently labelled as YFP$^+$. These cells constitute the cell cohort in which Foxp3 dynamics will be assessed. Continuous drug 2 administration via food permanently labels as RFP$^+$ the cells that, from $t = 0$ on, are Foxp3$^-$. At any later time point $t = t^*$, GFP$^+$YFP$^+$RFP$^-$ cells are the ones that have never lost Foxp3 expression since $t = 0$; GFP$^+$YFP$^+$RFP$^+$ ones are cells that have transiently lost Foxp3 expression, GFP$^-$YFP$^+$RFP$^+$ ones are cells that have lost Foxp3 expression without re-acquiring it. The frequency of GFP$^+$YFP$^+$RFP$^-$ cells within the YFP$^+$ cell cohort over time can be used to infer the Foxp3 residence time τ_{foxp3} and the frequency of committed Tregs.

an infinite Foxp3 mean residence time in lymphoreplete conditions (see blue dots in figure 4.8 A for $\tau_{foxp3} > 100$). For this extreme case, the prediction is that there is something in lymphoreplete context that prevents the down-regulation of Foxp3, irrespective of the slow and stochastic epigenetic remodelling that takes place. Yet, for this case, the model also predicts that the fraction of committed Tregs would be revealed by performing an adoptive transfer experiment into lymphodeficient host.

In this scenario there is no longer need for two systems of inducible and permanent labelling. It is enough that cells can be sorted based on Foxp3 expression (due to GFP reporter, for instance) and that permanent RFP labelling can be induced in the CD4 T cells that lose Foxp3 expression. Furthermore, in case of co-transfer of Tregs, together with Teffs, the experiment requires the two cell cohorts to be distinguishable, based on genetic marker, for instance. This is necessary in order to label the cohort of Foxp3$^+$ cells in which Foxp3 dynamics will be assessed.

The experiment consists in co-transferring, into lymphodeficient host, Tregs, sorted from the transgenic mouse, together with Teffs, sorted from a different and distinguishable donor (see Figure 5.3). The co-transfer is performed in proportion than ensures stability of Tregs (*e.g.* 1 Tregs : 10 Teffs [Komatsu et al., 2009]). Right after the transfer, at time $t = 0$ of the experiment, the host mice are provided with food containing drug 2. Drug 2 continuous administration induces the permanent labelling of cells that do not express Foxp3, from time $t = 0$ on. At any later time point t^*, cells that have never lost Foxp3 expression since time $t = 0$ are labelled as GFP$^+$Thy1.1$^+$RFP$^-$, cells that have transiently lost Foxp3 expression in the time window $[0, t^*]$ are labelled as GFP$^+$Thy1.1$^+$RFP$^+$, cells that have lost Foxp3 expression after time $t = 0$ without reacquiring it, are are labelled as GFP$^-$Thy1.1$^+$RFP$^-$. The frequency of GFP$^+$Thy1.1$^+$RFP$^-$ cells within the Thy1.1$^+$ cell cohort over time can be used to infer the Foxp3 residence time and the frequency of committed Tregs, as described in section 4.2.4 and Figures 4.12 and 4.12.

The model predicts that, even in case Foxp3 expression in lymphoreplete condition context-dependent yet permanent (as the blue dots in 4.12), the fraction of committed Tregs would be revealed in this experiment in a reasonable amount of time (see red dots in figure 4.12 that correspond to blue dots for $\tau_{foxp3} > 100$).

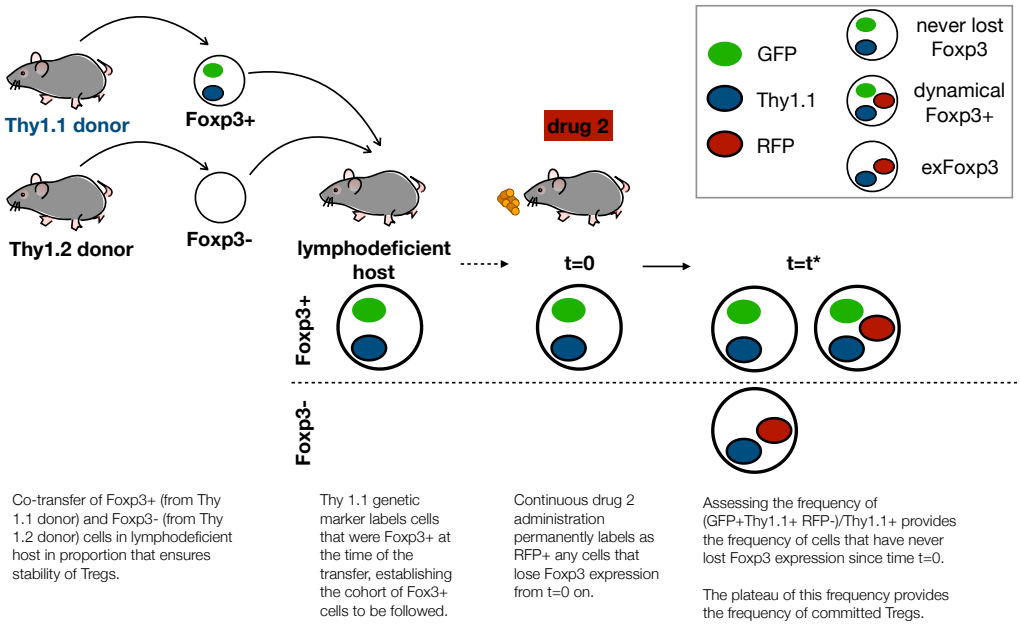

Figure 5.3: **Experimental design to quantify dynamical Foxp3 expression upon adoptive transfer.** Thy1.1 donor mice are the transgenic mice model proposed, in which Foxp3 expression is reported by GFP; Thy1.2 donor mice are Foxp3^{GFP} mice. GFP$^+$Thy1.1$^+$ CD4 T cells are sorted from Thy1.1 donor mice, while GFP$^+$Thy1.1$^-$ naive CD4 T cells are sorted from Thy1.2 donor mice. The two cohorts are transferred into lymphodeficient host mice in proportion (1:10), which ensures stable expression of Foxp3 in Foxp3$^+$ cells [Komatsu et al., 2009]. Thy1.1 genetic marker labels cell cohort in which Foxp3 dynamics will be assessed. Continuous drug 2 administration, via food, permanently labels as RFP$^+$ the cells that, from $t = 0$ on, are Foxp3$^-$. At any later time point $t = t^*$, GFP$^+$Thy1.1$^+$RFP$^-$ cells are the ones that have never lost Foxp3 expression since $t = 0$; GFP$^+$Thy1.1$^+$RFP$^+$ ones are cells that have transiently lost Foxp3 expression, GFP$^-$Thy1.1$^+$RFP$^+$ ones are cells that have lost Foxp3 expression without re-acquiring it. The frequency of GFP$^+$Thy1.1$^+$RFP$^-$ cells within the Thy1.1$^+$ cell cohort over time can be used to infer the Foxp3 residence time τ_{foxp3} and the frequency of committed Tregs.

5.7 Relationship between the repertoires of Tregs and Teffs

The analysis of populations dynamics carried out in chapter 2 motivates some considerations about Tregs repertoire. The model predicts that, in absence of thymic output, Teffs that are under the control of cognate Tregs, get progressively outcompeted by former Tregs that have lost Foxp3 expression. In other words, in thymectomised animals, the population of potential autoimmune Teffs cells eventually becomes constituted exclusively by exTregs. This means that, if one waits long enough after thymectomy, the sequence repertoire of Tregs would be included in that of Teffs.

This prediction depends on two model assumptions adopted in chapter 2. The first is that unstimulated Tregs lose Foxp3 expression with a constant rate, which means that all Tregs are plastic and there are no committed Tregs. The second assumption is that there is no up-regulation of Foxp3 in the periphery . As already mentioned, these assumptions are simplifications that we adopted with the aim of exploring, in the worst case scenario, what is the impact of Foxp3 lability in the maintenance of tolerance. The absence of committed Tregs is in part supported by the experimental data, according to the analysis of chapters 3 and 4, yet it remains an assumption which still lacks a definite empirical support. Under these two assumptions, as seen in chapter 2, as long the Foxp3 loss is slow, tolerance is not broken and Tregs coexist with Teffs populations they control. In the presence of thymic output, the Teffs that are controlled by Tregs, are of two kinds: the ones that come from the thymus, escaping the negative selection, and the ones that derive from Tregs that lost Foxp3 expression. Thymectomy would eliminate the source of thymic derived Teffs, which would be then gradually substituted by ex Tregs. This result could be experimentally tested, by sequencing TCR of Teff and Treg cells in adult thymectomised animals.

This prediction is compatible with previous claims. In the context of fate mapping (using the Foxp3$^{\text{GFP-Cre}}\times$ROSA26YFP transgenic mouse) Zhou et al. have shown that there is some overlap in the repertoire of exTregs and tTregs [Zhou et al., 2009]. Moreover, Sepulveda et al. suggested that Teffs thymocytes are more diverse than Tregs [Sepúlveda, 2011]. Yet their analysis did not rule out the possibility that Tregs and Teffs that are under Treg controll have the same

diversity.

What if any of the two assumptions is not fulfilled? The analysis of cellular Foxp3 dynamics (chapter 4) suggested the possibility that the net frequency of Tregs is dynamically maintained, with Foxp3 down- and up- regulation over time. If this is the case, in lymphoreplete animals, the slow Foxp3 loss to which un-stimulated Tregs are subjected is counter balanced by a context-dependent up-regulation of Foxp3. Furthermore, in line with the results of chapter 3, the possible up-regulation over time of Foxp3 would only affect the subset of CD4 T cells that we called context-dependent. Yet, we cannot rule out the possibility that even in case Foxp3 expression was dynamical, thymectomy would determine that the compartment of auto-reactive Teffs will be constituted by cells that at some point had expressed or will express Foxp3. Going back to the figure 3.7, this eventuality corresponds to an expansion, upon thymectomy, of the compartment depicted in light grey at the expenses of the dark one.

Also, there is another factor that could determine progressive substitution of the auto-reactive T cells by exTregs upon thymectomy, even under dynamical maintenance of the Foxp3$^+$ population. For this, it is worth to remember that, according to the population dynamics, in case the Tregs and cognate Teffs are in tolerance equilibrium, Tregs are characterised by a higher density than the Teffs they control. This result was first found in the original cross regulation model [Carneiro et al., 2007] and still holds in case of context-dependent Foxp3 expression (see chapter 2), where Tregs are subjected to continuous Foxp3 loss. High density of Tregs could favour the exTregs, whose source are the Tregs, in the competition against thymic derived auto-rective Teffs.

Further analysis of the population dynamics could shed some light on conditions required for competitive exclusion of auto-reactive Teffs by exTregs upon thymectomy.

Finally, in case there was a core of Tregs that is not subjected to any Foxp3 loss and in case their difference with respect to the context-dependent Tregs rely on TCR repertoire, then the sequence diversity in TCR of committed Tregs, would not be included in Teffs repertoire. This is another prediction that could be experimentally tested.

5.8 Foxp3$^-$ committed CD4 T cells and peripheral Foxp3 induction

As already mentioned, in chapter 2, we did not assume peripheral Tregs differentiation, mainly as a first approximation. On the other hand, one of the main results of chapters 3 is that the large majority of CD4 T cells are prevented from up-regulating Foxp3 in the periphery, and a main result from chapter 4 is that stable Foxp3 expression in lymphoreplete condition is likely achieved in a dynamical way that includes transient down-regulation of Foxp3 expression.

Modigliani *et al.* have proposed that when naive cells exit the thymus there is a limited period of time during which they can be "educated" to become Tregs, after which the cells lose this ability [Modigliani et al., 1996]. This proposal is in line with the finding that Recent Thymic Emigrants are the preferential precursors of Tregs which differentiate in the periphery [Paiva et al., 2013]. Also, Tregs that have lost Foxp3 expression can reacquire it *in vivo* if opportunely stimulated [Komatsu et al., 2009, Hori, 2014]. However, it is unclear whether this ability is confined to a limited time window after the Foxp3 loss.

More generally, it is known that CD4 T cells differentiate in the periphery in response to environmental cues such as TCR ligands stimuli and cytokines. Accordingly, naive T cells can differentiate *in vitro* into Th1 or Th2 type, if the appropriate cytokines are provided in the culture media, together with anti-CD3 and anti-CD28 antibodies. Cells that have differentiated *in vitro* into Th1 type and are subsequently switched into pro-Th2 medium, reprogram toward Th2 type, provided the change of medium is done early enough, but not later. The same happens for Th2 induced cells that can be reprogrammed to become Th1. These evidences fit with the more general idea that changing gene profiles and reprogramming in response to environmental cue is an ability that decreases with cell maturation and as cells differentiate along alternative pathways.

Also in case of Tregs, Foxp3$^+$ T cells stimulated *in vitro* together with anti-CD3 and CD28 antibodies and Th cell polarising cytokines, have shown how cytokines signals determine exFoxp3 fate. Several Th canonical like phenotypes have been observed, together with promiscuous phenotypes. Anti-TGF-β leads to IL-2 producing exFoxp3 cells [Komatsu et al., 2009]; IL-4 stimulations leads to IL-4 producing Th2-like exFoxp3 T cells, IL-6 stimulation leads to IL-17 se-

creting Th17-like exFoxp3 T cells; finally IL-2 stimulation does not affect Foxp3 expression, but induces the promiscous phenotype of INF-γ^+ Foxp3$^+$ T cells [Wei et al., 2009, Koch et al., 2012, Zhao et al., 2012].

A different view, arises from the modelling of the gene regulatory network underlying the CD4 T cells differentiation program in response to distinct micro-environments by Naldi et al.. They found hybrid phenotypes as transient states of the network, in a context-dependent way [Naldi et al., 2010, Abou-Jaoudé et al., 2015]. This result might contrast with the previous view, in the sense that it admits the possibility of transitioning from one phenotype to another one regardless the maturation state of the cells, as long as the external cues are available. Yet, it is worth to remember that gebe regulatory networks do not model cell maturation, nor time evolution.

Going back to the cells that we called committed Foxp3$^-$ as they cannot up-regulate Foxp3. The inability to express Foxp3 could be seen as a cell intrinsic property, either in terms of maturation and limited time window for plasticity, or in terms of up-regulation of other master genes that inhibit the up-regulation of Foxp3. Another possibility is that the incapacity of these cells to up-regulate Foxp3 is a cell extrinsic property, in the sense that depend on the context. This would imply that, although the cells could be induced to express the Foxp3 even later, the context is not able to provide enough pro-Foxp3 stimuli to the majority them, that continue their differentiation toward other phenotypes. This could reconcile the results of the gene regulatory network with the evidences of promiscuous phenotypes observed *in vivo* in pathologic conditions and the one that can be induced *in vitro*, where the conditions can be forced to resemble the pathologic ones. In any way, our modelling cannot discriminate between the two possibilities.

5.9 Concluding remarks

Robustness of tolerance can be explained as a result of non-linear and density dependent dynamics among populations of Teffs and Tregs of similar specificities, mediated by populations of cognate APCs ([Carneiro et al., 2007], here chapter 2). The CRM predicted two main subsets of clones in which CD4$^+$ T cells repertoire in the periphery is divided. A first set of highly diverse clones

that barely react to self-antigens and most likely are responsible for mounting immune responses against pathogens: their clonal expansion is limited and governed by the APCs availability; and a second set of less diverse clones that are auto-reactive. The set of auto-reactive clones contain both Tregs and Teffs: Teffs are the ones that can potentially cause autoimmunity by orchestrating the immune response against the bodies organs and tissues, whereas Tregs control their expansion, supported by high density of APCs presenting auto-antigens.

The main contribute of the present work is to propose that, within the entire pool of CD4 T cells, not all the cells are committed to be either Teffs, thus able to determine the cascade of events that give rise to immune responses, or Tregs, thus able to suppress the activation and clonal expansion of other immune cells. A large fraction of CD4 T cells is committed to be Foxp3^{-} and in no case can up-regulate Foxp3 and become suppressive. Within this compartment, we expect the majority of cells to be barely auto-reactive (therefore to belong to the first set of clones described) and only a minority to be the auto-reactive clones whose expansion is controlled by Tregs.

The remaining CD4 T cells, except at most a minor fraction of cells that are committed to Foxp3 expression, are auto-reactive cells whose phenotype and function is strongly determined by the context. Namely, for these cells, the inputs they receive from the density-dependent interactions ultimately determine their phenotype.

This means that for the majority of Tregs, that in lymphoreplete condition are observed to stably maintain Foxp3 expression and suppressive function, their stability is context-dependent. Also we have shown that stability of Foxp3 expression, is to be understood in a dynamical way, in the sense that within a stable cohort of Tregs, actually the cells transiently lose Foxp3 expression in a stochastic way, and quickly revert to the Foxp3^{+} state. In case the pro-Foxp3 stimuli are not adequately provided by the interactions, Tregs stop being suppressive and become pathogenic.

In this sense Treg identity, exemplified by stable Foxp3 expression which enables the suppressive function, is determined by the context, namely depends on the interactions Tregs make with the other cells of similar specificity. Treg identity ultimately depends on the population densities themselves.

The fact that Foxp3 expression depends on the context for large part of Tregs,

plays an important role in the robustness of tolerance: as long as the rate of Foxp3 loss is slow, plasticity of Tregs determines a smoother restoration of homeostasis after perturbation as compared to a scenario of purely committed Tregs. Furthermore, the slow time scale characteristic of Foxp3 expression loss, in Tregs that do not receive enough pro-Foxp3 stimuli, can be explained by the epigenetic remodelling of the Foxp3 locus. Data are compatible with epigenetic mechanisms being consequences of the transcriptional state of the cells, without being necessarily and directly determined by the inputs the cells receive.

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ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

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