CD4+ T cell homeostasis: the thymus, the cells and the environment

Afonso Almeida

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$CD4^+$ T CELL HOMEOSTASIS: THE THYMUS, THE CELLS AND THE ENVIRONMENT

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PRESENTED BY/APRESENTADA POR

Afonso Rocha Martins de Almeida

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Jury composition/Composição do Júri:

Professor Maria de Sousa (co-supervisor/co-orientadora)
Professor António Freitas (supervisor/orientador)
Professor Anneliese Schimpl
Doctor Margarida Correia-Neves
Doctor Alexandre Carmo

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SUMMARY-RESUMO-RÉSUMÉ

SUMMARY

The peripheral T cell number is under homeostatic control. Although T cells are produced daily in the thymus in significant numbers, and antigenic stimulation induces further T cell production through division, the peripheral T cell numbers are maintained constant. Thus, any newly produced T cell will only integrate the peripheral T cell pools if another T cell dies. In this way, the selection of the peripheral T cell repertoires is not only dependent on the interaction between each cell and its antigen but it is also dependent on interactions between different sub-populations of cells (Freitas and Rocha, 2000). In order to understand how peripheral T cell homeostasis is achieved, and to understand why is homeostasis attained at the observed equilibrium level, each of the contributing agents must be identified and the contribution of each determined.

T cells are originated in the thymus, resulting from a complex series of events. The thymic T cell production will be responsible throughout the lifetime of the organism for the daily export of newly generated T cells. However, the thymus involutes with age. This may have consequences for peripheral T cell homeostasis. Thus, the central T cell compartment should not be left out when peripheral T cell homeostasis is studied.

The peripheral T cell compartment comprises a number of smaller compartments, as not all T cells are born equal and many differentiate in the peripheral T cell pools into specific sub-populations with distinct proprieties and functions. The $CD4^+$ and the $CD8^+$ T cell subsets and the naïve, effector and memory compartments within the $CD4^+$ and the $CD8^+$ T cell compartments contribute differently for the individual's immunocompetence. Mechanisms involved in the control of the relative proportions of these sub-populations are also relevant for total T cell homeostasis.

The purpose of this thesis was to make advances in the understanding of the mechanisms responsible for peripheral T cell homeostasis in general and for peripheral $CD4^+$ T cell homeostasis in particular. The studies were divided into three parts.

In the first part, the Thymus and its role in the maintenance of T cell numbers were evaluated. We developed a novel experimental system that allowed us to obtain a quantitative assessment of the fraction of competent pre-T cell precursors required to restore thymus function and also to evaluate the contribution of the thymus to the peripheral T cell pools. With the help of a mathematical model we were able to interpret the data obtained in order to demonstrate that there are no compensatory homeostatic mechanisms during thymic development and that the size of the peripheral total T cell pool is fairly independent of thymic output. Thus, peripheral mechanisms compensate for a lack of thymic output. When the naïve and activated/memory T cell compartments were analysed separately, we found that the naïve T cell compartment was more prone to reflect the size of the thymic SP compartment. Thus, we concluded that these compensatory mechanisms are more efficient in the generation of activated/memory T cells.

In the second part, the subject of research was the importance of peripheral T cell interactions for the establishment of peripheral T cell homeostasis. We have studied the interactions between the $CD4^+CD25^+CD45RB^{low}$ T cells (the regulatory/suppressor $CD4^+$ T cells) and $CD4^+CD25^-CD45RB^{high}$ T cells (naïve $CD4^+$ T cells). We transferred these populations into immunodeficient hosts. We have

observed that the ratio $CD4^+CD25^+CD45RB^{low}$ to $CD4^+CD25^-CD45RB^{high}$ T cells present in the cells transferred was determinant for the numbers of cells recovered, and thus this interaction potentially determinant for peripheral T cell homeostasis. We have demonstrated this, re-introducing the $CD4^+CD25^+$ T cells in a mouse system (the $CD25^{-/-}$ BM chimeras) were the peripheral homeostasis is disturbed and this sub-population is absent. As we observed, the re-introduction of the $CD4^+CD25^+$ T cells in these BM chimeras had as a consequence the normalisation of the peripheral T cell pools. We have found proof that the presence of this sub-population is essential for the existence of homeostasis of the peripheral T cell numbers and thus, that peripheral T cell homeostasis is achieved also through sub-population structure.

In the third part of these studies, the importance of resources for the maintenance of the peripheral T cell sub-population structure was examined. Immediate candidates as resources are interleukins. The $IL2^{-/-}$ mice have reduced numbers of $CD4^+CD25^+$ T cells and develop autoimmune manifestations We postulated that the lack of IL2 was responsible for the decreased survival of the $CD4^+CD25^+$ regulatory T cells in the peripheral T cell pools, and thus that the autoimmune manifestations were again the consequence of a disruption in the peripheral sub-population structure, as these mice are devoid of this specific sub-population. We tested this hypothesis, by sorting the few $CD4^+CD25^+$ T cells present in the $IL2^{-/-}$ mice and testing these cells as suppressors *in vivo*. These cells proved to exert suppressor functions, suggesting that the $IL2^{-/-}$ mice are able to generate the $CD4^+CD25^+$ regulatory T cells. We confirmed this, by establishing BM chimeras using as donor BM cells a mixture of BM cells from $IL2^{-/-}$ and $CD25^{-/-}$ cells. These chimeras do not develop autoimmune manifestations and the peripheral T cell pools have the normal representation of the $CD4^+$ T cell sub-populations, including the $CD4^+CD25^+$ T cells. Thus, the $IL2^{-/-}$ BM precursors were able to generate a viable population of regulatory T cells, as long as IL2 was present in the periphery. This illustrates the role of cytokines as resources with a major importance for the establishment of the observed peripheral sub-population structure.

Returning to the main subject of this thesis, our results allow us to state that the observed peripheral T cell homeostasis reflects not only the thymic production but also peripheral phenomena, and that these include interactions between different sub-popoulations. Underrepresented peripheral sub-populations, like the $CD4^+CD25^+$ regulatory T cells, play a major role in the maintenance of peripheral T cell homeostasis.

RESUMO

Os números de células T periféricas são mantidos sob controlo homeostático. Apesar de as células T serem produzidas diariamente em números consideráveis no timo, e de novas células T serem geradas à periferia devido a estimulação antigénica, os números de células T periféricas são mantidos constantes. Assim sendo, uma nova célula poderá apenas integrar o compartimento periférico de células T substituindo uma outra. Por conseguinte, a seleção dos repertórios periféricos de células T não está apenas dependente da interação de cada célula com o antigénio, estando também dependente de interações com outras células (Freitas and Rocha, 2000). Para compreender a homeostasia das células T é necessário identificar os agentes intervenientes no processo e quantificar a contribuição de cada um deles.

As células T sao originadas no timo. A produção tímica de células T será exportada para a periferia, correspondendo a um potencial acrescento diário de novas células T. Por esta razão, é importante ter em conta a contribuição do timo para a homeostasia das células T periféricas.

O compartimento de células T periféricas inclui numerosos outros compartimentos, uma vez que diferentes categorias de células T são geradas no timo e, à periferia, é possível a diferenciação das células T em diversas outras sub-populações com características próprias. Assim, é importante diferenciar os compartimentos de células T $CD4^+$ e $CD8^+$ e, dentro destes, compartimentos naive ou activados/memória. Estas sub-populações contribuem diferentemente para a imunocompetência do indivíduo. Por esta razão, é também importante ter em conta os mecanismos involvidos no controlo das proporções relativas destas sub-populações.

O objectivo deste trabalho é contribuir para a compreensão dos mecanismos responsáveis pela homeostasia das populações de células T periféricas em geral, e das populacões de células T $CD4^+$ em particular. Os trabalhos foram divididos em três partes.

Numa primeira parte, o papel do timo na manutenção dos números de células T foi investigado. Desenvolvemos um novo sistema experimental que nos permitiu não apenas a avaliação da fração de células pre-T competentes necessária para assegurar a função tímica mas também a avaliação da contribuição da produção tímica de células T para a manutenção dos diferentes compartimentos de células T periféricas. Fomos capazes de demonstrar que não existem mecanismos de compensação homeostática durante o desenvolvimento tímico e que os números totais de células T periféricas não reflectem o número de células T exportadas pelo timo, o que implica a existência à periferia de mecanismos capazes de compensar a redução nos números de células exportadas pelo timo. Obtivemos também dados que nos permitiram concluir que os mecanismos compensatórios referidos são mais eficientes na geração de células T activadas/memória.

A segunda parte deste trabalho está relacionada com a relevância das interações entre populações de células T periféricas para a homeostasia das células T periféricas. Investigámos as interações entre as sub-populações $CD4^+CD25^+CD45RB^{low}$ (células reguladoras ou supressoras) e as células $CD4^+CD25^-CD45RB^{high}$ (células naives), transferindo as duas populacões isoladamente ou em conjunto para animais imunodeficientes. Observámos que a razão $CD25^+/CD25^-$ transferida é

determinante para o número de células T $CD4^+$ recuperadas nestes animais. Assim, esta interação pode estar implicada no estabelecimento da homeostasia das células T periféricas. Fomos capazes de demonstrar a importâcia da presença desta sub-população reguladora para a manutenção do equilíbrio normal dos números de células T $CD4^+$ periféricos, reintroduzindo a população $CD4^+CD25^+$ en animais quimeras de medula óssea ($Rag_2^{-/-}$ reconstituídos com células de medula óssea de dadores $CD25^{-/-}$) onde esta população está ausente. Estes animais apresentam distúrbios homeostáticos graves, que resultam no desenvolvimento de doenças autoimunes acompanhadas de importantes acumulações de linfócitos. A reintrodução da sub-população de células reguladoras é suficiente para impedir estas anomalias, restitutindo a normalidade às populações de linfócitos T $CD4^+$ periféricas.

Na parte final da tese, a relevância dos recursos para a manutenção da estructura populacional das células T $CD4^+$ periféricas foi investigada. A proporção de células $CD4^+CD25^+$ é reduzida em animais $IL2^{-/-}$. Estes animais apresentam um fenótipo semelhante ao dos animais $CD25^{-/-}$. Formulámos a hipótese de a falta de IL2 à periferia nos animais $IL2^{-/-}$ ser a causa da redução na proporção de células $CD4^+CD25^+$ observada e de ser esta a causa das anomalias verificadas nos animais IL2. Testámos esta hipótese, separando as células $CD4^+CD25^+$ existentes nos animais $IL2^{-/-}$ e testando estas células enquanto células supressoras *in vivo*. Verificámos que estas células são capazes de exercer funções supressoras. Estabelecemos também quimeras de medula óssea utilizando animais hospedeiros $Rag_2^{-/-}$ irradiados e reconstituindo estes com misturas de células de medula óssea provenientes de animais $CD25^{-/-}$ e $IL2^{-/-}$. Estas quimeras não apresentam manifestações de natureza autoimune e a observação da composição das populações de células T $CD4^+$ periféricas revela a presença da proporção normal de células reguladoras $CD4^+CD25^+$, que apenas podem ter origem nas células de medula óssea provenienetes de dadores $IL2^{-/-}$. Demonstrámos assim que os percursores $IL2^{-/-}$ são capazes de gerar uma população normal de células $CD4^+CD25^+$, na condição de a IL2 estar presente à periferia. Estes resultados demonstram o papel dos recursos, neste caso da IL2, no estabelecimento da estructura populacional observada nos compartimentos de células T periféricos.

No seu conjunto, os resultados obtidos permitem-nos afirmar que a homeostasia das populações de células T observada não é apenas o resultado da exportação de células T por parte do timo, sendo também o resultado de fenómenos que ocorrem à periferia, nomeadamente, interações entre as diferentes sub-populações. Populações com fraca representação, tal como as células reguladoras $CD4^+CD25^+$, são determinantes para o estabelecimento do equilíbrio homeostático dos números de células T $CD4^+$ periféricos.

RÉSUMÉ

Chez les vertébrés adultes, le nombre de cellules T périphériques est soumis à un contrôle strict. Bien qu'un grand nombre de cellules T soient produites chaque jour, le nombre de cellules T à la périphérie reste constant. Chaque nouveau lymphocyte T produit ne pourra donc s'établir à la périphérie qu'après la mort d'un lymphocyte déjà établi. Dans cette optique, la sélection du répertoire des cellules T périphériques n'est pas uniquement dépendant des interactions entre une cellule et son antigène, mais elle est également dépendante d'interactions entre différentes sous-populations cellulaires (Freitas and Rocha, 2000). Dans le but de comprendre comment l'homéostasie des cellules T périphériques est atteinte, et afin de comprendre pourquoi l'homéostasie parvient à un certain niveau d'équilibre, il est important de déterminer chaque facteur intervenant dans ce processus et la contribution de chacun d'entre eux.

Les cellules T sont générées dans le thymus. La production thymique de cellules T est responsable, chaque jour, de l'export de nouvelles cellules T. Cependant la taille du thymus n'est pas constante, puisque le thymus involue avec l'âge, ce qui peut avoir des conséquences sur l'homéostasie des cellules T périphériques. Il faut donc tenir compte du compartiment T central, le thymus, lorsque l'on étudie l'homéostasie des compartiments périphériques.

Le compartiment T périphérique est composé d'un certain nombre de sous-compartiments, puisque chaque cellule T n'est pas identique, et de nombreuses cellules vont se différencier à la périphérie en des sous-populations spécifiques, possédant des fonctions et des propriétés différentes. Par exemple, les sous-populations $CD4^+$ et $CD8^+$ devront être considérées séparément puisqu'elles sont impliquées dans différents types de réponses immunitaires et ont des mécanismes d'action différents. De la même façon, les compartiments naïfs, effecteurs et mémoires contribuent différemment à l'immuno-compétence de chaque individu. Les mécanismes impliqués dans le contrôle du nombre de chacune de ces sous-populations sont également importants pour la compréhension de l'homéostasie cellulaire T totale.

L'objectif de cette thèse a été de comprendre les mécanismes responsables de l'homéostasie périphérique T en général, et de l'homéostasie des cellules T $CD4^+$ périphériques en particulier. Ce travail a été divisé en trois parties.

Dans la première partie de cette thèse, nous avons évalué le rôle du thymus dans la maintenance du nombre de cellules T. Nous avons développé un nouveau système expérimental nous permettant d'obtenir une estimation quantitative de la fraction des cellules précurseures pré-T compétentes, nécessaire pour assurer la fonction thymique mais aussi d'évaluer la contribution thymique à l'établissement du compartiment T périphérique. Nous avons montré qu'il n'existe pas de mécanismes homéostatiques compensatoires au cours du développement thymique. Ce résultat nous a ensuite conduit à évaluer l'effet d'un export thymique réduit sur l'établissement du compartiment T périphérique. Nous avons montré que la taille du compartiment T périphérique est indépendante du thymus, suggérant que des mécanismes compensatoires se mettent en place à la périphérie. Lorsque nous avons étudié les compartiments naïfs et activées/mémoires séparément, nous avons observé

que les mécanismes compensatoires sont plus efficaces pour les sous-populations activées/mémoires.

Dans la seconde partie de cette thèse, nous avons étudié le rôle des interactions entre les cellules T périphériques dans l'établissement de l'homéostasie T périphérique. Nous avons analysé l'interaction entre les cellules T $CD4^+CD25^+CD45RB^{low}$ (également appelées cellules T $CD4^+$ régulatrices) et les cellules $CD4^+CD25^-CD45RB^{high}$, dans des expériences de transfert chez la souris. Nous avons observé que le ratio entre les cellules $CD4^+CD25^+CD45RB^{low}$ et les cellules $CD4^+CD25^-$ $CD45RB^{high}$ transférées était déterminant pour le nombre de cellules recouvrées suggérant donc que l'interaction entre ces deux populations pourrait être déterminante pour l'homéostasie périphérique T. Nous avons testé cette hypothèse en transférant des cellules T $CD4^+CD25^+$ dans un modèle murin (les chimères de moelle osseuse $CD25^{-/-}$) où l'homéostasie périphérique est perturbée et où cette sous-population $CD25^+$ est absente. Nous avons observé que la présence de ces cellules T $CD4^+CD25^+$ dans ces chimères de moelle osseuse a pour conséquence la normalisation du compartiment T périphérique. Nous avons montré donc que l'homéostasie des cellules T périphériques est atteinte aussi grâce à la structure des sous-populations qui la constitue.

Dans la troisiéme partie de cette thèse, nous avons étudié l'importance des ressources pour la maintenance de la structure des sous-populations T périphériques. Il a été montré que le nombre de cellules T $CD4^+CD25^+$ est réduit chez les souris invalidées pour le gène de l'IL-2. Il a aussi été montré que ces souris développent des maladies auto-immunes avec des caractéristiques communes à celles développées par les souris $CD25^{-/-}$. Nous avons fait l'hypothèse que le manque d'IL-2 serait responsable de la diminution de la survie des cellules T régulatrices $CD4^+CD25^+$ dans le compartiment T périphérique, et que donc les manifestations auto-immunes seraient la conséquence de la perturbation de la structure des sous-populations périphériques, puisque ces animaux ne contiennent pas cette sous-population spécifique. Nous avons testé cette hypothèse en triant les quelques cellules T $CD4^+CD25^+$ présentes chez les animaux $IL2^{-/-}$ et en testant leur fonction de cellules suppressives *in vivo*. Ces cellules ont montré leur capacité à exercer une fonction suppressive, suggérant que les souris $IL\text{-}2^{-/-}$ sont capable de produire des cellules régulatrices T $CD4^+CD25^+$. Nous avons confirmé ces résultats en établissant des chimères de moelle osseuse, avec des cellules provenant de la moelle osseuse d'animaux $IL\text{-}2^{-/-}$ et d'animaux $CD25^{-/-}$. Ces animaux chimériques ne développent pas de maladies auto-immunes et le compartiment T périphérique est constitué d'une proportion normale des différentes sous-populations $CD4^+$, notamment les $CD4^+CD25^+$. Les précurseurs issus de la moelle osseuse des animaux $IL\text{-}2^{-/-}$ ont donc été capables de générer une population viable de cellules T régulatrices, capable d'utiliser pour leur survie l'IL-2 produite de façon paracrine. Ces résultats illustrent bien le rôle des cytokines comme ressources majeures, notamment pour l'établissement de la structure des populations périphériques.

L'ensemble des résultats obtenus au cours de cette thèse nous a conduit à formuler que l'homéostasie des cellules T périphériques est le résultat, non seulement de l'impact thymique, mais aussi de mécanismes périphériques. Les populations sous représentées, comme la population de cellules T régulatrices $CD4^+CD25^+$, pourraient exercer un rôle important dans la maintenance de l'homéostasie des cellules T à la périphérie.

ABBREVIATIONS

AICD- Activation Induced Cell Death

Ag- Antigen

APC- Antigen Presenting Cell

BM- Bone Marrow

BrDU- Bromodioxyuridine

CFSE- 5,6-Carboxyfluorescein diacetate succinimidil ester

CLP- Common Lymphoid Precursor

DC- Dendritic Cells

DN- Double Negative

DP- Double Positive

IBD- Inflammatory Bowel Disease

FTOC- Fetal Thymus Organ Culture

HSA- Heat Stable Antigen

HU- Hidroxyurea

MHC- Major Histocompatibility Complex

NK- Natural Killer

RTE- Recent Thymus Emigrants

SP- Single Positive

TCR- T Cell Receptor

Tg – Transgenic

TN- Triple Negative

TREC- Thymus Recombination Excision Circles

WT- Wild Type

SECTION A

INTRODUCTION

1- RELEVANCE OF LYMPHOCYTE HOMEOSTASIS

The ability of the Immune system to cope with infections and to do it safely is directly related to the individual's lymphocyte pool at any given time instant and throughout the individual's lifetime. As the total number of lymphocytes is kept constant, it follows that any new lymphocyte, in order to integrate the peripheral pool, must replace an existing one. As each lymphocyte bears a unique receptor, the specificities present in the peripheral pool of an individual will be the result of this selection. Thus, the immunocompetence of an individual is directly dependent on the mechanisms and rules responsible for the maintenance of lymphocyte numbers.

1.1- Homeostasis in the Immune System

As in complex ecological systems, the immune system shows a « return tendency, due to density dependent processes, to reach a stationary distribution of population densities » (Hanski, 1999). This is usually referred to as lymphocyte homeostasis (Freitas and Rocha, 2000).

Lymphocytes are produced daily in significant numbers in the Thymus (T cells) and Bone Marrow (B cells), and export from the primary lymphoid organs results in a daily input to the peripheral lymphocyte pools (Scollay and Godfrey, 1995). More cells are generated in the periphery as a result of antigen encounter or non-Ag dependent processes (Doherty et al., 1997; Tough et al., 1996). As numbers are kept constant, we must assume that an equal number of cells leave the peripheral lymphocyte pool, due to migration into tissues or death (Freitas and Rocha, 2000). To predict which cells will be able to integrate the peripheral lymphocyte pools and to understand why these pools are kept constant at size n=x and not n=y we must identify the mechanisms responsible for the homeostatic regulation of peripheral lymphocyte numbers.

1.2- B and T cell pools represent lymphocyte pools with independent homeostatic regulation

The first sub-division of the peripheral lymphocyte pool is a separation between the peripheral T and B cell pools. These two lymphocyte sub-populations are responsible for the specific immune responses and are thus essential for the efficacy of the immune system, providing an adaptive immune response. They differ in the ontogeny and also in the nature of their effector mechanisms. Thus, B cells are generated in the BM (Bursa in birds) while T cells owe their name to their thymic origin. The B cell effector mechanisms are dependent on the production of antibody, while T cell effector mechanisms are more diverse and include direct cell-cell interactions but also production and release of soluble factors (cytokines) (for an overview see Janeway et al., 1999). In addition, these T and B populations also have independent homeostatic regulations. This conclusion can be drawn from the simple observation of B cell numbers in animals devoid of T cells ($CD3\varepsilon^{-/-}$ (Malissen et al., 1995) or $TCR\alpha^{-/-}$ (Mombaerts et al., 1992a) mice). The fact that B cell numbers in these animals do not significantly differ from those found in normal mice strongly suggests that the homeostatic regulation of the B cell pool is independent of the presence or absence of T cells. This conclusion is further supported by the fact that in the inverse situation, *i.e.* in animals devoid of B cells (μKO), the size of the T cell pool is not significantly affected either (Kitamura et al., 1991). This observation will allow us to split the problem of peripheral lymphocyte homeostasis in peripheral B cell homeostasis and peripheral T cell homeostasis. The latter will be the object of this thesis.

Most of the information we dispose of today concerning lymphocyte development or even peripheral selection and survival events comes from studies performed in the mouse model. The following sections concern the murine immune system, the direct object of this thesis, though pertinent human data may be referred to. In the final section of this thesis the implications for the human case will be briefly discussed.

PART I

T CELL GENERATION AND EXPORT

2- GENERATION OF T CELLS

The role of the thymus as the T cell production site was firstly observed after thymectomy studies. The observation was that after thymectomy, specific areas of the peripheral lymphois organs were absent. Subsequent thymic graft studies shwon that cells originating in the grafts were indeed preferentially found in the thymus-dependent areas of the peripheral lympohid organs. The existence of thymus-dependent and thymus-independent areas of the peripheral lymphoid organs led to the designation of the thymus-dependent cells as T cells (see early work revision in Parrott and De Sousa, 1971). Thus, although some T cells with an extrathymic origin exist (*e.g.* originated in the gut (Saito et al., 1998) the large majority of T cells originate in the thymus, developing from a BM derived precursor. The thymic T cell development occurs in a sequence of steps that correspond to a functional and morphological transformation. The resulting cell has a functional specific receptor and can have different classes of effector mechanisms. These cells are exported to the periphery.

2.1- The Thymus: Histology

The Thymus consists of numerous lobes. We can distinguish in each an outer cortical region (thymic cortex) and an inner medulla (fig.1). A higher magnification shows the thymic stroma, consisting of epithelial cells and connective tissue, and the presence of scattered Macrophages and Dendritic Cells. The large majority of the other cells present are thymocytes, the developing T cells (fig.1). Thymocytes result from the expansion in the thymus of colonizing cells coming from the BM (see 2.2). During the course of their development thymocytes will distribute along the different areas of the thymus (fig.1). The more immature cells are found in the outer cortex, and the more mature ones in the medulla. A very brief description of the events involved follows in the next sections.

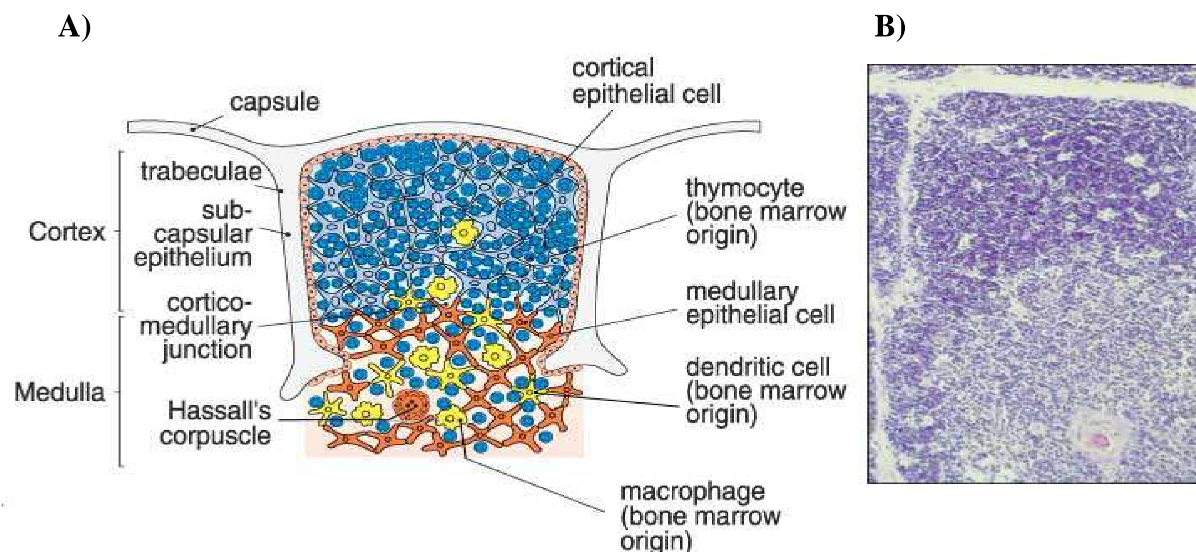

Figure 1- The Thymus: Its structure and its cellular content. The figure shows the histology of the organ (B), and the distribution of the different cell types in the identifiable areas (A).
(From Janeway, C. et al., 1999)

2.2- Colonization of the Thymus: Bone Marrow Precursors

The T cell precursors must migrate from the BM into a functional thymus in order to develop. This conclusion can be drawn from the nude mice analysis, a situation where a failure in the development of the thymus results in the almost complete absence of T cells. BM precursors from these mice are able to develop fully, when transferred into recipients with a normal thymus. Conversely, BM precursors from normal congenic mice are not able to generate T cells after transfer into nude mice (for an overview see Janeway et al., 1999). This sets up the basis for an interactive view of T cell development: the microenvironment has a crucial role on the delivery of the signals that will drive intrathymic T cell development (Anderson and Jenkinson, 2001; Savino et al. 2002).

An *i.v.* injection of 5 x 10^5 BM cells into irradiated mice is clearly sufficient to reconstitute the thymus of these mice, and the intrathymic injection of much larger numbers of donor thymocytes provides only transient reconstitution, confirming the requirement for BM derived precursors to achieve permanent thymic reconstitution (Scollay et al., 1986). While it is clear that there must be a thymic colonization by BM derived precursors, whether this occurs continuously (Scollay et al., 1986) or in waves (Foss et al., 2001) is still under debate, though the first view is clearly favoured.

2.3- Lineage commitment in lymphopoiesis

The nature of the BM precursor cell and the signals that drive its differentiation along the T cell pathway have been object of numerous studies. A common lymphoid precursor (CLP) has been identified, first as a cell with the ability to differentiate into the T and B cell lineages (Wu et al., 1991), then also into DCs (Ardavin et al., 1993) and NK cells (Kondo et al., 1997) but that it does not give origin to myeloid cells (reviewed in Akashi et al., 2000). This cell does not seem to have self-renewing ability, as reconstitution with CLPs provides only transient reconstitution (Akashi et al., 2000).

The branching in the development of the CLP into the different lymphoid lineages is dependent on the action of transcription factors (*e.g.* Pax-5 or GATA-3) (Nutt et al., 1999; Ting et al., 1996), transmembrane ligands or receptors (*e.g.* Notch and its ligands) (Pui et al., 1999; Radtke et al., 1999) and on cytokine signalling (*e.g.* IL7 or IL15) (Di Santo et al., 2000; Peschon et al., 1994). From now, only T cell development will be considered in this introduction. (For a more detailed description of the factors and mechanisms involved in lineage commitment see revisions in Akashi et al., 2000; Busslinger et al., 2000; Deftos and Bevan, 2000; Di Santo et al., 2000; Kuo and Leiden, 1999).

3- DEVELOPMENT OF T CELLS

After colonizing the thymus T cell precursors, upon interaction with the thymic microenvironment, will undergo a number of developmental changes, that are mostly related with the expression of the TCR ($\alpha\beta$ –the large majority- or $\gamma\delta$) and the assembly of its signalling apparatus (CD3). The TCR is a clonally variable $\alpha\beta$ heterodimer while the CD3 complex is a group of invariant polypeptides (γ, δ, ε, ζ and η). The TCR genes (both α and β chain genes) will go through a series of programmed rearrangements of germ line V D and J genes, a process referred to as V(D)J recombination (for an overview see Janeway et al., 1999). The enzyme responsible for these rearrangements is the RAG (1 and 2) and mice lacking this enzyme lack T (and B) cells (Mombaerts et al., 1992b; Shinkai et al., 1992).

We can characterize the developmental stages by the expression of cell-surface markers. The expression of the coreceptors CD4 and CD8 correlates with the state of development of T cells: from immature ($CD4^-CD8^-$ Double Negative) to mature ($CD4^+CD8^-$ or $CD4^-CD8^+$ Single Positive), passing through an intermediate immature ($CD4^+CD8^+$ Double Positive) stage (fig.2). When reaching the thymus, precursors do not express the markers characteristic of T cells and their receptor genes are not rearranged. At this stage, the

precursor cell can still differentiate into B cells, NK cells and αβ or γδ T lymphocytes (Akashi et al., 2000).

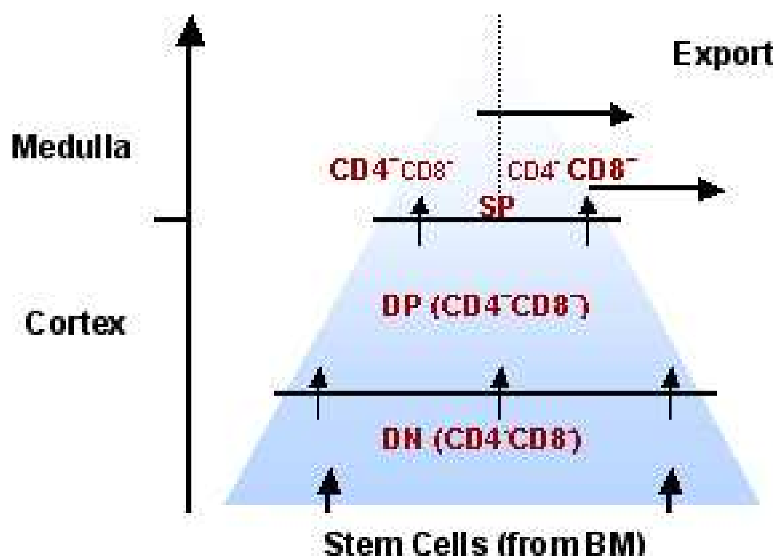

Figure 2: Differentiation along the T cell pathway in the Thymus

3.1- The Double Negative Thymic compartment

After the first interactions with the thymic microenvironment, the first rounds of proliferation will take place and characteristic markers (Thy1 and HSA in mice or CD2 in humans) of the T cell lineage will be expressed. At this point thymocytes are immature triple negative ($CD4^-CD8^-CD3^-$), or Double Negative (DN), regarding the coreceptors. DN thymocytes account for about 5% of the total number of thymocytes and comprise the more immature stages of T cell development, along with some other cells (some γδ and some minor populations of αβ T cells). 60% of DN thymocytes will develop into γδ or αβ T cells (for an overview see Janeway et al., 1999) (γδ T cells are a minority that we are not going to discuss further as they are not the object of this thesis).

The DN (or TN) immature thymocyte stage can be subdivided further with the help of cell surface markers, corresponding to sequential stages of T cell development. These markers are the adhesion molecule CD44 and the α chain of the IL2 receptor -CD25. The maturation sequence is: $CD44^+CD25^- \rightarrow CD44^+CD25^+ \rightarrow CD44^-CD25^+ \rightarrow CD44^-CD25^-$. These stages are also known as DN1, DN2, DN3 and DN4, respectively (or TN1, TN2, TN3 and TN4) (Godfrey and Zlotnik, 1993) (fig.3).

As mentioned previously, most of the changes occurring at this stage are related to the expression of the TCR. TCRβ gene rearrangement precedes TCRα rearrangement and starts at the DN3 stage, or at the transition into the DN3 stage (Godfrey and Zlotnik, 1993). It occurs in two consecutive steps, involving an initial D→J joining event, followed by a V→ DJ rearrangement. Depending on the ability to form a productive TCRβ rearrangement, a cell will proceed or not in it's development (β selection) (Godfrey and Zlotnik, 1993). The monitoring of this process is done through the expression of a preTCR, resulting from the association of the rearranged TCRβ chain with a preTα (pTα) chain and the CD3 complex molecules (von Boehmer and Fehling, 1997). The pTα is a surrogate chain that is encoded by a non-rearranging gene. The signalling provided by the preTCR seems to rescue DN3 cells from apoptosis (von Boehmer and Fehling, 1997). If the preTCR is not successively assembled, the T cell development is blocked at this DN3 stage. Consistent with this, the $Rag_2^{-/-}$ (Shinkai et al., 1992), unable to perform TCR gene rearrangement, and the $CD3\varepsilon^{-/-}$ mice (Malissen et al., 1995), unable to mount a functional CD3 complex, both display a developmental arrest at this stage.

The fraction of thymocytes producing in-frame β rearrangements has been calculated to be 5/9 (Malissen et al., 1992), so a large proportion of thymocytes fail in preTCR formation and do not proceed further in development. If the conditions apply, developing thymocytes will loose the CD25 expression and will acquire low levels of coreceptor expression. These events, along with extensive proliferation (see below for the quantitative aspects of thymic differentiation) characterize the DN4 stage that precedes the DP immature stage.

A summary of the events occurring in the DN Thymic compartment is presented in figure 3, including some markers not discussed in detail here.

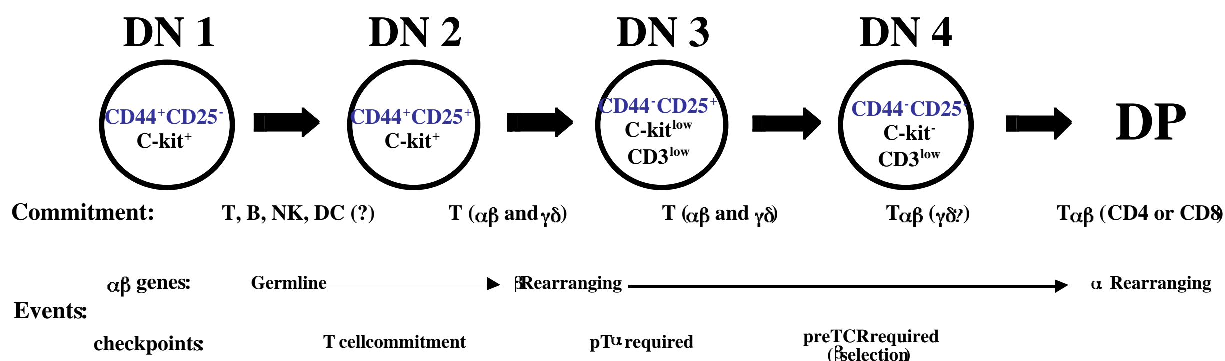

Figure 3:**Developmental events in the DN compartment** - Summary

3.2- The Double Positive Thymic compartment

After the final stage of development in the DN compartment, the TCRβ chains are rearranged and expressed, the CD3 complex is apparent at the cell surface and the coreceptors are expressed, rendering thymocytes Double Positive.

Each of the β selected cells can independently start to rearrange their α chain genes so that each productive TCRβ rearranged chain can test many different α chains for positive selection (see below). The expression of the preTCR is also responsible for the phenomenon of allelic exclusion, meaning that only one chromosome TCRβ chain will be expressed (von Boehmer and Fehling, 1997), and implying that T cells will express one single TCRβ chain. The TCR α chain genes do not have D gene segments, so recombination is done with Vα and Jα genes only and there is no allelic exclusion on the TCR α chain locus, so the two chromosome sequences will have the opportunity to rearrange, increasing the probability of producing a functional α chain. Therefore, many T cells will produce valid α chain rearrangements from both chromosomes, and will be able to express two different α chains (Malissen et al., 1992). This is also referred to as allelic inclusion of the TCRα chain locus. The TCR expressing cells will then pass through the processes of thymic selection.

3.2.1-Positive and Negative Selection

The T cell function is dependent upon the recognition by a given TCR of specific peptides bound to specific MHC molecules (see review on the history of the discovery of MHC restriction in Zinkernagel and Doherty, 1997). For a T cell to respond to a given Ag, the Ag must first be processed in the intracellular compartments of an Antigen Presenting Cell (APC) where it is coupled to MHC molecules. There are two classes of MHC molecules: Class I MHC molecules, which present peptides derived from intracytosolic antigens, and Class II MHC molecules that present peptides derived from antigens captured in vesicles (for an overview see Janeway et al., 1999). Class I molecules will be recognized by $CD8^+$ T cells. Class II MHC molecules will be recognized by $CD4^+$ T cells. MHC recognition determines the characteristic types of responses of T cells. Thus, the TCR must be MHC restricted, recognizing the presenting MHC molecules of the individual (Zinkernagel and Doherty, 1997) and should allow for enough diversity to be able to respond to unpredictable Ags. However, as the MHC can equally bind peptides derived from the individual itself (self peptides) it is equally important that the selected TCRs are selected in such a way that they do not respond to presented self peptides, as this would result in the destruction of the individual. The selected TCRs must be self- tolerant.

The processes that ensure both conditions occur in the thymus and are named positive (matching TCR with self MHC) and negative (deleting TCRs specific for self peptide-self MHC complexes) selection (for an overview see Janeway et al., 1999). The exact mechanisms and processes responsible for positive and negative selection, as well as the mechanisms that are responsible for $CD4^+$ vs $CD8^+$ T cell lineage commitment are complex, have been object of a large body of work and are still under intense investigation (see revisions in Benoist and Mathis, 1997; Sebzda et al., 1999; Amsen and Kruisbeek, 1998; Marrack and Kapler,1997; Hogquist, 2001). As these subjects are not directly related to the results and concepts that are central to this thesis, I will not describe them here.

3.3- The Single Positive Thymic Compartment

The final stages of thymic differentiation occur in the thymic medulla, where thymocytes are found after downregulation of one of the coreceptors. Phenotypically, SP thymocytes have been shown to undergo changes in the expression of cell-surface markers like CD24 (HSA), CD62L, Qa-2, CD69 and CD45RB (Lucas et al., 1994), and in chemokine receptors like CCR7 or CCR9 (Campbell et al., 1999; Norment et al., 2000; Wurbel et al., 2000). Thus, some differentiation events take place in the SP thymic stage. The thymic medulla has also been identified as a location of tolerance induction (Anderson and Jenkinson, 2001; Klein and Kyewski, 2000), though it is not clear if this means that positive/negative selection can occur at this stage or if tolerance in the thymic medulla is achieved through non-deletional processes (Anderson and Jenkinson, 2001). Interestingly, SP thymocytes have been shown to proliferate before export to the peripheral pool, an event that should increase the numbers of exportable cells (Ernst et al., 1995; Penit and Vasseur, 1997).

Hence, the final stages of the Single Positive pool represent the pool of selected lymphocytes and receptors that will be exported to the periphery and that will be responsible for the T cell immunocompetence of the individual. The importance of the size of this compartment and of the export rate will be discussed below.

3.4- Kinetics of T cell Development

The duration of all the developmental processes necessary to complete T cell development is of 4 to 5 weeks. Most studies, relying on DNA labelling techniques (namely $[H^3]$ Thymidine

(Egerton et al., 1990) or BrDU incorporation (Huesmann et al., 1991; Penit et al., 1995; Penit and Vasseur, 1997), have allowed the study of the duration of thymic development and have also allowed the study of the magnitude of expansion and the identification of the developmental stages where expansion is occurring.

The BrDU technique is now the most widely spread method to determine the proportion of cycling cells in a population. BrDU is a halogenated nucleotide that incorporates into the DNA as a thymidine analogue. As antibodies against the BrDU are available, the cycling cells can be identified. A single pulse of labelling is used to determine the fraction of cycling cells in a population, as well as the time taken for these pulse-labelled cells to progress to subsequent developmental stages. Continuous labelling studies are useful to determine the turnover time of a population, as labelled cells replace their unlabelled counterparts (Scollay and Godfrey, 1995). The principle of the [H^3] Thymidine technique is the same, but the detection method is different (Egerton et al., 1990). The majority of the most recent studies on these subjects use the BrDU incorporation method.

3.4.1- The DN compartment

During the early stages of thymocyte development, a small number of precursor cells will not only undergo developmental changes and choices but also massive expansion. The minimal number of BM cells capable of providing precursors for thymic reconstitution in irradiated animals has been estimated at about 3 x 10^5 cells (Scollay et al., 1986). The number of real T cell precursors responsible for thymus colonization has not been easy to evaluate, as reliable markers for these precursors are not available and they may develop into T cell committed precursors already in the thymus (Akashi et al., 2000). The DN developmental process seems to take about 2 weeks. It was found that after one BrDU pulse, 20 to 30 % of the whole DN compartment was $BrDU^+$ (Penit et al., 1995). At the most immature DN1 stage ($CD44^+CD25^-$), little division is taking place, with only 4% BrDU+ cells (Penit et al., 1995). An increase was found to occur at the DN2 ($CD44^+CD25^+$) stage, with 20 % of $BrDU^+$ cells after a single BrDU pulse. The majority of the labelled DN thymocytes were equally distributed between the DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) stages, as a result of the higher representativity of these later subsets. However, in the DN3 stage, a more reduced percentage of the cells incorporated BrDU, with only 10% of $BrDU^+$ cells. The highest proportion (35%) of dividing cells was found in the later DN4 stage, and this value was no different from the one found for the earliest of the DP cells ($CD4^{low}CD8^{low}$) (Penit et al., 1995). Theese results suggest that cell proliferation starts during or just after CD25 expression, stops after CD44 down-regulation (this fits with the fact that only a fraction of the DN3 cells will make productive βrearrangements and with the observed disappearance of an important

progeny of these DN3 stage cells) and restarts during CD25 loss (Penit et al., 1995). It had been calculated that in the early stages of Thymic development thymocyte precursors will expand in such a way that one single precursor will give rise to up to 4 000 daughter cells (Shortman et al., 1990). However, estimates made after analysis of the BrDU data referred above give an estimate in the order of a 300 fold expansion, corresponding to a total of 9 to 10 divisions (Penit et al., 1995). The differences found can be partially due to the different methods used (thymidine incorporation vs. BrDU) or to the difficulty in the evaluation of the number of thymic precursors that colonize the thymus (Shortman et al., 1990). A summary is shown in figure 4.

3.4.2- The DP compartment

After βselection, and as a direct consequence of βselection (Fehling and von Boehmer, 1997) the last proliferative stage of DN4 and of early DP thymocytes will take place. As a result of these early stage proliferative phases, the DP compartment will make up for about 85% of the total thymocyte number. The effects of positive and negative selection on the thymic transit duration and on thymocyte number have been evaluated in Tg and WT mice, using the BrDU technique. BrDU incorporation studies in normal C57Bl6 mice have clearly shown that the daily generation of DP thymocytes largely exceeds the generation of mature SP thymocytes (Huesmann et al., 1991). Similar studies performed using Tg mice have also revealed that the lifespan or the transit time of cells in the DP compartment is between 3 and 4 days, and the value of 3.5 days has been used to describe the duration of this procedure. It was possible too, to conclude from these studies, that positive selection occurs without cell division and that the same holds true for the DP→SP transition (Ernst et al., 1995; Huesmann et al., 1991). The linear kinetics observed in the cell-labelling experiments suggests that the bulk of the cells moves in the DP compartment as if in a conveyor belt, on a first in - first out basis (Scollay and Godfrey, 1995). When evaluating the efficiency of the selection processes, it is also obvious that the large majority of the cells will not be able to reach final maturation. Most DP thymocytes will die by neglect, as a result of a failure to produce a TCR that is able to react with self MHC-peptide complexes to originate TCR mediated signalling above lower limit threshold levels (reviewed in Sebzda et al., 1999). These thymocytes will undergo death by apoptosis (Surh and Sprent, 1994). The proportion of DP thymocytes that die by neglect has been calculated to be 90% (Egerton et al., 1990; Huesmann et al., 1991).

As referred above, the final SP repertoire is dependent on positive and negative selection. The proportion of the positively selected thymocytes transiting to the SP compartment has been estimated to be below 5% (Egerton et al., 1990).

3.4.3- The SP compartment

Thymocytes remain for as long as 2 weeks in the medullary stage of T cell development, before exit to peripheral pools (Egerton et al., 1990). The daily rate of production of SP thymocytes seems to represent 1% of the thymus cellularity (Egerton et al., 1990), a value that corresponds approximately to the daily rates of thymic export into the peripheral compartments (Scollay et al., 1980 and see chapter 6). It has been shown that thymocytes in the SP Thymic compartment can proliferate, a last thymic expansion phase that has been suggested to be responsible for an increase in the positively selected repertoire numbers before peripheral colonization (Ernst et al., 1995; Penit and Vasseur, 1997). This post selection expansion phase was suggested to be independent of TCR-MHC interactions and dependent on IL7R expression (Hare et al., 2000; Hare et al., 1998) and could originate an increase in the thymic output of up to 30% (Penit and Vasseur, 1997). In absolute terms, the rate of production of mature thymocytes has been calculated as 3% of the number of DP thymocytes, equivalent to 1% of the total thymocyte number (Egerton et al., 1990). This number is in agreement with estimates on thymic export (Scollay et al., 1980) (chapter 6). An attempt to give a general overview of T cell developmental kinetics is shown in figure 4.

DN1 → DN2 → DN3 → (70% †) DN4 → DP → (90% †) SP

	DN1	DN2	DN3	DN4	DP	SP	CD4 / CD8	Total
Absolute Numbers ($x10^6$)	≠0.04	≠0.04	≠3.6	≠3.6	≠170	≠22	≠16 / ≠6	200
% of Thymocytes	≠0.02	≠0.02	≠1.8	≠1.8	≠85	≠11	≠8 / ≠3	100
Proliferation (% cycling)	Low (4%)	High (20%)	Low (10%)	High (35%)	Low (10%)	Low (5%)		
Duration in the Compartment	8 Days		5 Days		3-4 Days	7-14 Days		30 Days

Figure 4: **Kinetics and quantitative aspects of T cell development**. Absolute numbers are calculated for a young adult mouse ($200x10^6$ thymocytes). The % of cycling cells is given as found after one single pulse labeling of BrDU. Data compiled from several studies (Egerton, 1990; Ernst, 1995; Huesmann, 1991; Lucas, 1994; Penit, 1995; Scollay, 1995).

4-HOMEOSTASIS WITHIN THE THYMUS

Though the sequence of events in thymocyte development is characterized in some detail, less detailed information is available that relates to the existence of homeostasis within the thymus. If the idea that the thymocyte number is kept under control is questioned by the fact that the thymus involutes with age (chapter 5), it is equally true that the events in thymic selection take place at a considerably faster time-scale. The many selection and expansion phases in thymic development could thus be the "target" for homeostatic processes, if the number of thymocytes was under control in any of the developmental stages. One group has assessed this directly, and reached the conclusion that the mature $CD8^+$ but not the $CD4^+$ SP compartment was under homeostatic control (van Meerwijk et al., 1998). The magnitude of the "homeostatic" compensation found was, however, not very significant.

Another piece of information that could be related to homeostasis-like phenomena inside the thymus is the availability of selection "niches" for positive selection (Huesmann et al., 1991; Merkenschlager, 1996; Merkenschlager et al., 1994). When most of the DP thymocytes express a selectable transgenic TCR, the formation of mature SP cells is 10 to 20 times more efficient than observed in normal mice. However, this means that only 20% of the DP thymocytes mature (Huesmann et al., 1991). This is due to the limited availability of stromal cells (Merkenschlager, 1996; Merkenschlager et al., 1994) capable of mediating positive selection, as most DP thymocytes with a selectable transgenic TCR will undergo maturation when they represent only 5% or less of the total DP pool (Huesmann et al., 1991). This observation suggests that there is a rate-limiting step for the number of positively selectable thymocytes. It is not clear if this is a mechanism that could be responsible for the maintenance of thymocyte numbers (or of SP thymocyte numbers), as the transgenic mouse situation may be too dissimilar to the physiological condition, and this kind of competition can be extremely rare in the physiological situation. Thus, the existence of homeostasis inside the thymus is (was) still an open question. We have investigated into this, developing a novel system and analysis for this purpose. (Section B, article #1).

5- THE THYMUS AND AGING

The thymus involutes with age. This important observation has been verified in several animal models and in the human situation. It has received attention not only because of it's implications for the reconstitution of the immune system in situations where the lymphocyte pool is depleted due to irradiation or disease but also because thymic involution correlates

with an immunological decline, reflected in an increase in both the susceptibility to infections and in the incidence of autoimmune disorders. We therefore knew for a long time that the thymus exerts some functional activity even in the adult (Metcalf, 1965a; Miller, 1965; Miller, 1962; Taylor, 1965). The ability to reconstitute an individual's lymphocyte pool after peripheral depletion also correlates inversely with age. Older people and animals do not completely reconstitute the peripheral lymphocyte pool, while much younger patients and animals do (Mackall and Gress, 1997). In humans, the reduction of Thymic mass starts at the age of 1 year (when the organ attains its maximal size) and results in an important reduction of Thymic mass by the time of puberty (George and Ritter, 1996). In mice, declines in the capacity to promote thymocyte proliferation are noted as early as 2 weeks after birth (Hirokawa et al., 1994) and a reduction in the thymic size is visible from week 6 after birth (Hirokawa and Makinodan, 1975). However, children of up to 15 years and mice of 3-4 months are still able to regenerate the peripheral T cell pool to a normal size, which has contributed to the general idea that Thymic involution starts at puberty, an idea that has been challenged (George and Ritter, 1996; Steinmann et al., 1985).

In humans, the decrease in thymus size is masked by changes in the architecture of the organ. In a child, the thymic lobes are separated by thin septa of connective tissue. In the thymus of an elderly person, the septa have greatly expanded and mostly comprise fat cells. Adipose tissue also develops under the capsule, separating it from the true thymic tissue. Thus, this increase in fat, connective tissue and perivascular space counterbalances the diminution of the lymphoepithelial areas of the thymus and the overall size of the organ remains constant throughout life. In mice this does not happen and the size of the thymus decreases with age. In the thymus of an old (24 month) mouse, the thymic T cell production has been estimated to be 0,7% of the number of T cells produced by a newborn mouse (George and Ritter, 1996).

Thymic involution can be derived from factors intrinsic to the immune system or can be a response to extrinsic factors. In the first situation, thymic involution could be either due to a deficient supply of BM precursors or secondary to alterations on thymic stroma. A third possibility was that these two factors were acting at the same time. This was tested in BM chimera systems, where BM from old donors was grafted into irradiated young hosts or where BM from young donors was grafted into irradiated old hosts (Hirokawa et al., 1994). In other experimental setups, neonatal thymi were grafted under the kidney capsule of old mice (Mackall and Gress, 1997; Metcalf, 1965b). All the results obtained point to a more relevant role of the thymic stroma, even if the capacity of old BM to repopulate the thymus seems to be slightly reduced (Mackall and Gress, 1997; Metcalf, 1965b). Hence, when aged irradiated mice were reconstituted with BM from young donors, the thymic abnormalities were not reversed and the thymic size and cellularity remained reduced (Hirokawa et al., 1994;

Mackall et al., 1998). When aged mice received a neonatal Thymic transplant and were then irradiated and reconstituted with neonatal BM cells, normal Thymic regenerative capacity was observed (Mackall and Gress, 1997). Thus, the age of the thymus and not extrathymic factors present in the aged milieu is the major factor contributing to the reduced generation of T cells from aged thymi.

The general notion that thymic involution is linked to puberty had suggested that hormonal factors could be the primary cause for age related thymic involution. Interactions between the gonadal steroids and the immune system have been documented and include the occurrence of thymic hyperplasia after gonadectomy or after destruction of the anterior portion of the hypothalamus (Hirokawa et al., 1994). Additional data suggest that age-related changes within the thymus itself may increase the susceptibility to inhibition via the extrathymic hormonal milieu (Mackall and Gress, 1997). Thus, though the extrathymic milieu does exert some influence on thymic involution, the primary cause seems to lie in the thymus itself. When irradiated aged mice were reconstituted with BM from young donors, the thymic reconstitution was reduced but the thymocyte subset representation was normal, confirming that the aged thymus is able to function and to generate substantial numbers of T cells. This is also being confirmed in aged humans, where recent measures of TRECs or of TCR rearrangement in old individuals or HIV infected adults has also provided evidence for the continuous production of T cells late in life (Douek et al., 1998; Jamieson et al., 1999). This last point is of major relevance for the reconstitution after depletion of the peripheral T cell pool and points out that thymic export is a component of peripheral homeostasis that is present throughout life. The next section deals with thymic export.

6- THYMIC EXPORT AND MIGRATION

After the developmental processes referred above (see chapter 3), mature T cells are exported to the periphery where they will constitute the peripheral T cell pool. Thymic emigrants will be part of the daily input of T cells incorporating into the peripheral T cell pool. As the T cell number is kept constant, it follows that a newcoming T cell will only integrate into these peripheral pools if another T cell is being replaced. The quantification of the thymic output is thus crucial for the understanding of the T cell homeostasis' dynamics. The number of cells exported each day will not only be responsible for the renewing of the available repertoire, adding new specificities to the peripheral pools, but will also be responsible for the replacement of at least part of the cells previously installed in the peripheral pools. The question that arises is: to what extent? To advance in the resolution of this problem, the

thymic output and its impact on the observed peripheral homeostasis were evaluated. Five basic strategies have been used:

1- The evaluation of the impact of thymic ablation (thymectomy) on the maintenance of peripheral numbers (Metcalf, 1965a; Miller, 1965; Miller, 1962; Rocha et al., 1983; Mackall, 1993; Parrott and de Sousa, 1971; Taylor, 1965).

2- The ability of peripheral T cells to expand after transfer into athymic hosts (Rocha et al., 1989; Tanchot and Rocha, 1995).

3- The evaluation of the impact of an increase in thymic mass (or thymic export) on the peripheral T cell numbers (Berzins et al., 1998; Berzins et al., 1999; Leuchars et al., 1978; Metcalf, 1965b).

4- The direct measurement of the number of thymic emigrants after intrathymic injection of fluorescent dyes (Graziano et al., 1998; Kelly et al., 1993; Scollay et al., 1980) or after the identification of the thymic migrant phenotype (Douek et al., 1998; Kong et al., 1998; Kong et al., 1999; McFarland et al., 2000).

5- The evaluation of the thymic output after the induction of peripheral T cell depletion by administration of anti-thy1 antibodies. (Gabor et al., 1997).

In parallel, and as seen above (Chapter 5), the thymic emigration was evaluated in the aging thymus situation.

6.1- Quantitative aspects of thymic output

Adult thymectomy was shown to be responsible for a 40% reduction in the size of the peripheral pools (Rocha et al., 1983) and the presence of a thymus was shown to be essential for peripheral T cell reconstitution after T cell depletion (Mackall et al., 1997; Metcalf, 1965a; Miller, 1965; Miller, 1962; Parrott and de Sousa, 1967). Accordingly, direct measurements of thymic export using intrathymic FITC injection in wild type (Scollay et al., 1980) or in TCR Tg mice (Kelly et al., 1993) have shown that a relatively constant fraction of the thymocyte number (1%) is exported daily into the peripheral pools in young adult mice. This translates into a number between 1 to 2 x 10^6 cells that are exported daily. With age, the fraction of thymocytes exported daily is reduced (0,1% at 6 months of age), and thus, an increasingly smaller number of thymocytes are exported (Scollay et al., 1980). These estimates of thymic export have been directly or indirectly confirmed in a number of later studies (Berzins et al., 1998; Berzins et al., 1999; Gabor et al., 1997; Tanchot and Rocha, 1997) and studies using CFSE intrathymic injection gave slightly higher but comparable

values for daily export in young adult mice (2 - 3 x 10^6) (Graziano et al., 1998). Thus, thymic export alone is responsible for an input of 50 x 10^7 cells per month into the peripheral T cell pool. In order to achieve homeostasis, the incorporation of these T cell emigrants into the peripheral T cell pool must either be restrained, by some kind of feedback mechanism acting on thymic export or by pre-emptive selection at the time of incorporation, or compensated by death of T cells from the already established peripheral pools.

In studies of hyperthymic mice (mice receiving grafts of thymic lobes under the kidney capsule), it was found that the rates of thymic export by individual grafted lobes were independent of the number of thymuses grafted and were constant, independently of the degree of replenishment of the peripheral T cell pool (Berzins et al., 1998; Leuchars et al., 1978). This suggests that there is no feedback control of the peripheral T cell pool over thymic export (Berzins et al., 1998; Leuchars et al., 1978; Tanchot and Rocha, 1997). Studies on the reverse situation, where thymic export was evaluated after T cell depletion was induced by the administration of anti Thy 1 antibodies, also suggested that in a situation of demand due to peripheral depletion, the thymus is not able to compensate by increasing the thymic output (Gabor et al., 1997). We have developed a system that allows the study of thymic export in a situation where the peripheral compartment is not full and the thymus should be able to support an increase in thymic export (see results section, article #1).

Another issue is the incorporation of thymic emigrants into the peripheral T cell pools. To study how the peripheral T cell pool reacts to thymic export, experiments were performed using the thymic graft protocol. The major conclusion is that the size of the peripheral pool is largely independent of the thymic output or mass. Mice receiving an additional thymus, grafted under the kidney capsule, will double the number of T cells exported daily, yet peripheral T cell numbers are kept at similar levels (Berzins et al., 1998). This situation is overcome by grafting a much larger thymic tissue (9 thymic lobes). These results were interpreted as proof for the existence of peripheral homeostasis mechanisms, that were responsible for the non-increase of peripheral T cell number in the first experiment but that these mechanisms could be overcome in extreme situations, as in the second experiment (Berzins et al., 1998). In a complementary study, the same group has suggested the existence of a separate peripheral pool for Recent Thymic Emigrants (RTE), and that these are “exempt from peripheral T cell homeostasis”, for a period of three weeks (Berzins et al., 1999). It could also be true that homeostasis was just reset for an equilibrium value around a higher steady-state number.

It was further suggested that this “exclusion” of RTEs from peripheral T cell homeostasis allows repertoire turnover throughout adult life, an important role for thymic output (Berzins et al., 1999). This leads to a second issue on the relevance of thymic output for the peripheral T cell pool: the qualitative role of thymic output.

6.2- Qualitative aspects of thymic output

It is known that peripheral T cells are capable of considerable expansion, of a magnitude similar to that of colony forming units (Miller and Stutman, 1984; Rocha et al., 1989). The evaluation of the proportion of peripheral T cells in cycle, incorporating BrDU in a 24 hour period, shows that peripheral expansion is an important mechanism of mature T cell production in the adult mouse (Rocha et al., 1990; Sprent, 1993), independently of whether cycling cells represent a large (Rocha et al., 1990) or a small (Tough and Sprent, 1994) fraction of the total peripheral T cells. However, peripheral division mechanisms are not able to generate new TCRs, thus, peripheral division will give rise to a less diverse repertoire. Accordingly, it has been shown that the peripheral compartments obtained after peripheral expansion are biased towards an activated/memory phenotype (Mackall et al., 1993). Thus, thymic output seems to be essential for the renewing of the specificities present at the peripheral pools, being the only provider of naïve T cells. Importantly, as we will see below, the naïve and the activated peripheral T cell pool sizes are independently regulated (Tanchot and Rocha, 1995).

It has been discussed whether, inside this peripheral naïve pool, RTEs are preferentially selected for entry into the peripheral naïve T cell pool (Berzins et al., 1998), representing a sub-division in the peripheral naïve pool for a period of seeding of 3 weeks (Berzins et al., 1999) or whether the entry of RTEs into the peripheral naïve pool is a random event (Tanchot and Rocha, 1997), being the replacement of the naïve pool T cells independent of cell age. The cell-age independent replacement of the peripheral naïve T cells would enable rapid contraction of large clones and a longer survival of rare ones, reinforcing the role of continuous thymic outputs in the maintenance of repertoire diversity in the naïve pools (Tanchot and Rocha, 1997). In the absence of thymic output, the size of the naïve pool would not necessarily decrease, as peripheral expansion concerns the activated/memory pool but has no influence on the size of the naïve pools (Tanchot and Rocha, 1995) but the individual life-spans of the naïve cells will increase as a result of a lack of competing cells (Freitas et al., 1996). However, as larger clones are allowed to persist, the diversity of the naïve pool will decrease.

Thus, although thymic export does not seem to play a very important role for the maintenance of peripheral T cell numbers after initial seeding, it does seem to play an essential role for the peripheral naïve T cell pool, being the only source of new specificities.

6.3- Migration

The information concerning the signals triggering mature thymocyte migration from the thymus is sparse. Chemokines are obvious candidates for molecules involved in thymocyte exit from the thymus. Indeed, a recent report (Ueno et al., 2002) has shown a role for the chemokine CCL19 in the emigration of mature thymocytes in Fetal Thymus Organ Cultures (FTOCs). Importantly, mature thymocytes express CCR7, the receptor for CCL19 and CCL21 and neutralization of CCL19 but not of CCL21 was shown to result in impaired thymic emigration (Ueno et al., 2002). In CCR7$^{-/-}$ mice, thymic emigration and peripheral seeding are reduced in newborn mice, when compared to the wild type situation (Ueno et al., 2002). In adult CCR7$^{-/-}$ mice, however, the circulating T cell pool is not reduced (Forster et al., 1999; Ueno et al., 2002), suggesting that alternative pathways operate and allow the emigration of mature thymocytes (Ueno et al., 2002).

Thus, more information is required to identify the signals involved and elucidate the mechanisms resulting in thymic emigration. Whatever these are, a fraction of the mature SP T cells generated will be exported and will be confronted with the peripheral T cell compartment. How this peripheral T cell compartment is organized is the subject of the next chapter.

PART II

PERIPHERAL T CELL POOLS

7- THE ORGANIZATION OF THE MATURE T CELL POOLS

The periphery of the immune system is the sum of the secondary lymphoid tissues. It is thus in the secondary lymphoid organs that most of the immune system events will take place and it is in the secondary lymphoid organs that T cell numbers are maintained constant, said to be under homeostatic control. The encounter of T cells with antigen must be mediated by special antigen presenting cells, and these are usually present in organized tissues, being the special structure of this tissue determinant for the necessary interactions involved in immune events (for an overview see Janeway et al., 1999).

7.1 - T cells in the Spleen and Lymph Nodes

The secondary lymphoid organs comprise the Spleen (fig.5), the Lymph nodes (fig.6) and the mucosa-associated lymphoid tissues. It can be said that these tissues operate on the same principle, trapping antigens from the sites of infection and presenting them to the lymphocytes, thus inducing immune responses (for an overview see Janeway et al., 1999). The majority of lymphocytes are present in the Spleen and lymph nodes.

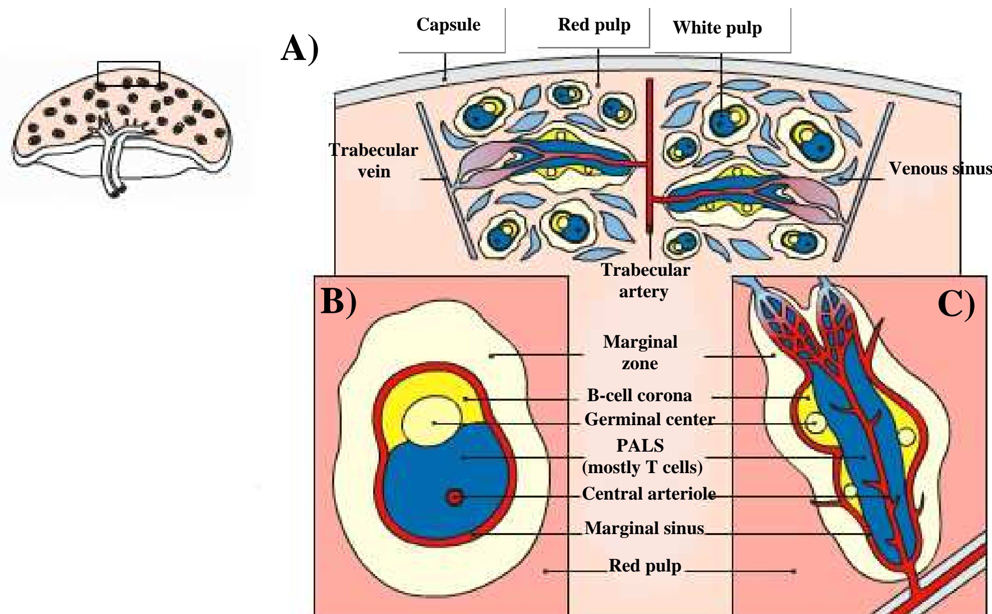

Figure 5: The Spleen. The figure shows a schematic representation of the selected area of the spleen. A) Structure of the spleen. The true lymphoid tisuue of the spleen is contained in the white pulp. The bulk of the white pulp is arranged around a central arteriole and inside a marginal sinus (see also B and C, transvrese and longitudinal sections of the whithe pulp, respectively). We can also distinguish the periarteriolar lymphoid sheath (PALS) consisted by a majority of T cells, and flanked by B cells. B cells can be organized in follicles. Secondary follicles form germinal centers (B and C). (Modified from Janeway et al., 1999).

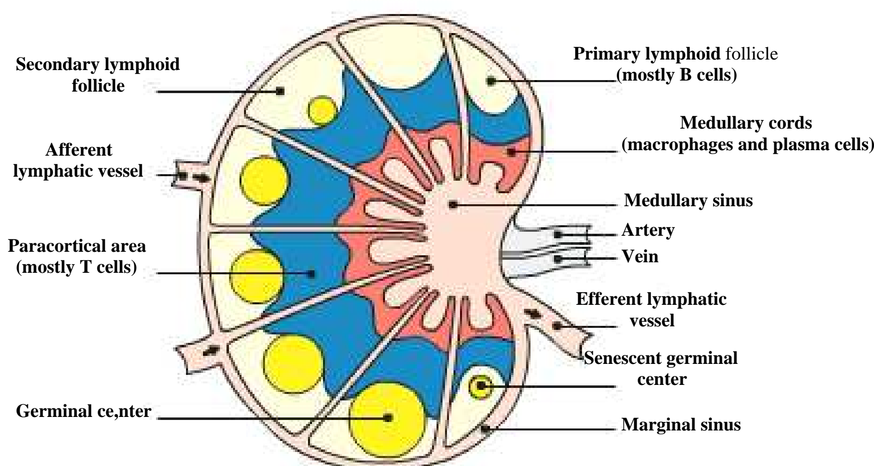

Figure 6: The Lymph Node. The figure shows a schematic representation of a lymph node. We can distinguish in the lymph node four regions (left to right, outside to inside): the cortex, paracortex, the medulla and the hilus or medullary sinus. B cells are localized in follicles in the cortex, where germinal centers are often found. T cells are distributed in the paracortical areas, along with Dendritic cells. Both T and B cells can be found in the medulla. Lymphocytes enter the lymph nodes through HEVs, present in the paracortex and can only move through the efferent lymphatic vessel (Modified from Janeway et al., 1999).

7.1.1-T cells in the Spleen

B cells are the majority of the spleen's lymphocytes. If we consider an average sized spleen (100 x 10^6, in an 8 weeks old C57Bl6) mouse, B cells represent roughly 60% of the splenic lymphocytes. $CD4^+$ T cells represent 15 to 20% of the spleen's lymphocytes and $CD8^+$ T cells represent roughly 10 to 15% of the lymphocytes present in the spleen (fig.7).

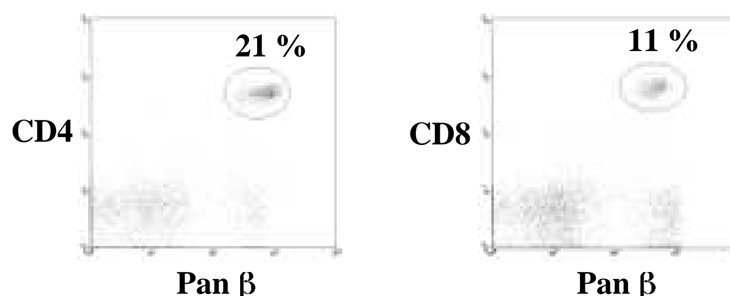

Figure 7: T cell populations in the Spleen. The figure shows the FACS analysis of a Spleen cell suspention obtained from a 8 weeks old C57Bl6 mouse, after staining with the indicated antibodies. The numbers represent the percentages of cells in the regions displayed.

7.1.2-T cells in the Lymph Nodes

T cells make up the majority of lymph node lymphocytes. Considering the total number of Lymphocytes found in the lymph nodes (roughly 60 x 10^6 in the same 8 weeks old C57Bl6 mouse), 30-40% are $CD4^+$ T lymphocytes and 20 to 30% are $CD8^+$ T cells. B cells account for about 40% of the lymph node lymphocytes (fig. 8).

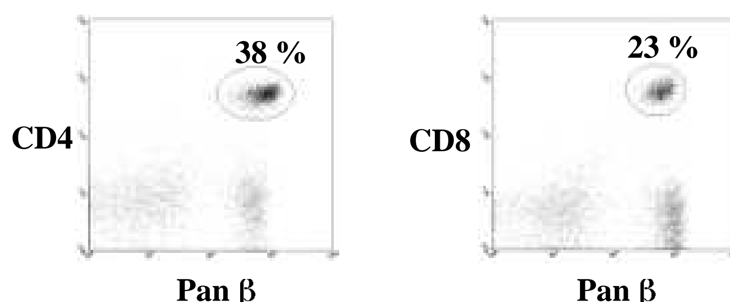

Figure 8: T cell populations in the Lymph nodes. The figure shows the FACS analysis of a Lymph node cell suspention obtained from a 8 weeks old C57Bl6 mouse, after staining with the indicated antibodies. The numbers represent the percentages of cells in the regions displayed.

7.1.3- Lymphocyte traffic

We have seen (chapter 6) that T lymphocytes, once produced, are exported into the peripheral pools. In these peripheral pools, T lymphocytes are not sessile, and the ability to move from one lymphoid organ to another is another important feature of the peripheral lymphocyte pool. This lymphocyte trafficking is responsible for the dispersion of the available repertoire of lymphocytes and directs lymphocyte subsets to specific microenvironments which will be responsible for survival or differentiation, what has been termed ecotaxis (de Sousa, 1971). Lymphocyte circulation is also responsible for the targeting of the immune effector cells to the sites of antigenic or microbial infection (reviewed in Butcher and Picker, 1996).

Traffic patterns may be distinctive features of distinct sub-populations of lymphocytes. In general, it can be said that naïve cells are programmed to recirculate through secondary lymphoid organs, increasing the chances of antigen encounter while memory cells also traffic through secondary organs but can also access and recirculate through extra lymphoid immune effector sites (inflamed skin, joints or the intestinal lamina propria, for example). Also, whereas the naive cell traffic pattern is homogenous, the memory and effector cell pool includes a heterogeneous pattern of circulation, with different subsets, that express different homing receptors (Butcher and Picker, 1996; Sallusto et al., 1999; Weninger et al., 2001).

These homing patterns are relevant for peripheral T cell homeostasis, as they will be responsible for the access to defined peripheral niches (Butcher and Picker, 1996), including homing mechanisms amongst the factors that play a role in peripheral niche competition between lymphocytes (Butcher and Picker, 1996; Freitas and Rocha, 2000).

7.2- Peripheral Sub-population Structure

While the first sub-division to be considered in the peripheral lymphocyte pool is between the T and the B cell pools, that have independent homeostatic controls (see chapter 1), the second sub-division concerns the $CD4^+$ and the $CD8^+$ T cell pools. Are these pools regulated independently? The answer to this question is no, to a certain extent. In MHC class II deficient mice, though $CD4^+$ T cells are not present (or are present in very reduced numbers) the T cell number is normal (Cosgrove et al., 1991), and in $CD4^{-/-}$ mice a similar phenomenon takes place, (Rahemtulla et al., 1991) thus, the $CD8^+$ T cell pool is able to compensate the lack of $CD4^+$ T cells by an increase in numbers. Conversely, the $CD4^+$ T cell pool seems to compensate the absence of $CD8^+$ T cells in $CD8^{-/-}$ mice, increasing their representativity in

the lymph nodes of these mice (Fung-Leung et al., 1991). There is, however, a regulation of the $CD4^+/CD8^+$ ratios, as observed after the expansion of cells after transfer into immunodeficient hosts (Rocha et al., 1989). Independently of the $CD4^+/CD8^+$ ratio initially transferred, the recovered $CD4^+/CD8^+$ ratio was fixed after peripheral expansion (Rocha et al., 1989). Thus, it matters to identify the independent and the common pathways that lead to the homeostasis of the total peripheral T cell pool or of discrete sub-populations of $CD4^+$ or $CD8^+$ T lymphocytes.

The $CD4^+$ vs. $CD8^+$ T cell pool represents one relevant sub-division of the peripheral T cell pools, but other sub-divisions must be considered when describing the structure of the peripheral T cell pools (fig. 9). These sub-divisions are related to the state of activation and differentiation of the T cells found at the periphery. Thus, naïve, activated (effector) and memory sub-populations must be defined. In short, we can say that naïve T cells are T cells that have not encountered antigen, they are said to be virgin T cells. Effector cells are considered to be activated T cells, engaged in immune responses and memory cells are considered to be the T cells responsible for secondary immune responses, they are more efficient and require previous priming. These sub-populations are all present at a given moment in the peripheral T cell pool, but the proportions may vary, depending of the immune status and age of the animal. The characteristics of these sub-populations will be further described below

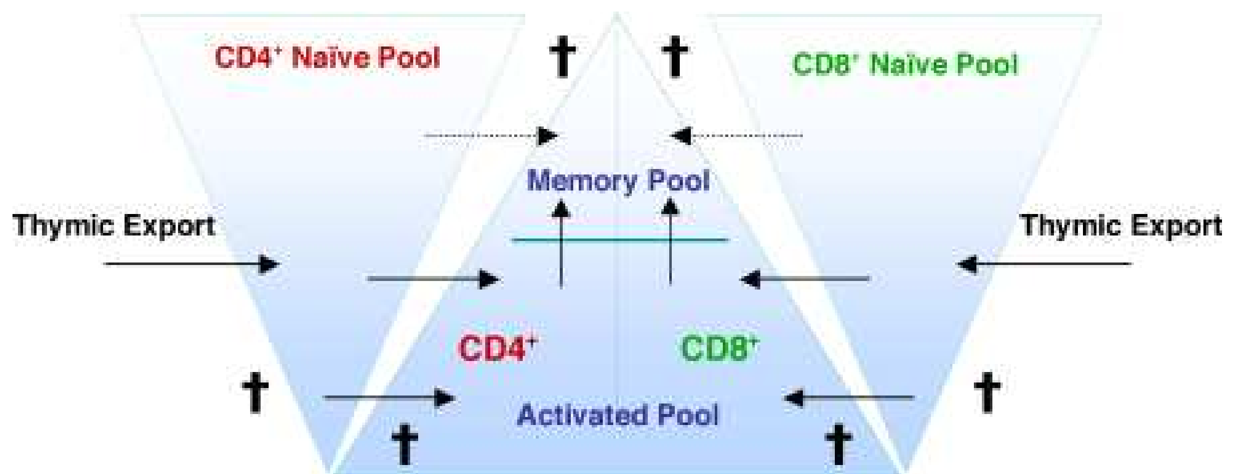

Figure 9: The peripheral T cell pool. Naïve $CD4^+$, naïve $CD8^+$ and activated and memory compartments of $CD4^+$ and $CD8^+$ T cells must be considered. The proportion of the different compartments varies according to the immune status or age of the animal. Considering age, it could be said that the variation on the reresentation of each of these compartments follows a top to bottom sense. ✝: Output from the respective T cell pools due to cell death. Arrows represent migration or differentiation from one pool to another.

From the definitions above, we may readily deduce that the naïve T cell pool provides the ability to cope with new antigens, being the source of diversity, but including a certain number of cells that will never encounter antigen. On the other hand, the memory pool will be composed of clones of cells having previously encountered antigen, thus antigenically selected to enter the memory pool. This selection opposes diversity, but provides efficiency. How the immune system deals with the problem of maintaining versatility while providing efficiency will be discussed next.

7.2.1- Naïve and activated T cell pools have independent homeostatic regulation

The separation of the homeostatic regulation of the peripheral naïve and activated T cell pools (Tanchot and Rocha, 1995) is of major relevance for the understanding of peripheral homeostasis and of the relationship between peripheral T cell homeostasis and the immune system's functions. This was shown using a $CD8^+$ TCR Tg mouse, specific for a male peptide (aHY). In female $B6Rag_2^{-/-}$ $CD8^+$ TCR Tg, all the peripheral T cells show a naïve phenotype, as the Ag is not present in the mouse. In this situation, the size of the peripheral T cell pool is about half the size of the peripheral T cell pool of the female Tg $Rag^{+/-}$ mouse. Thus, continuous output of naïve T cells for 10-12 weeks was not able to fill up the peripheral T cell pool in this situation. In the female Tg $Rag^{+/-}$ mouse a considerable fraction of the peripheral $CD8^+$ T cells express a TCR constituted by an endogenous αchain and the transgenic βchain. The number of naïve cells expressing the Tg TCR is the same in both situations, but the $Rag^{-/-}$ Tg mouse lacks an activated compartment, present in the $Rag^{+/-}$ Tg mouse (Tanchot and Rocha, 1995). Conversely, the transfer of peripheral T cells into immunodeficient nude mice was not sufficient to accomplish full reconstitution of the peripheral T cell pool, as the naïve pool could never be reconstituted in this way (Tanchot and Rocha, 1995).

This suggests that these two sub-populations belong to two different ecological niches, exploring different resources for which competition should be expected within each sub-population but not between them (Freitas and Rocha, 2000). In other terms, competition occurs in such a way that a naïve T cell will compete with another naïve T cell while a memory T cell will compete with another memory T cell. This assures the preservation of both sub-populations and is the answer to the problem of assuring efficiency while maintaining diversity. In the next pages, we will try to characterize the major sub-populations to be considered in the peripheral T cell pool.

7.2.2- The naïve T cell pool

Naïve T cells are the direct export product from the thymus. These cells have, by definition, not encountered antigen. Thus, these cells represent the naïve repertoire present in the peripheral pool, that should allow a response to newly encountered antigens. In order to perform, these cells should be able to encounter the antigen, and it is then important that naïve cells re-circulate, migrating continuously from one secondary lymphoid organ to another (reviewed in Sprent and Surh, 2002). Importantly, these cells have been shown to express receptors for entry into the lymph nodes (CD62L) or for chemokines (CCR7), suggesting the type of signals responsible for the circulation pattern of naïve T cells (Sprent and Surh, 2002).

Accordingly, the phenotype of the naïve T cells (for now we will consider both $CD4^+$ and $CD8^+$ T cells) includes some of these molecules. In C57Bl6 mice (some markers vary depending on the mouse strain), naïve T cells are typically defined as $CD44^-$, $CD62L^{high}$, $CD45RB^{high}$, $CD25^-$, $CD69^-$, small, resting cells. However, some of these markers can be shared with subsets of activated or memory cells, as is true for $CD62L^{high}$, described in a subset of human memory $CD4^+$ and $CD8^+$ T cells (Sallusto et al., 1999) and some cells can be "activated" as a result of homeostatic proliferation (homeostatic proliferation will be discussed later in this introduction) defying the definition of both naïve and of effector T cells (Murali-Krishna and Ahmed, 2000). The result is a difficulty to define a naïve T cell using a single cell surface marker. Thus, in most situations, combinations of the markers referred to above are used. When only a single marker is used the CD45RB marker for $CD4^+$ T cells and the CD44 marker for $CD8^+$ T cells are the ones most currently used.

The function of naïve T cells depends on their activation which, in turn, depends on the encounter of these cells with antigen, processed and presented as MHC-peptide complexes at the surface of APCs. Together with signalling provided by costimulatory molecules and cytokines, activation will result in proliferation and differentiation of T cells along the pathway to cytotoxic or helper cells (for $CD8^+$ T and $CD4^+$ T cells, respectively).

Finally, regarding the replenishment of the naïve pool and naïve T cell substitution, two characteristics should be considered, firstly the life-span and the signals necessary for the survival of the naïve T cell (this will be considered below, in more detail) and secondly, the probability of substitution of the naïve T cell. Naïve T cells have been described as non-cycling cells (Swain et al., 1996; von Boehmer and Hafen, 1993) or as including a very small proportion of cycling cells (Bruno et al., 1996; Tough and Sprent, 1994), thus newly originated naïve T cells from the thymus are the main source of naïve T cells. Accordingly, in situations of T cell depletion and of peripheral reconstitution, the reconstitution of the naïve T cell pool is dependent on the presence of a functional thymus (Mackall et al., 1997), and the

size of the naïve pool declines with age (Barrat et al., 1997), a decline that has been suggested to be antigen dependent (Linton et al., 1997). The incorporation of thymic emigrants into the peripheral naïve pool has been described (see chapter 6), and represents thus, the entry of new specificities into the peripheral T cell pool and the major source of new naïve T cells.

What is the representativity of the naïve T cell pool? As we have seen, this depends on the age and on health condition (degree of infection), but in a young adult mouse (6 to 8 weeks), the naïve pool accounts for 50 to 60% of the peripheral $CD4^+$ or $CD8^+$ T cell pools.

7.2.3- The Effector pool

Upon TCR mediated activation, T cells loose their naïve status and integrate the effector compartment, differentiating into $CD8^+$ T cytotoxic killer or $CD4^+$ helper T cells. Here, we will again concentrate on the $CD4^+$ T helper subset, the direct object of this thesis. When considering the $CD4^+$ T helper effector pool, two main subdivisions must be mentioned. As the effector pool is responsible for the direct effector functions of T cells, these subdivisions take into account the major functional products of these cells, that are, for $CD4^+$ T helper cells, cytokines.

The definition of the phenotype of effector cells is difficult, as it often coincides with that of the phenotype of memory cells. Effector T cells are large blastic cells, expressing activation markers, like CD25 or CD69 (often transitory for the first and short term for the second), express CD44 and have down-regulated CD45RB (some variations in this marker are dependent on the mouse strain used) and CD62-L expression (Swain et al., 1996). This phenotype is shared with some memory cells and in part, with cells that are activated as a result of homeostatic proliferation (Ernst et al., 1999; Kieper and Jameson, 1999; Murali-Krishna and Ahmed, 2000). For this reason, it is useful to consider in the peripheral T cell pool, naïve versus activated/memory cells, thereby avoiding the difficulty in defining the line that phenotypically separates effector from memory cells.

The homing of effector cells also reflects their function and, here again, part of the phenotypic alterations observed, namely CD62L down-regulation are related to homing (reviewed in Sprent and Surh, 2002). Thus, contrary to naïve T cells, effector cells have the ability to circulate through extra-lymphoid immune effector sites (Butcher and Picker, 1996), and as opposed to naïve T cells, effector (and memory) T cells display great heterogeneity, with subsets often displaying a tissue-specific pattern of circulation (Butcher and Picker, 1996).

When considering the effector function of T cells, and of $CD4^+$ T cells in particular, the pattern of cytokines secreted is the main readout of effector function, as it was observed that

the cytokine secretion pattern is associated with a specific function, namely the cell mediated/inflammatory immunity or humoral responses (reviewed in Glimcher and Murphy, 2000). An organism tends to mount one kind or the other of immune response, not both. Hence, two subsets of $CD4^+$ T cell effectors were defined, according to both the referred functions and the specific cytokine secretion pattern that was associated (Glimcher and Murphy, 2000). The factors driving the observed polarization were also an object of investigation. A very brief account of the characteristics of the two subsets follows.

7.2.3.1 – TH1 $CD4^+$ T cells

TH1 (T helper 1) cells are associated with cell mediated/inflammatory immune responses. The cytokine that is more characteristic of TH1 cells is IFNγ, but TH1 cells also produce IL2, and TNF (α or β). Here again, attempts were made to identify a phenotype (other than cytokine secretion), and some cell-surface markers have been ascribed to TH1 cells. Hence, it was shown that TH1 cells display preferential expression of the IFNγ receptor β chain, the β chain of the IL12 receptor, the receptor for IL18 and the chemokine receptors CXCR3 and CCR5 (reviewed in Glimcher and Murphy, 2000).

The differentiation (or polarization) of the $CD4^+$ T cells relies on the presence of key cytokines that drive the differentiation towards a given direction and suppress at the same time differentiation into the other subset. For TH1 $CD4^+$ T cells, the key cytokine seems to be IL12 (reviewed in Glimcher and Murphy, 2000). Mice deficient in IL12 or in STAT4, the downstream signalling molecule for IL12, do not have TH1 cells (Glimcher and Murphy, 2000; Kaplan et al., 1996; Thierfelder et al., 1996). More recently, it has been demonstrated that IL12 derived signals drive differentiation towards a TH1 fate at least in part by inducing the transcription factor T-bet (Glimcher and Murphy, 2000). T-bet is expressed in NK cells and in TH1 cells, both producers of IFNγ, and is thus correlated with IFNγ production. At the same time, T-bet expression correlates with the simultaneous shut off of IL4 and IL5, supporting the notion that T-bet is a master regulator of TH1 differentiation (Glimcher and Murphy, 2000).

7.2.3.2 – TH2 $CD4^+$ T cells

TH2 $CD4^+$ T cells are responsible for the humoral immune responses. The hallmark cytokine for TH2 $CD4^+$ T cells is IL4, but TH2 cells also secrete IL5, IL9, IL10 and IL13, cytokines that provide help for B cells and are important for the allergic response (Glimcher and Murphy, 2000). TH2 associated markers include T1/ST2 (an IL1-like molecule) as well as the chemokine receptors CCR3, CCR4 and CCR8 (reviewed in Glimcher and Murphy, 2000).

The cytokine that seems to be responsible for the development along the TH2 pathway is IL4. Downstream signalling, via STAT6 seems to be involved, as TH2 responses are severely impaired in STAT6$^{-/-}$ mice (Shimoda et al., 1996; Takeda et al., 1996a). However, STAT6 dependent signalling may not be absolutely necessary for TH2 development, as TH2 responses may be generated in the absence of STAT6 (reviewed in Glimcher and Murphy, 2000). Here again, it was demonstrated that transcription factors play a role in the differentiation. The transcription factors c-maf and GATA-3 are involved in IL4 and IL5 production, respectively (Glimcher and Murphy, 2000).

Effector cells represent a variable proportion of the peripheral T cell pool, depending on the infectious status of the mouse (or individual). Thus the activated/memory T cell pool could be said to make up, in the same 6 to 8 weeks old C57Bl6 mouse, 40 to 50% of the peripheral T cell pool. It should be pointed out that at any given instant there is a basal degree of immune responses, with cytokine production and immune response kinetics, implying that some cells are undergoing clonal expansion and others are dying in the clonal contraction phase. It is one of the important features of peripheral T cell homeostasis, that this variation occurs in such a way that T cell numbers are conserved.

7.2.4- The memory T cell pool.

Immunological memory can be defined as an antigen-induced alteration in the reactive state of the immune system, occurring in such a way that the memory responses are more rapid on inset and more effective in antigen clearance (Bruno et al., 1995). Thus, it is important to consider the factors that are behind this faster and more efficient response. Two levels must be probed, namely the alterations in the frequency of antigen specific T cells and the qualitative differences that distinguish a memory T cell. By definition, a memory response is a secondary response. This means that a primary response occurred and thus, that responding T cell clones underwent considerable expansion (fig. 10). This expansion is followed by a contraction phase, responsible for the death of >90% of the effector cells (reviewed in Ahmed and Gray, 1996). After the contraction phase of an immune response, the memory phase follows (fig.10). By the end of the contraction phase, there are still enough cells from the responding T cell clone to increase the frequency of the T cell specificity in the order of 5 to 100 fold (reviewed in Ahmed and Gray, 1996), it is thus a quantitative transformation. From this it can also be deduced that the repertoire of the memory compartment is less diverse than the repertoire of the naïve compartment (Arstila et al., 1999).

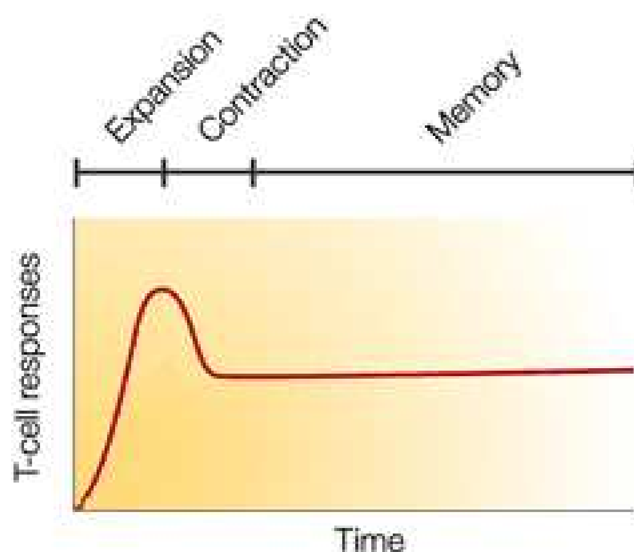

Figure 10: Immune responses. The Imuune response consists of three distinct phases. See text for the quantitative correspondence. (Modified from Kaech et al, 2002).

While the higher frequency of specific T cells can account for some of the qualitative changes in the memory responses, qualitative changes in the T cells account for most of the increased efficiency of secondary responses (Garcia et al., 1999; Rogers et al., 2000; Veiga-Fernandes et al., 2000) and reviewed in (Kaech et al., 2002). The phenotype of memory T cells is not easy to distinguish from the phenotype of effector cells. Thus (considering C57Bl6 strain mice), memory T cells maintain high expression levels of CD44 and low expression levels of CD45RB, the markers most currently used. The use of the CD62L marker that was considered to be downregulated in memory cells is now less reliable, as subsets of CD62L^{high} memory cells have been described (Sallusto et al., 1999). Qualitative changes define a memory cell as a different entity from a naïve or effector cell, and are related to the gene-expression profile (namely cytokine expression), the homing behaviour (Sallusto et al., 1999; Weninger et al., 2001), with the constitutive expression of different molecules involved in adhesion or chemotaxis (like CD62L and CCR7; (Sallusto et al., 1999) , the cell cycle status (Veiga-Fernandes et al., 2000) and last but not least, the survival requirements (Garcia et al., 1999; Lantz et al., 2000; Murali-Krishna et al., 1999; Tanchot et al., 1997).

As for the origin of memory cells, it is the subject of some controversy. It is under debate whether these memory cells arise directly from effector cells or whether memory cells are a separate lineage arising from naïve precursors (reviewed in Kaech et al., 2002). There is strong evidence to support both sides. More recently, memory cells were subdivided into central memory and effector memory cells (Sallusto et al., 1999) and it was suggested that the central memory cells do not participate in the primary responses, becoming a reservoir of

memory T cells responding to antigen reencounter (Kaech et al., 2002). Thus, the memory T cell compartment is not viewed as a homogeneous compartment and its sub-divisions may arise from different pathways, depending on the way T cells are activated (namely on the nature of the APC and of co-stimulation) and on the microenvironment (namely on the cytokines present). Considering the CD4^{+} T cell compartment, it should be mentioned that the TH1/TH2 division seems to be sustained in the memory T cells generated. The memory T cells generated seem to be reminiscent of the type of response generated (Hu et al., 2001; Swain et al., 1996), but here again the polarity may be restricted to subsets of memory cells (Sprent and Surh, 2002).

The replacement of memory T cells is determinant for long-term memory, as memory will disappear if memory T cells disappear. As referred above, the memory pool has independent homeostatic regulation, thus, new responses should generate new memory T cells that will only subsist in the peripheral memory pool by replacing other memory cells (Tanchot and Rocha, 1995). Thus, as new responses occur, the established memory pools must be affected, a phenomenon that, if independent of the cell age, would again contribute to the survival of smaller clones. Indeed, it was found that new infections can alter the previous established memory pools (Selin et al., 1996), and that this happens in a selective way, allowing the permanence of cross-reactive clones (Selin et al., 1999).

It matters to consider three final points concerning memory pool turnover: first, that the memory T cell pool includes cycling cells, as evaluated by BrDU incorporation (Bruno et al., 1996; Tough and Sprent, 1994), representing another source of memory cells. This new input of memory phenotype T cells may arise from non-antigen induced proliferation, occurring in the peripheral memory pool- bystander proliferation (reviewed in Sprent and Surh, 2002). Bystander proliferation seems to reflect the influence of cytokines as survival factors and inducers of division, and particular cytokines may have opposing roles in the control of bystander proliferation and of memory T cell survival (Ku et al., 2000).

Secondly, more input of memory cells can also result from homeostatic proliferation, that results in expression of memory markers but that requires a lymphopenic condition (see 8.4). The process seems to be analogous to the expansion occurring after transfer of T cells into immunodeficient mice, that results in the generation of T cells with a memory phenotype (Mackall et al., 1993).

Finally, it should be referred that with age, the size of the activated/memory pools shows a slight increase (Barrat et al., 1997) that may compensate the decrease in the size of the naïve pool, without being related to it. As described above, the naïve and memory pools have independent homeostatic regulations.

To conclude, I will just refer that though all these processes may induce changes in the composition of the memory T cell compartment, some T cells seem to maintain without

division and that important heterogeneity must be considered in the memory pool, with an important possibility of including effector T cells, that do not necessarily “qualify” as memory T cells. It is useful to consider an activated/memory compartment that makes up to 50% of the peripheral T cell pool of our 6 to 8 weeks old C57Bl6 mouse.

7.3- Peripheral Repertoire

From the enormous amount of variability and diversity possible when assembling a TCR, and from the actual number of peripheral T lymphocytes, it is immediately deducible that not all of the potential repertoire may be represented in an individual at a specific time point. Thus, we must consider a potential repertoire and an available repertoire. Depending on the specificities present at a given instant, the peripheral T cell pool will or will not be capable of responding to antigen. Thus, the difference between the potential repertoire and the available repertoire can give us a measure of the vulnerability of the immune system.

The theoretical diversity of the mouse or human T cell repertoire has been calculated at 10^{15} (Davis and Bjorkman, 1988). The size of the available repertoire is limited by the total number of peripheral T cells, but the presence of T cell clones should be admitted. For instance, with the use of the immunoscope technique, it was found that the mouse spleen contains 2×10^6 clones of about 10 cells each (Casrouge et al., 2000), and similar studies performed in human blood lymphocytes gave an estimate in the order of 25×10^6 different TCRs minimum (Arstila et al., 1999).

Part of the reduction observed in the available repertoire must be considered as the result of the impossibility to represent all possible TCRs in a peripheral pool of reduced size, but some of this difference is accounted for by selection events. As referred (chapter 3), a large majority of the produced TCRs wont be able to reach full maturation as a result of a failure to pass positive and negative selection. The repertoire originated by thymic selection will reflect this, showing a marked overrepresentation of specific sequences (Correia-Neves et al., 2001). More, post-emigration events at the periphery will further shape the peripheral repertoire, with an impact in the diversity and on the specific sequences and families present (Blish et al., 1999; Correia-Neves et al., 2001; Rocha and von Boehmer, 1991; Viret et al., 1999). These events will be reflected in the differential frequency of specific TCRs in the naïve repertoires when compared to memory repertoires. To an increase in the precursor frequency of specific T cells after priming (Blattman et al., 2002; McHeyzer-Williams et al., 1999), will correspond a decrease in the repertoire diversity in the memory compartment,

which has been shown that can represent less than 1% of the peripheral diversity (Arstila et al., 1999).

From the overall analysis, it seems again to be significant the separation of the homeostatic regulation of the naïve and memory pools, as the means to preserve the diversity of the peripheral repertoire while ensuring the efficiency of the memory responses (Antia et al., 1998; Tanchot and Rocha, 1998).

PART III

T CELL HOMEOSTASIS

8- HOMEOSTASIS OF THE PERIPHERAL T CELL POOL

The peripheral T cell numbers in the specific compartments are dependent on the cellular input and output. Seeding from the thymus, transition from one pool to another and division supply the input. Death must balance input representing, together with transition-out of the given lymphocyte pool, output. In between, T cells integrating each of the peripheral T cell pools survive. When confronted with questions concerning the turnover of the T cell populations, we tend to ask a very basic question: what is the life span of a T cell? The formulation of this question supposes that the lifespan of a T cell is a cell-intrinsic propriety.

In this chapter I will consider the definitions of life span, the survival requirements of the different peripheral T cell sub-populations (cell-intrinsic component) and the influence of the environment and of cell interactions (cell extrinsic component) on the life span of the peripheral T cells.

8.1- Lymphocyte life spans

The main difficulty in the definition of lymphocyte life spans resides in the distinction between considering that the lifespan of a cell ends when this cell divides or dies and considering that the lifespan of a cell ends when this cell dies, but not when it divides (reviewed in Freitas et al., 1986). Both definitions present problems: in the first case, the persistence of a specificity through clonal expansion is ignored; in the second case changes in the characteristics of the progeny cells are ignored (Freitas and Rocha, 1993). The first definition, that considers that a cell dies when it becomes two cells, is the most widespread (Freitas and Rocha, 1993; von Boehmer and Hafen, 1993), however, at the population level, the persistence of a cell or of its progeny is not distinguished and thus cycling and non-cycling components are usually referred (Freitas and Rocha, 1993).

The strategies used to evaluate lymphocyte life spans can be divided into three main categories:

1-Evaluation of the rate of cell division, using BrDU or thymidine incorporation techniques.

2- Evaluation of cell persistence after arrest of cell production.

3- Evaluation of persistence after cell transfer into adequate hosts.

All these strategies have limitations. The Administration of exogenous DNA precursors suffers from the necessity of using important doses of these exogenous DNA precursors, to ensure labelling of the highest proportion of the dividing cells (cell division does not occur in a synchronous fashion in the peripheral pools). These high doses often produce toxic effects (reviewed in Freitas and Rocha, 1993), and estimates based on these methods should be taken with caution, as they may reflect the fact that not all of the peripheral cycling cells have been labelled or that cycling cells have been eliminated due to selective toxic effects. The arrest of cell production, using cytostatic drugs like hidroxyurea, may have selective toxic effects and affect non-lymphoid components, introducing error through general effects. The arrest in thymic cell production after thymectomy induces surgical stress. Finally, the evaluation of lymphocyte life spans after transfer of peripheral T cells into immunodeficient or differing in allotypic markers, besides possible effects due to cell manipulation, is hampered, in the first case by proliferation induced after transfer into lymphopenic hosts (homeostatic proliferation, see 8.4) and by ignoring the impact of thymic export and, in the second case, by strong competition with the established cells.

Thus, when considering lymphocyte life span, the very notion of lymphocyte life span as an intrinsic propriety of the cell should be questioned: lymphocyte life spans are conditioned by the presence or absence of other cells and cannot be considered a cell intrinsic propriety (Freitas and Rocha, 1993). Thus, the estimates obtained are revealing of the potential life span of a lymphocyte at a population level in the studied conditions and the conclusions obtained refer to probability of survival in given conditions, or to the renewal rates of populations.

And what are these conclusions? Probably reflecting the referred difficulties and ambiguities, the life span of lymphocytes is a controversial issue. While it is generally agreed on that the peripheral T cell pool is composed of both a long lived and a short lived component, the representation of each is under debate (reviewed in Freitas and Rocha, 1993). Two opposing views emerged: The first view, obtained after BrDU incorporation studies and HU administration, considers that a majority of the peripheral T cells have a short life span of 3 to 7 days, as after 3 to 7 days of BrDU administration 30 to 50% of the peripheral lymphocytes were labelled (Freitas and Rocha, 1993; Rocha et al., 1990) and

50% of the peripheral T cell pools were depleted after 3 days of administration of the cytostatic drugs HU or ganciclovir (reviewed in Freitas and Rocha, 1993). The second view, based on BrDU or thymidine incorporation studies or in persistence of transferred T cells into immunodeficient hosts, considers that most peripheral T cells are long-lived, with a life span of several weeks or months (Sprent, 1993; Sprent and Basten, 1973; Sprent et al., 1991).

Note that these initial studies and conclusions do not distinguish between $CD4^+$ and $CD8^+$ or between naïve and memory T cells. The introduction of the naïve vs. memory distinction (with the help of cell surface markers and of transgenic mouse models) has provided more information. Thus, the life span of naïve anti-HY TCR Tg $CD8^+$ T cells has been calculated at 8 weeks minimum and very little division was observed in these cells (von Boehmer and Hafen, 1993), but the situation was shown to be more complicated, as the proportion of cycling cells in $CD4^+$ TCR Tg T cells depends on the TCR concerned (Bruno et al., 1996), suggesting that interactions of the TCR with unknown ligands are behind the cycling of some naïve TCRs but not others, in the peripheral pools (Bruno et al., 1996). In similar studies performed with polyclonal populations, the turnover of naïve and memory T cell populations was confirmed to have a multitude of components, as cycling and non cycling naïve and memory phenotype T cells were found. The latter had, however, a more important cycling component (Tough and Sprent, 1994).

Altogether, these data, many times conflicting, reflect the difficulty to treat the lymphocyte life span concept. A crucial advance was obtained when the lymphocyte life span was viewed not as a cell-intrinsic propriety but dependent on the probability to meet the survival requirements of the specific sub-populations of lymphocytes. If these survival requirements are present in limited supply this probability is then dependent on the presence or absence of other cells. Thus, lymphocyte survival is an active rather than passive phenomenon and lymphocytes may compete for survival signals. In the next two chapters, evidence for the existence of lymphocyte competition is presented and the survival requirements of naïve and memory T lymphocytes are discussed.

8.2- Survival requirements of naive and memory T lymphocytes

If lymphocyte survival is an active process, what kind of signals mediate T cell survival? The obvious candidate for delivering the signal that T cells require for survival is the TCR.

For the $CD8^+$ T cell compartment, compelling evidence for the role of TCR-MHC interactions in peripheral naïve and memory T cell survival came from experiments (again) in the HY system (Tanchot et al., 1997). Monoclonal anti-HY Tg $CD8^+$ T cells were transferred

into host mice expressing no class I molecules, class I molecules from a non-restricting haplotype ("wrong MHC") or class I molecules of the restricting MHC element ("correct" MHC). Naïve or memory CD8$^+$ T cells were transferred, and the requirements for survival and proliferation were evaluated. The results have shown that not only the TCR-MHC interaction is indeed required for peripheral T cell survival, but that the survival and proliferation requirements differ for naïve and memory T cells: while naïve T cells required the presence of the correct MHC in order to survive, memory T cells could survive in presence of the wrong MHC (Tanchot et al., 1997). These differential requirements for naïve versus memory survival can be part of the explanation for the niche differentiation that takes place in the peripheral pools (Tanchot and Rocha, 1995).

These differences in the survival requirements of naïve and memory T cell sub-populations provide a cell-intrinsic component of peripheral T cell homeostasis, placing sub-populations with different survival requirements in segregated niches. Niche segregation of the different sub-populations provides a way to conciliate competition for survival signals present in limited supply with the need to maintain different sub-populations, with different functions or proprieties (Freitas and Rocha, 2000).

The CD4$^+$ T cell compartment has been shown to obey to similar principles, as peripheral CD4$^+$ T cell survival depends on peripheral expression of MHC class II molecules. These conclusions were drawn from several experimental systems, relying either in the transient expression of class II in the thymus in otherwise class II deficient hosts, by targeted complementation of Class II deficiency using intrathymic delivery of recombinant adenoviruses (Rooke et al., 1997) or tetracycline controllable Class II expression in the thymus (Witherden et al., 2000), by grafting class II$^+$ thymic lobes under the kidney capsule of Class II KO hosts (Takeda et al., 1996b) (these strategies allow thymic selection of a cohort of CD4$^+$ T cells, that are then confronted with a Class II barren periphery), expressing class II in Dendritic cells only (Brocker, 1997) or by the transfer of CD4$^+$ T cells into Class II KO hosts (Boursalian and Bottomly, 1999; Kirberg et al., 1997; Viret et al., 1999). Though all these studies confirm that Class II molecules are essential for CD4$^+$ T cell survival, the half-life of the CD4$^+$ T cells in the absence of Class II varied from 3-8 weeks (Kirberg et al., 1997; Rooke et al., 1997; Witherden et al., 2000) to > 16 weeks (Takeda et al., 1996b), probably reflecting differences in the experimental designs used.

The conclusions drawn above, place the peripheral survival of CD4$^+$ T cells reminiscent of thymic positive selection, a process also dependent on TCR – MHC-peptide interactions (see chapter 3). Accordingly, polyclonal CD4$^+$ T cells do not survive when transferred to H2M$\alpha^{-/-}$ mice (where class II molecules bind one single peptide), or when transferred to mice expressing a different class II allotype, or to class II deficient hosts (Viret et al., 1999). Thus, peptide recognition is also necessary for peripheral CD4$^+$ T cell survival

and the peptides responsible may be the same necessary to mediate positive selection, as illustrated by the ability of $CD4^+$ T cells selected in $H2M\alpha^{-/-}$ mice to survive when transferred into $H2M\alpha^{-/-}$ peripheral pools but not when transferred into MHC Class II deficient hosts (Viret et al., 1999). More recent reports, using the reverse approach (Labrecque et al., 2001; Polic et al., 2001), have reached similar conclusions. In these studies the peripheral $CD4^+$ and $CD8^+$ T cells were stripped of their TCRs in a controllable fashion, using a tetracycline based (Labrecque et al., 2001) or an inducible knock-out system (Polic et al., 2001), and the decay of the peripheral T cells was then evaluated. The conclusions match most of the previous knowledge: $CD4^+$ T cells decay more slowly than $CD8^+$ T cells (Labrecque et al., 2001; Polic et al., 2001) and memory cells decay is slower than in the naïve T cell compartment (Polic et al., 2001). Results also suggest that $CD4^+$ T cell memory survival is not dependent on TCR mediated signals (Polic et al., 2001).

Regarding the differential survival requirements for memory and naïve $CD4^+$ T cells, there is less available information than for $CD8^+$ T cells. Still, the available data point for a similar distinction in the survival requirements for naïve versus memory T cell compartments. The memory compartment seems to require much less or even not at all (Polic et al., 2001; Swain et al., 1999) contact with Class II molecules. These differential requirements can be due to a pre-activated state of memory cells, that allows the existence of much lower thresholds for survival (Garcia et al., 1999), as has been shown for the $CD8^+$ T cell compartment (Murali-Krishna et al., 1999; Tanchot et al., 1997; Veiga-Fernandes et al., 2000).

This pre-activated state and the phenotypic distinction between memory and naïve T cells are also the result of changes in the expression of different adhesion molecules and cytokine receptors. It is then not surprising that molecules like cytokines are also involved in peripheral T cell survival and in the differential survival requirements for naïve and memory T cells (Freitas and Rocha, 2000). Thus, the requirement for γc dependent cytokines has been shown to be different for naïve (dependent) or memory (independent) $CD4^+$ T cells (DiSanto et al., 1996; Lantz et al., 2000). Also, IL15 has been implicated in the survival and proliferation of $CD8^+$ memory T cells (Ku et al., 2000; Lodolce et al., 1998; Sprent and Surh, 2002) and IL7 in the survival of naïve T cells both from mice (Schluns et al., 2000; Tan et al., 2001; Vivien et al., 2001) and humans (Fry et al., 2001), for example. The role of cytokines in peripheral T cell homeostasis will be discussed further below.

These survival signals may translate into the expression of survival proteins like Bcl-2 family members, key regulators of apoptosis (Adams and Cory, 1998). Failure to express the correct protein would have as a consequence the death of the cell by apoptosis. The Bcl-2 family includes both molecules that essentially prevent apoptosis (Bcl-2 itself, Bcl-X_L, MCL-1) and molecules that promote apoptosis (BAX, BCL-X_S, Bak, BAD or Bim) and it seems that it

is the overall ratio of anti-apoptotic to pro-apoptotic molecules that determines the susceptibility to a death stimulus (reviewed in Chao and Korsmeyer, 1998). The anti-apoptotic molecules BCL-2 and BCL-X_L have been shown to be expressed in several T cell developmental stages, in a reciprocal pattern; thus, Bcl-2 is highly expressed in the thymus in the mature SP thymocytes and in the peripheral mature T cells (Chao and Korsmeyer, 1998), while BCL-X_L is expressed in the immature DP stages in the thymus and it is induced upon activation in the peripheral pools (Chao and Korsmeyer, 1998). Importantly, mice over-expressing these molecules displayed an increased cell survival, and mice deficient in the pro-apoptotic molecule Bim displayed lymphocyte accumulation (Bouillet et al., 1999), suggesting that the balance of pro and anti apoptotic molecules is relevant for peripheral T cell homeostasis. The expression of these survival molecules has been linked to signalling through the TCR or through cytokine receptors such as the IL7R (Chao and Korsmeyer, 1998), and the balance between pro and anti apoptotic molecules has been shown to be more in the anti-apoptotic side in memory $CD4^+$ T cells, when compared to effector $CD4^+$ T cells (Garcia et al., 1999).

Other molecules involved in peripheral T cell survival are transcription factors. One in particular, lung Kruppel-like factor (LKLF) has been shown to be expressed in naïve T cells only, and to be down regulated upon activation. Mice deficient in this molecule have strongly reduced (90%) peripheral T cell compartments (Kuo et al., 1997). Thus, genes controlled by this transcription factor are actively engaged in the survival of the cell. This active engagement of the cell in its own survival has been designated the “Red Queen Hypothesis” of lymphocyte survival (Freitas and Rocha, 1997), as lymphocytes seem to be engaged in a fight for survival, competing for survival signals. Competition and its relevance for peripheral T cell homeostasis are discussed next.

8.3- Competition and Homeostasis

Competition may be defined as “an interaction between two populations, in which, for each, the birth rates are depressed or the death rates increased by the presence of the other population” (Begon et al., 1990). In order to apply this definition to the immune system, and to lymphocyte populations in particular, we should observe if the potential for lymphocyte expansion and survival is in any way limited in the peripheral pools. As referred above (and reviewed in Freitas and Rocha, 2000), both the excess of lymphocyte production in the central lymphoid organs and the recognized potential for expansion of peripheral T cells suggest that in the normal homeostasis situation T and B cell numbers are limited by other

constraints. In order to point lymphocyte competition as one of the major factors, two major criteria must be fulfilled: the size of a population should be dependent on the presence or absence of competitors and the presence of these should alter the dynamics (or life span) of the populations.

Two simple examples can be used to show where lymphocyte competition applies or not: if the presence or absence of a lymphocyte population does not have any effect on the size of another, these populations are not competing. This is the case for T and B lymphocytes: as we have seen (see 1.2) B cell deficient mice do not have different T cell population sizes than wild type mice, and the same for the reverse situation. Thus, T and B cells are not competing populations. The demonstration of lymphocyte competition has been firstly observed for the B cell compartment (Freitas et al., 1995). In this study the size of a population of transgenic B cells was compared in a situation where the Tg B cells are the sole components of the peripheral B cell pool of an irradiated $Rag_2^{-/-}$ BM chimera reconstituted with BM from Tg origin or when the same Tg BM precursor B cells are only a fraction of the total reconstituting BM, being diluted in normal non-Tg BM cells. Although in the BM chimeras reconstituted with a single kind of BM precursors (all Tg or all wild type) the peripheral B cell pools are of the same size, when the two BM precursor types are mixed the reconstituted peripheral pools are again of the same size but display a bias towards an higher representation of the B cell from wild type origin than the proportion in the donor BM mix (Freitas et al., 1995). This selection is not observed in the precursor proB or preB pools, and it is stronger in the IgM secreting sub-population. These results show that the life span and the population sizes can be interfered with by the presence or absence of other populations (Freitas et al., 1995). Thus, the peripheral B cells are competing.

The same strategy was used to extend these conclusions to the T cell compartment, namely to the $CD8^+$ T cell compartment (Freitas et al., 1996). Here again, when Tg or wild type populations were present alone they generated T cell compartments of similar size. When mixed, the recovery of the wild type origin population was at higher proportions than injected, thus the wild type population showed a competitive advantage in the repopulation of the peripheral T cell pools of the mouse chimeras (Freitas et al., 1996). Hence, lymphocytes compete, but what are lymphocytes competing for?

Another important information came from the referred B cell study (Freitas et al., 1995): In the kinetics of reconstitution of the peripheral pools, the selection for wild type B cells was only apparent when the peripheral pool was close to maximum size: the shape of the curve followed a density-dependent growth curve that resembles the Monod growth function: it increases in a saturating manner with resource availability (Monod, 1950). What happens is that during the initial expansion phase of reconstitution resources are abundant, thus competition will only operate later, when resources are limiting. Thus, lymphocytes

seem to be competing for resources. It must be pointed out that competition may arise from different phenomena. It can either be direct competition for a shared resource or, alternatively, one population may exclude the other from the habitat where the resource is available, competition not being directly related to the resource.

How can we define resources? Resources are factors that can lead to increased cell survival or growth through at least some range of their availability (Pianka, 1976). Thus, a resource is any factor that is used by a cell and that because of this is no longer available for usage by other cells. As a result of competition, character displacement is also observed (McLean et al., 1997). This means that as a reaction to the presence of other populations there are changes in the populations considered, a phenomenon associated with the mechanisms of speciation (Schluter, 1994). This was verified to be the case for B lymphocytes, as the binding pattern of a population of diverse B cells was altered in the presence of a competing transgenic population (McLean et al., 1997). Also, by manipulating the levels of resources we should be able to alter cell survival or cell growth.

One example of resource is antigen. In the experimental systems referred (Freitas et al., 1996; Freitas et al., 1995; McLean et al., 1997) the administration of the cognate Ag of the respective Tg strains could reverse the situation, favouring dominance by the Tg populations (Freitas et al., 1996; Freitas et al., 1995; McLean et al., 1997). More recently, it was shown that even during an immune response T cells do actually compete for antigen bearing APCs (Kedl et al., 2000; Smith et al., 2000), confirming the role of T cell competition in immune responses.

Other examples of resources are MHC molecules, cytokines (cytokines will be referred in one of the following sections), chemokines, APCs, costimulatory molecules, hormones, growth factors, etc. Some of those are external to the immune system; others may be produced by the lymphocytes themselves, some can be survival signals others may be proliferation or inhibitory signals and some will act on specific sub-populations of lymphocytes while others are pleiotropic, being used by many different subsets. In this view, it should be noted that the differential expression by different subsets of lymphocytes of receptors for these resources may represent an ability to use them, ability that may turn out to be a competitive advantage for sub-populations of lymphocytes, and that can be the driving force of differentiation in resource usage, leading the sub-population to explore a specialized niche. For all of the referred above, competition is an appealing mechanism for the maintenance of peripheral T cell homeostasis (Raff, 1992).

8.4- Homeostatic proliferation

The ability of peripheral T cells to respond to a situation of T cell deficiency by peripheral proliferation is known for long to be responsible for the recovery of T cell numbers in adult mice suffering transient T cell depletion (Piguet et al., 1981; Rocha et al., 1983; Stutman, 1986). It was also clear that the peripheral T cell pools were maintained by mechanisms that were thymic independent, as could be inferred by the constant T cell numbers in aging mice or humans (see chapter 6). The use of cytostatic drugs confirmed important division rates in the peripheral pools (Freitas et al., 1986; Rocha et al., 1983), as did direct measurements of cycling cells using thymidine or BrDU (Bruno et al., 1996; Freitas and Rocha, 1993; Rocha et al., 1990; Sprent, 1993; Tough and Sprent, 1994). The ability of peripheral T cells to expand was confirmed in transfer experiments in athymic or thymectomized mice reconstituted with peripheral T cells (Freitas et al., 1986; Pereira and Rocha, 1991; Rocha et al., 1989; Sprent et al., 1991). The enormous expansion potential of peripheral T cells (Pereira and Rocha, 1991; Rocha et al., 1989) further suggested that in the physiological situation this proliferation was responsible for the turnover of part of the peripheral populations but that proliferation was restrained. Thus, it could be deduced that the extent of peripheral T cell proliferation was determined by the presence or absence of other peripheral T cells, as T cells were able to "sense" T cell deficiency and engage in proliferation. If that was the case, then this sensing mechanism could be one of the major players in peripheral T cell homeostasis. The mechanisms responsible for this peripheral proliferation and how peripheral T cells perceived deficiency were the big questions arising from this reasoning.

From studies of the survival requirements for T cells at the periphery, it is now clear that peripheral T cell survival is dependent on TCR- MHC interactions (Brocker, 1997; Freitas and Rocha, 1997; Garcia et al., 1999; Kirberg et al., 1997; Labrecque et al., 2001; Polic et al., 2001; Rooke et al., 1997; Takeda et al., 1996b; Tanchot et al., 1997; Viret et al., 1999; Witherden et al., 2000) (and see 8.3) and it is not surprising that proliferation of $CD4^+$ T cells in a T cell deficient mouse is also dependent on TCR-MHC class II interaction (Beutner and MacDonald, 1998; Ernst et al., 1999; Viret et al., 1999). With the introduction of recent techniques and experimental models (namely TCR Tg mouse models and BrDU or CFSE staining techniques) the factors leading to T cell proliferation in immunodeficient hosts were re-evaluated, and the definition of homeostatic proliferation or lymphopenia-induced proliferation could be advanced: homeostatic proliferation is the proliferation of T cells in a lymphopenic environment without intentional immunization (Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999).

The transfer of CFSE labelled (the intensity of the CFSE signal is halved at each division, allowing the distinction of cells in different rounds of division (Lyons and Parish, 1994) T cells (both $CD4^+$ and $CD8^+$) into hosts turned immunodeficient by sub-lethal irradiation (or antibody depleted of T cells) showed that the transferred cells expanded, as shown by the dilution of the CFSE labelling (Ernst et al., 1999). The degree of expansion was correlated with the amount of host radio-resistant cells: more division was observed in mice with less radio-resistant cells (Ernst et al., 1999). Accordingly, when the T cell deficient mice were supplemented with large amounts of T cells, homeostatic expansion was decreased, and this was related to the presence of T cells, as it was not observed when these mice were supplemented with B cells (Ernst et al., 1999).

TCR-MHC interactions are thus consensually considered to be involved in these events, but the nature of the peptide ligands is another important issue. Interactions with self-peptides similar to those involved in positive selection were known to be involved in peripheral survival of $CD4^+$ T cells (Viret et al., 1999). The involvement of these same interactions in homeostatic proliferation was investigated, using as hosts irradiated $H2\text{-}M^{-/-}$ mice (Martin et al., 1996; Miyazaki et al., 1996), that present the CLIP peptide as the only peptide bound to MHC class II molecules. In these hosts, $CD8^+$ T cells showed normal homeostatic proliferation, while $CD4^+$ T cells were not able to proliferate (Ernst et al., 1999). Significantly, when donor $CD4^+$ T cells were from $H2M^{-/-}$ mice homeostatic proliferation was again observed. This led to the conclusion that peptides similar (if not the same) to those involved in positive selection were responsible for homeostatic proliferation (Ernst et al., 1999).

In a parallel study (Goldrath and Bevan, 1999), very similar findings were described and similar conclusions were drawn, this time from experiments using transgenic $CD8^+$ T cells. In these experiments OT-1 TG $CD8^+$ T cells were labelled with CFSE and transferred into B6, irradiated B6, antibody depleted B6 or irradiated $TAP^{-/-}$ (expressing low levels of Class I molecules), and cell division was followed. The requirement for class I was deduced from the inexistence of cell division in the irradiated $TAP^{-/-}$ mice, as opposed to all the other hosts (except non-irradiated hosts) (Goldrath and Bevan, 1999). In order to investigate the nature of the peptide that was involved in this observation, transgenic mice were created, expressing different MHC-Class I binding peptides, of different affinity (agonist, antagonist or irrelevant) to the OT-1 TCR and bred into the $Tap^{-/-}$ background. The transfer of the OT-1 $CD8^+$ T cells to the mice expressing the agonist peptide resulted in important expansion and dilution of the dye, seen as complete dilution (>8 divisions) in the 5-day duration of the experiment. When the cells were transferred into the mice expressing Class I molecules bound to an irrelevant peptide, homeostatic proliferation was not observed. When the $CD8^+$ T cells were transferred to mice expressing an antagonist peptide bound to class I molecules,

homeostatic proliferation was observed, suggesting that low affinity interactions promote division in lymphopenic hosts in the absence of conventional antigenic stimulation (Goldrath and Bevan, 1999). Although the nature of the self-peptides intervening was debated, the major results and conclusions were further supported by other similar studies (Bender et al., 1999; Kieper and Jameson, 1999), establishing homeostatic proliferation as one of the mechanisms responsible for T cell recovery in situations of T cell depletion and, maybe, for peripheral T cell homeostasis.

In these studies, another interesting observation was made, namely that homeostatic proliferation was accompanied by a phenotype shift, from a naïve to a memory-like phenotype, characterized by the up-regulation of the CD44 marker (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999). It was suggested that this phenotype shift occurred without acquisition of effector function (Goldrath and Bevan, 1999; Kieper and Jameson, 1999), suggesting a pre-activated state. It was also suggested that a restricted repertoire results from homeostatic proliferation (La Gruta et al., 2000).

If the kind of interactions responsible for homeostatic proliferation seem to hinge upon the TCR-MHC peptide interaction, why homeostatic proliferation only occurs in a situation of T cell deficiency is still an open question. Do other T cells inhibit proliferation of neighbour cells, or is this a result of competition for APCs, costimulatory molecules, antigen (MHC-peptide ligands) or soluble factors?

Soluble factors (the cytokines IL7 and IL15 and the chemokine CCL21) have been involved in homeostatic proliferation (Schluns et al., 2000; Sprent and Surh, 2002; Tan et al., 2001), as have been DC numbers (Ge et al., 2002b). One report (Dummer et al., 2001) has identified the T cell areas of the secondary lymphoid organs as the microenvironment where both homeostatic proliferation and inhibition of homeostatic proliferation takes place (Dummer et al., 2001). The inhibition of homeostatic proliferation by other T cells seems to be independent of TCR mediated interactions, as $CD4^+$ T cells were able to inhibit homeostatic proliferation of $CD8^+$ T cells in MHC Class II^- hosts (Dummer et al., 2001), thus inhibition does not seem to be derived from competition for MHC-peptide ligands on APCs. Competition for other factors (soluble or not) in the APC's vicinity is still a possibility to explain the inhibition of homeostatic proliferation by other T cells. Another possibility is some unknown kind of direct cell-cell inhibition mechanism.

Thus, if the ligands involved in the proliferation occurring in a situation of T cell deficiency seem to be identified, the mechanisms by which the T cell senses this situation of lymphopenia are far from being known. Also, if the importance of homeostatic proliferation for the recovery of T cell numbers in a situation of T cell depletion seems unquestionable, its role in the maintenance of T cell numbers in normal homeostasis is not clear, as we do not know the extent of T cell deficiency needed to trigger homeostatic proliferation, or if this is

sensed in local microenvironments or at the whole organism level. One recent report suggests that homeostatic proliferation is indeed relevant for the first wave of migrating lymphocytes in neonatal mice (Le Campion et al., 2002), a situation where the migrating T cells are confronted with an empty peripheral T cell pool. We do not know if in normal physiology this is the only situation where homeostatic proliferation takes place.

Another important consequence seems to be the phenotype conversion of T cells undergoing homeostatic proliferation. If, in the large majority, reports point to a phenotype conversion, from naïve to memory-like phenotype (Ernst et al., 1999; Goldrath and Bevan, 1999; Kirberg et al., 1997; La Gruta et al., 2000; Le Campion et al., 2002), some reports have identified some rounds of division in T cells that keep naïve phenotypic markers (Seddon et al., 2000), and others referred that the upregulation of activation markers was a transient phenomenon (Goldrath et al., 2000), but it is not clear if these few rounds of division inside the naïve compartment represent a delay in the acquisition of the activation markers. The suggested transient acquisition of activation markers that precedes reconstitution of a naïve compartment is probably the consequence of the reconstitution in irradiated host mice of thymic derived naïve pools from precursors contained in the spleen or lymph node transferred cells (Ge et al., 2002a; Tanchot et al., 2002).

Finally, the link between the more recent studies, identifying an expansion of naïve T cells in response to a lymphopenic situation and older studies where unseparated naïve and memory T cells were transferred into empty hosts is not necessarily direct, as the contribution of the transferred memory and naïve T cells may differ. Also, in studies where polyclonal populations of T cells are transferred, contribution of possible autoreactive T cells present in the transferred cells should operate differently, and the control of their expansion operate independently (see next chapter), providing another source of expanding cells or another source of inhibiting processes. These subjects will be probably addressed in future studies.

8.5- Cellular interactions- Suppressor and Regulatory T cells

Another situation where the presence of absence of other cells is relevant for the expansion of sub-populations of cells is the mechanism involving suppressor or regulatory T cells, described as acting in the maintenance of peripheral tolerance. Tolerance can be defined as a situation where the immune system does not mount a pathologic response against a specific antigen, but responses to other antigens are not affected (Li et al., 2001). It is accepted that the immune system is tolerant to self-components. In some cases, however,

we also know that autoimmune diseases exist, thus that the mechanisms responsible for peripheral tolerance to self may fail.

There are two major classes of tolerance mechanisms: a) deletional mechanisms, that rely in the elimination of the self-reactive clones and b) non-deletional mechanisms, that act in spite of the presence of potentially self-reactive cells. Regarding T cells, key mediators of many autoimmune diseases (Sakaguchi, 2000) two important facts have been noticed: first, that the thymic selection process responsible for central deletional tolerance to many self components is not fail-proof, as self-reactive T cells can be found in the peripheral T cell pool (reviewed in Sakaguchi, 2000; Seddon and Mason, 2000; Shevach, 2000) and second, that these cells were controlled and “control” could be transferred by T cells from tolerant donors, thus the designation of dominant tolerance (as it acts in spite of the presence of the autoreactive T cells) (Davies et al., 1996; Qin et al., 1993; Waldmann and Cobbold, 1998; Waldmann and Cobbold, 2001). The T cells responsible for this suppression of potentially aggressive autoimmune responses are named suppressor (or regulatory T cells).

The existence of suppressor T cells had been postulated after the observation that the response of certain cell combinations was not only of less magnitude that the sum of the response of the individual cell populations but also of less magnitude that the response of one of the individual cell populations (Gershon et al., 1972). These reports were followed by other reports on the mechanism that was responsible for the observed suppression (Green et al., 1983). The suggested mechanism, however, relied on the supposed existence of soluble molecules of a nature that was proven to be incompatible with the discovered molecular structure of the TCR and of the MHC (reviewed in Shevach, 2000). This delayed research in this area but other studies would provide further evidence for the existence of regulatory T cells. In this introduction I will be referring mostly to one of the sub-populations of suppressor T cells (the $CD4^+CD25^+$ regulatory T cells) and to the experimental models directly related to this sub-population.(A more detailed review is found in Shevach, 2000).

8.5.1- $CD4^+CD45RB^{low}$, $CD4^+CD45RB^{high}$ T cells and the Colitis model

The first relevant combination of cell surface markers to define distinct subpopulations of $CD4^+$ T cells with effector and regulatory proprieties relied on the differential expression of the CD45RB marker. This marker defines subsets of naïve ($CD4^+CD45RB^{high}$) and primed T cells ($CD4^+CD45RB^{low}$) (Lee et al., 1990). After transfer into immunodeficient SCID mice, it was found that these markers also defined a subpopulation of $CD4^+CD45RB^{high}$ T cells capable of inducing wasting disease in the hosts and a population of $CD4^+CD45RB^{low}$ T cells that not only did not induced wasting disease or colitis in the SCID hosts, but also prevented disease provoked by the $CD4^+CD45RB^{high}$ T cells when the co-transfer of the two

subpopulations was performed (Morrissey et al., 1993; Powrie et al., 1993). Similar results had already been found in the rat (Powrie and Mason, 1990). These results suggested that in the peripheral T cell pools there were cells with autoimmune-like features and that T cells with immunoregulatory proprieties were also present. However, as the antigen was not identified it could not be concluded if the lesions observed were a manifestation of autoimmune disease or the result of uncontrolled responses to environmental antigens (Morrissey et al., 1993).

The disease was characterized also by an important IFNγ production by the activated $CD4^+CD45RB^{high}$ originated T cells (Powrie et al., 1993) and it was observed that this regulatory interaction between $CD4^+CD45RB^{low}$ and $CD4^+CD45RB^{high}$ T cells was also important for the balance between protective and pathogenic cell mediated immunity (Powrie et al., 1994a). The IBD could be prevented in the SCID hosts by the inhibition of the TH1 responses (Powrie et al., 1994b), identifying TNF and IFNγ as the mediating molecules in the disease process, and the disease could be abrogated by the systemic administration of rIL10 (Powrie et al., 1994b) but not of rIL4. Subsequent studies have also ascribed an important role to TGF-β in the regulatory process, as administration of an anti-TGF-β antibody was sufficient to abrogate the suppressive abilities of the $CD4^+CD45RB^{low}$ population (Powrie et al., 1996). This placed the regulatory function of the $CD4^+CD45RB^{low}$ T cells out of the scope of a simple TH2 versus TH1 mechanism. In agreement with a role of IL10 and of TGF-β in the prevention of colitis, the $IL10^{-/-}$ mice develop colitis (Kuhn et al., 1993) and the TGF-β 1 deficient mice (Christ et al., 1994; Shull et al., 1992) or mice expressing a dominant negative TGF-β II receptor (Gorelik and Flavell, 2000; Lucas et al., 2000) also develop autoimmune disease and wasting disease. The role of IL10 was confirmed in other reports where the transfer of $CD4^+CD45RB^{high}$ T cells transgenic for the expression of IL10 (Hagenbaugh et al., 1997) was shown not to induce colitis or where the transfer of $CD4^+CD45RB^{low}$ from $IL10^{-/-}$ mice not only did not conferred protection from disease induced by T cells but also induced colitis (Asseman et al., 1999). Interestingly, in an *in vitro* system, it was found that the repetitive stimulation of $CD4^+$ T cell clones from both mice and humans in the presence of IL10 would drive the differentiation of T cells with regulatory proprieties, named Tr1 (Groux et al., 1997). Thus, IL10 seems to be important for both the generation and function of regulatory T cells (Groux and Powrie, 1999). Note that the Tr1 and the $CD4^+CD45RB^{low}$ regulatory sub-populations may be unrelated sub-populations.

8.5.2- $CD4^+CD25^+$ Regulatory T cells

In parallel with the referred studies, other reports were highlighting the presence of regulatory T cells in the peripheral T cell pools. In studies where early thymectomy (day 3 after birth) was performed it was reported that the mice would develop autoimmune diseases, namely oophoritis (Nishizuka and Sakakura, 1969). It was also reported that the disease would not develop if thymectomy was performed at later time points (day 7) (Nishizuka and Sakakura, 1969). The development of disease would also be abolished if day 7 thymocyte suspensions, adult spleen cell suspentions or lymph node cell suspensions were given to the mice but not day 7 peripheral T cell suspensions (Kojima et al., 1976). These observations were extended to other mouse strains, where the process was similar but the type of autoimmune manifestation varied slightly, thus, tiroiditis was observed in C3H strain mice (Kojima et al., 1976) and BALB/C mice had a tendency to develop gastritis (Kojima et al., 1980). Subsequent studies shown that the disease could be transferred to syngeneic nude mice by the transfer of spleen cells from the sick mice and prevented by cell suspensions from healthy individuals. Later studies identified both the effector and the regulatory T cells as $CD4^+CD8^-$ T cells (Sakaguchi et al., 1982a; Sakaguchi et al., 1982b) (and reviewed in (Shevach, 2000).

Sakaguchi and colleagues did the first steps on the identification of the CD4+ T cell subpopulation with regulatory activity in this system. The strategy consisted in the search of the sub-population whose removal would be responsible for the development of autoimmune diseases and whose reconstitution would be resulting in the prevention of the autoimmune phenomena. The first advance was the identification of $CD4^+CD5^+$T cells as the fraction of the peripheral $CD4^+$ T cells containing regulatory T cells. Cell suspensions depleted of $CD4^+CD5^+$T cells caused the development of autoimmune diseases in syngeneic recipient nude mice and co-transfer with the $CD4^+CD5^+$T cells prevented the disease development (Sakaguchi et al., 1985). However, the $CD4^+CD5^+$T cells are a large majority and there was clearly the need to find better markers for this regulatory $CD4^+$ T cell subpopulation (Shevach, 2000). Hence, when 10 years later the same group, using the same strategy (Sakaguchi et al., 1995), identified a much smaller component of the peripheral $CD4^+$ T cell subpopulation (10%), defined by the expression of the IL2Rα chain (CD25) as the regulatory T cell, a major advance had been done. In this study, transfer of $CD4^+$ T cells depleted of the $CD25^+$ sub-population induced autoimmune disease and in some cases wasting disease when transferred into nude mice. Reconstitution of the population prevented the autoimmune manifestations in a dose-dependent manner (Sakaguchi et al., 1995). Interestingly, these cells where characterized as $CD4^+CD5^+CD45RB^{low}CD25^+$, thus they were included in the previously identified regulatory sub-populations (Powrie et al., 1994a; Powrie et al., 1993;

Sakaguchi et al., 1985), were found to be absent from the spleens of day 3 NTx mice and were shown to appear in the peripheral pools immediately after day 3 (Asano et al., 1996). It is also not surprising that the regulatory T cells capable of inhibiting IBD in the colitis model are predominantly found in the $CD4^{+}CD45RB^{low}CD25^{+}$ population (Maloy and Powrie, 2001).

It is now assumed that regulatory T cells are present in the peripheral repertoire of normal animals, that they may suppress harmful responses to self or foreign antigens and that they reside mainly in the $CD4^{+}CD25^{+}$ subpopulation (Maloy and Powrie, 2001). Importantly, the $CD4^{+}CD25^{+}$ regulatory population has been clearly identified in humans (Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Shevach, 2001; Stephens et al., 2001; Taams et al., 2001), thus the therapeutic use of regulatory T cells is a promising area, assuring further research in the study of these cells.

8.5.2.1- Characteristics of $CD4^{+}CD25^{+}$ regulatory T cells

8.5.2.1.1- Phenotype. The $CD4^{+}CD25^{+}$ are the better-characterized regulatory population. This is also in part due to their established regulatory proprieties both *in vivo* (Asano et al., 1996; Read et al., 2000; Sakaguchi et al., 1995; Suri-Payer et al., 1998) and *in vitro* (Takahashi et al., 1998; Thornton and Shevach, 1998), what allows diversified approaches in the experimental systems used. Thus, apart from the described cell surface phenotype ($CD4^{+}CD5^{+}CD45RB^{low}CD25^{+}$) $CD4^{+}CD25^{+}$ regulatory T cells are characterized by the constitutive expression of CTLA4 (Read et al., 2000; Takahashi et al., 2000) and of GITR, the Glucocorticoid-induced TNF receptor (McHugh et al., 2002; Shimizu et al., 2002). Recent reports, using DNA array analysis identified, besides GITR (McHugh et al., 2002), other genes, some of which may be involved in the suggested "anergic" phenotype of the $CD4^{+}CD25^{+}$ regulatory T cells (Gavin et al., 2002; McHugh et al., 2002).

8.5.2.1.2- $CD4^{+}CD25^{+}$ regulatory T cells are "anergic". In the first studies where the *in vitro* suppressive ability of the $CD4^{+}CD25^{+}$ regulatory T cells was shown (Takahashi et al., 1998; Thornton and Shevach, 1998) it was also found that these cells had an anergic phenotype, as described by their poor proliferative response upon TCR stimulation (Thornton and Shevach, 1998) and that these cells were dependent on exogenous IL-2 for growth (Papiernik et al., 1998). Indeed, other reports refer to anergic T cells as regulatory T cells, either without reference to CD25 expression (Chai et al., 1999), or clearly showing that these anergic regulatory T cells express the CD25 marker (Jordan et al., 2000).

8.5.2.1.3- Cytokine profile. Other important characteristic of the $CD4^{+}CD25^{+}$ regulatory T cells is the cytokine profile. These cells were reported to produce larger amounts of TGFβ, IL4 and of IL10 (Asano et al., 1996) than their $CD25^{-}$ counterpart. This has

been confirmed for IL10 but not for IL4 (Thornton and Shevach, 1998). The cytokine expression has been associated with their function in some situations but not in others (see below) but the cytokine profile, IL10 production in particular, is a distinctive feature of these cells.

8.5.2.1.4- Caveats of the CD25 marker. The major difficulty when using the CD25 marker as a marker for the regulatory $CD4^+$ T cells is the fact that this marker is for long known as an activation marker, thus that it is induced upon TCR stimulation (Nelson and Willerford, 1998), and thus, the general assumption is that a fraction of the 10% $CD4^+CD25^+$ T cells found in the peripheral pool represents a contaminant population of activated T cells, and not a regulatory T cell (Maloy and Powrie, 2001). Accordingly, when CD25 was induced on a CD25- population upon activation the resulting cells were devoid of suppressive ability (Thornton and Shevach, 1998).

The CD25 designation stands for the αchain of the IL2R and the expression of this protein does not seem to be related with the function of these cells, thus the search for cell surface markers specific for the regulatory T cells continues (Sakaguchi, 2000). In accordance, reports of $CD4^+$ regulatory T cells in the $CD25^-$ fraction exist (Annacker et al., 2001; Olivares-Villagomez et al., 1998; Read et al., 2000) what suggests that either there are really several regulatory subpopulations or that the CD25 is not the definitive marker for these cells (Maloy and Powrie, 2001).

8.5.2.2- Mechanism of suppression by Regulatory T cells

The mechanism by which the regulatory T cells exert their suppressive and regulatory activity has been under intense investigation but it is still far from being elucidated. This situation may be reflecting the different experimental systems used but most probably reflects heterogeneity not only in the regulatory $CD4^+$ populations studied but also in the mechanisms by which regulatory T cells regulate. Interestingly, the two major categories of regulatory mechanisms described so far segregate with the two major categories of experimental systems used: in studies performed *in vivo*, the most commonly suggested mechanisms are related to the secretion of suppressive cytokines while *in vitro* studies point for a cell-contact dependent mechanism. Importantly, the readout for the regulatory activity is not necessarily the same for *in vitro* and *in vivo* studies: the usual readout for *in vitro* regulatory activity is suppression of proliferation of other populations while the readout for *in vivo* regulatory activity is more complex but usually involves protection from disease induced by other populations (reviewed in Maloy and Powrie, 2001).

8.5.2.2.1- Cytokine mediated regulation. Strong evidence exists for the role of cytokines in the effector function of regulatory T cells *in vivo*. As discussed above (see 8.5.1)

IL10 and TGF-β are involved in the protection conferred by $CD4^+CD45RB^{low}$ regulatory T cells and both these cytokines seem to be expressed at higher levels in the $CD4^+CD25^+$ population as well (Asano et al., 1996). These cytokines can be mediating suppression directly on the target "pathogenic" T cell or act indirectly, via suppressive effects on APC function (Maloy and Powrie, 2001) or by supplying the correct cytokine milieu for the development of regulatory T cells (Groux et al., 1997). The regulatory activity was shown not to be mediated by IL4, as regulatory T cells from $IL4^{-/-}$ mice were effective in the suppression of $CD45RB^{high}$ mediated colitis (Powrie et al., 1996; Powrie et al., 1994b). The non-involvement of IL4 clearly separates the regulatory T cells from a possible confusion with TH2 cells, and adds to the evidence suggesting that these cells are a lineage apart from other effector populations.

8.5.2.2.2- Cell-contact dependent regulation. The major body of evidence against the role of cytokines in the regulatory function of $CD4^+CD25^+$ regulatory T cells concentrates on the *in vitro* studies of suppression (Nakamura et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000). As referred, the ability of the $CD4^+CD25^+$ regulatory T cells to suppress the proliferation of other cell populations *in vitro* has allowed the identification of important requirements for the regulatory activity. The suppressor activity was found to be independent of cytokines *in vitro*, as the addition to the cultures of anti-IL10, anti-IL4, anti-TGF-β or anti-IL10 and anti-TGF-β antibody did not abrogate suppression (Takahashi et al., 1998; Thornton and Shevach, 1998) and $CD4^+CD25^+$ regulatory T cells from $IL4^{-/-}$ or from $IL10^{-/-}$ mice were able to suppress $CD4^+CD25^-$ proliferation (Takahashi et al., 1998; Thornton and Shevach, 1998). Most interestingly, when the two sub-populations were separated by a semi-permeable membrane suppression was abrogated (Takahashi et al., 1998; Thornton and Shevach, 1998), suggesting that regulation was mediated by a cell-contact dependent mechanism (Takahashi et al., 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000). Whether this contact is a direct cell-contact between the suppressor and the suppressed cell, or via a third-party cell (APC), is still in debate, as suppressor activity was observed when irradiated or fixed APCs were present (Takahashi et al., 1998; Thornton and Shevach, 2000) but it was also shown that the regulatory T cells act on the APC, down-regulating the expression pf MHC Class II, CD80 and CD86 (Cederbom et al., 2000; Vendetti et al., 2000) opening the way for two possible mechanisms of cell-contact dependent suppression. A recent report suggested another intriguing possibility, namely that cell-contact dependent suppression is mediated by cell surface-bound TGF-β (Nakamura et al., 2001), building a bridge linking two categories of suppressive mechanisms.

8.5.2.2.3- Role of CTLA-4 in suppression. The costimulatory molecule CTLA4 has also been suggested as relevant in the mechanism of suppression by $CD4^+CD25^+$ regulatory T cells (Read et al., 2000; Takahashi et al., 2000), as blockade of CTLA4 interaction resulted in inhibition of suppression by $CD4^+CD25^+$ regulatory T cells. However, *in vitro* studies failed to reach the same conclusion (Jonuleit et al., 2001; Thornton and Shevach, 1998). Together with the report of suppressive activity of $CD4^+CD25^+$ cells from $CTLA4^{-/-}$ mice (Takahashi et al., 2000) these reports suggest that CTLA4 is not involved in the mechanism of regulation by $CD4^+CD25^+$ regulatory T cells. Thus, more experiments are needed in order to clarify the role of CTLA4 in $CD4^+CD25^+$ regulatory activity.

Altogether, the reported data and the suggested mechanisms seem to be closer to suggest that the $CD4^+CD25^+$ regulatory T cells exert their suppressive and regulatory functions by more than one mechanism than to identify the mechanism responsible for their abilities. This can also reflect the other side of the problem: the nature of the responses that are being suppressed in these experimental systems may not be the same in all cases. We have addressed some of these questions (section B, article #2 and additional results) and this subject will deserve more space in the discussion section.

8.5.2.3- Specificity of $CD4^+CD25^+$ regulatory T cells

One well established feature of the $CD4^+CD25^+$ regulatory T cells is that although these cells need to be activated via their TCR, after activation the regulatory capacity is antigen non-specific (Takahashi et al., 2000; Thornton and Shevach, 2000). Thus, $CD4^+CD25^+$ regulatory T cells isolated from TCR Tg mice inhibited the responses of $CD4^+CD25^-$ cells to the same antigen but also inhibited responses to other antigens, without requirement for antigen recognition or MHC restriction (Takahashi et al., 2000; Thornton and Shevach, 2000) but depending on their own activation, as the Tg $CD4^+CD25^+$ regulatory T cells inhibited the response of their $CD25^-$ counterpart when activation was done by the cognate peptide or with anti-CD3 antibody, while $CD4^+CD25^+$ regulatory T cells from normal BALB/C mice could only inhibit responses induced by anti-CD3 (Thornton and Shevach, 2000). Importantly, the $CD4^+CD25^+$ regulatory T cells require a much lower concentration of peptide than required to activate the $CD4^+CD25^-$ responder T cells (Takahashi et al., 1998). It was also reported that these cells inhibit responses of $CD8^+$ T cells (Takahashi et al., 2000), reinforcing the non-specific nature of the *in vitro* suppressive ability of the $CD4^+CD25^+$ regulatory T cells.

8.5.2.4- Generation of $CD4^{+}CD25^{+}$ regulatory T cells

There is enough evidence to consider the generation of these cells as one of the thymic functions (Maloy and Powrie, 2001; Seddon and Mason, 2000; Shevach, 2000). SP $CD4^{+}$ thymocytes contain 5-10% of $CD25^{+}$ cells with suppressive capacity in humans (Stephens et al., 2001), rats (Stephens and Mason, 2000) and mice (Itoh et al., 1999; Papiernik et al., 1998). These findings also pose interesting questions regarding the developmental process that leads to the generation of these cells. The $CD4^{+}CD25^{+}$ regulatory T cells are absent from Tg mice bred on the $Rag_2^{-/-}$ background but present in Tg mice on a conventional background, what indicates that their generation is dependent on the endogenous expression of TCR α chains (Itoh et al., 1999; Thornton and Shevach, 2000) and is dependent on thymic generation. More recent reports suggested that the generation of these cells was dependent on MHC Class II^{+} thymic epithelium (Bensinger et al., 2001) and dependent on the nature of the peptide mediating positive selection, thus that these cells could be induced by an agonist peptide (Jordan et al., 2001). Another recent report suggests that these cells are selected as a proportion of the naturally generated thymocytes, suffering normal positive and negative selection, as $CD4^{+}CD25^{+}$ cells were found in similar proportions in mice expressing reduced diversity of peptides bound to class II molecules (Pacholczyk et al., 2002).

One possibility is that inside the window of avidity allowing positive selection, there is another small window of higher avidity TCR self-peptide interactions that drives selection of thymocytes into the $CD4^{+}CD25^{+}$ lineage (Modigliani et al., 1996) (fig. 11), this would allow the selection of a small fraction of regulatory T cells, that could be “anergic” in their proliferative responses but would be nevertheless activated by lower amounts of MHC bound self-antigen (Itoh et al., 1999).

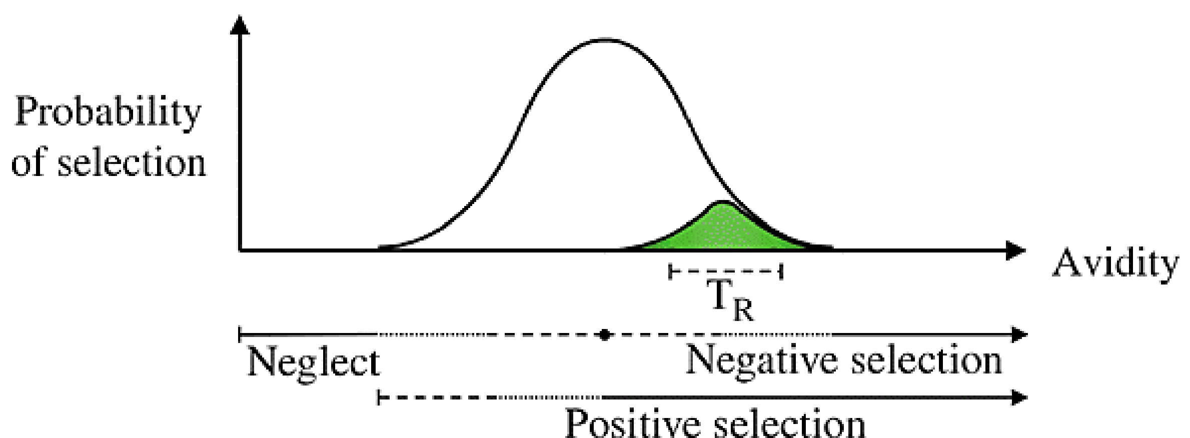

Figure 11: Thymic selection of regulatory T cells. The figure illustrates a theory for thymic selection of regulatory T cells (see text). (Modified from Maloy et al., 2001).

8.5.3 – Other regulatory T cells

Other sub-populations of lymphocytes with regulatory activity have been described both inside and outside the $CD4^+$ T cell compartment. Non-$CD4^+$ regulatory cells include $CD4^-$ $CD8^-$ T cells (Zhang et al., 2000), NK T cells (Hammond et al., 1998; MacDonald, 1995), NK cells (Homann et al., 2002) and $CD8^+$ T cells (Garba et al., 2002; Suzuki et al., 1999). Other regulatory $CD4^+$ T cells include the referred Tr1 cells, generated after repetitive stimulation in the presence of IL10 (Groux et al., 1997) or the T_H3 subpopulation, that arises after oral administration of and antigen and that produce high amounts of TGF-β (Neurath et al., 1996; Weiner, 1997). The overlap in these $CD4^+$ subpopulations is still an open question, to be addressed in future studies.

8.5.4 - Homeostasis and $CD4^+CD25^+$ regulatory T cells

What are the consequences or implications of the presence of $CD4^+CD25^+$ regulatory T cells in the peripheral T cell pools?

The implications of the interactions responsible for this mechanism of control have already been extended beyond the field of tolerance. For instance, in one report (Shimizu et al., 1999) it has been shown that the removal of the $CD4^+CD25^+$ regulatory subpopulation could evoke effective tumour immunity in otherwise non-responding mice (Shimizu et al., 1999). Thus, the $CD4^+CD25^+$ regulatory subpopulation may have a much broader role in the control of immune responses, or in the control of T cell proliferation. These cells may be major players in the maintenance of population sizes and occupy a central position in the organization of the mature T cell pools. Interestingly, the proportion of $CD25^+$ (5-10%) cells seem to be highly conserved in the thymic and peripheral $CD4^+CD8^-$ T cell pools of humans (Dieckmann et al., 2001; Levings et al., 2001; Stephens et al., 2001; Taams et al., 2001), and mice (Papiernik et al., 1998; Sakaguchi et al., 1995; Shevach, 2000) and mice deficient in the CD25 molecule (Willerford et al., 1995) have a perturbed peripheral homeostasis.

We have addressed these issues (see results section, article #2 and additional results), and the results and conclusions can be found in the articles themselves and in the discussion section.

8.6- Resources: The role of Cytokines

As referred, resources are factors that can lead to increased cell survival or growth through at least some range of their availability (Pianka, 1976). Different kinds of molecules can function as resources for T lymphocytes and can, in some situations become limiting, determining the size and the composition of the peripheral T cell pools.

Among these molecules, cytokines are one of the most commonly suggested, due to their role in differentiation and in the effector mechanisms of lymphocytes. Cytokines can be defined as small soluble proteins secreted by one cell that can alter the behaviour or properties of the same or other cells (for an overview see Janeway et al., 1999). Cytokines can be produced by the components of the immune system or by other cells. Cytokines can act locally or at a distance, and thus the concentration of these molecules in specific environments can reflect immunological states of the whole individual or local responses. In all cases, the cytokine milieu is an important part of the peripheral niches supporting peripheral pools of lymphocytes. Thus, cytokines can function as resources and can also be produced by lymphocytes. This means that lymphocytes can play a role in the creation of their own niches (Freitas and Rocha, 2000). It also means that cytokines are important mediators of interactions between lymphocytes. Finally, the expression of receptors for these cytokines can be correlated with the niche occupancy of different sub-populations; thus, the expression of cytokine receptors can be important for the definition of specialized sub-populations of lymphocytes.

Some cytokines are involved in the effector function of lymphocytes and others are involved preferentially in the differentiation of lymphocytes. In some cases, the same cytokine may be involved in both. Also, some cytokines have been identified as relevant for survival of lymphocytes, others as relevant for expansion of lymphocytes and others seem to be inhibitors of T cell expansion. It would be fastidious to describe here the features of all cytokines. It would also be too extensive to name all cytokines with a possible role as resources or in homeostasis of the peripheral T cell pools. I will concentrate in this introduction in three cytokines shown to be involved in the survival of lymphocytes: IL2, IL7 and IL15, as these cytokines are related in the receptor usage and are or can be implicated in the homeostatic mechanisms directly studied in this thesis (see results and discussion sections). Other cytokines have already been referred in this introduction, namely effector cytokines secreted by $CD4^+$ T cells (see 7.2.3) and others involved in regulatory T cell function (see 8.5).

8.6.1- The IL2 Receptor

The IL2R has shared components with the receptors for a number of other cytokines, namely IL4, IL7, IL9 and IL15. This implies that overlapping effects may occur.

The IL2R is a multimeric receptor, composed of three different subunits: IL2Rα (CD25), IL2Rβ (CD122) and IL2Rγ or γ_c (CD132) (reviewed in Nelson and Willerford, 1998). The IL2Rβ and the IL2Rγ are the signalling components, while the IL2Rα regulates affinity for IL2. Thus, the IKL2Rα is the “private” chain for IL2, while the IL2Rβ chain is shared with the receptor for IL15 and the IL2Rγ chain is shared with the receptors for IL4, IL7, IL9 and IL15, the reason why it is also referred to as the common γ chain or γ_c (Nelson and Willerford, 1998)

In the T cell lineage, the IL2Rα is expressed in immature $CD4^-CD8^-CD3^-$ (triple negative) thymocytes, and its expression is turned off after TCRβ rearrangement. It is again expressed in a fraction of SP thymocytes. In the periphery 10% of $CD4^+$ T cells and 1% of $CD8^+$ T cells express this molecule, and its expression can be further induced by TCR stimulation (Nelson and Willerford, 1998). It is at this time unclear weather the peripheral expression of the IL2Rα chain (CD25) reflects ongoing T cell activation or thymic export of CD25 expressing cells or what is the proportion from each origin. The IL2Rα chain is also expressed in other lineages, namely in the B cell lineage, in immature pre-B cells and also in some mature B cells after activation (Nelson and Willerford, 1998).

The IL2Rβ chain has a different pattern of expression, being constitutively expressed at low levels in resting T cells, B cells, NK cells, macrophages and neutrophils (Nelson and Willerford, 1998). In T cells, Its expression is upregulated upon activation through the TCR or not (Nelson and Willerford, 1998).

The IL2Rγ chain is expressed constitutively in T and B cells, NK cells, macrophages, neutrophils and granulocytes, reflecting its usage by several different cytokine receptors. In opposition to what has been observed for the two other chains, its expression decreases with T cell activation (Nelson and Willerford, 1998).

The relevance of these different components of the IL2R is appreciated when the phenotypes of the existing deficient mice for each of the receptors' constituents are considered.

As had been found in humans with mutations in the IL2Rγ chain (Nelson and Willerford, 1998), the phenotype of the IL2R$\gamma^{-/-}$ mouse is characterized by a severe immunodeficiency (DiSanto et al., 1995), with an important reduction in pre-B cell numbers and in thymocyte numbers (22 fold reduced) (DiSanto et al., 1995). In the periphery, T and B cells pools were also reduced (3 and 12 fold, respectively), and NK cells were absent (DiSanto et al., 1995). Thus the absence of γ_c resulted in an incomplete block in T and B cell

development and in an incomplete replenishment of the peripheral pools (DiSanto et al., 1995). These results were consistent with an important role for IL7 mediated signals in T cell development, as had been shown with $IL7R^{-/-}$ mice (Peschon et al., 1994). When aHY transgenic γ_c deficient mice were generated, the thymic hypoplasia was partially reversed in female mice, but the peripheral numbers of Tg cells were severely reduced, indicating that besides of the role of γ_c signals in T cell development, these signals are also critical for T cell maintenance or expansion in the peripheral pools (DiSanto et al., 1996). In a subsequent study, using Class II restricted transgenic anti-HY TCR $CD4^+$ T cells, it was shown that γ_c signals are essential for naïve T cell survival but not for memory T cell survival or for antigen-APC stimulated activation (Lantz et al., 2000). However, these results do not allow the identification of the interleukin responsible for the observed effect. As we will see below, IL7 is the most probable cytokine involved in this result. These results are also relevant as they provide further support for the niche differentiation between naïve and memory T cells (see 7.2.1 and 8.2).

The $IL2R\beta$ chain is shared by the IL2 and the IL15 receptors only (Nelson and Willerford, 1998). The phenotype of the $IL2R\beta^{-/-}$ mouse is very different from the phenotype of the $IL2R\gamma^{-/-}$ mouse. The main characteristic of the $IL2R\beta^{-/-}$ mouse is a deregulated T cell activation, and consequent autoimmunity (Suzuki et al., 1995). The autoimmune B cell phenotype could be rescued by $CD4^+$ T cell depletion; thus, $CD4^+$ T cells were the trigger of $IL2R\beta$ deficiency derived autoimmunity (Suzuki et al., 1995). At 3 weeks of age these mice suffered from splenomegaly and lymphoadenopathy, as a result of important T and B cell expansion. $CD4^+$ T cell depletion could rescue mice from autoimmunity. However, at later time points myeloproliferative disorders still developed, suggesting an independent intrinsic abnormality in granulocytes (Suzuki et al., 1995). In common with the $IL2R\gamma$ deficiency, $IL2R\beta^{-/-}$ mice also showed an abnormal development in intestinal intraepithelial lymphocytes and in NK cells (Suzuki et al., 1997a). The thymic selection events were apparently not affected, discarding a role for IL15 or IL2 in thymic function (what was already apparent from the $IL2^{-/-}$ and $IL15^{-/-}$ mouse analysis, see below) and, in contrast to what had been described for the $IL2R\alpha$ deficiency (see below), Fas-mediated apoptosis was normal (Suzuki et al., 1997b). Thus, the $IL2R\beta^{-/-}$ mouse revealed a role similar to the $IL2R\gamma$ chain, when NK or certain sub-populations of intestinal intraepithelial lymphocytes are concerned, but it also revealed an almost opposed role in the peripheral T cell pools, as the $IL2R\beta^{-/-}$ mouse was characterized by an uncontrolled proliferation of T cells and resulting autoimmunity. This phenotype was later ascribed to a role of $IL2R\beta$ mediated signals in the development of a $CD8^+$ T cell regulatory population, that was suggested to act either by preventing T cell activation or by elimination of activated T cells (Suzuki et al., 1999). Here again, the results

are not conclusive regarding the roles of the two cytokines capable of using the IL2Rβ chain as part of their receptor complex.

The IL2Rα$^{-/-}$ mice have also been described (Willerford et al., 1995) and share some features with the IL2Rβ$^{-/-}$ mice but not others. Note that the IL2Rα chain (or CD25) is part only of the IL2 receptor, thus the phenotype of the IL2Rα chain deficient mouse should correlate with the phenotype of the IL2 deficient mouse. Thus, the IL2Rα deficient mice have an apparently normal development of T and B cells (like the IL2Rβ$^{-/-}$ mice) but develop when adults a massive enlargement of the peripheral lymphoid organs, associated with peripheral T and B cell activation and leading to autoimmune manifestations (including anaemia and inflammatory bowel disease) and death (Willerford et al., 1995). Here again it was suggested that the B cell abnormalities were secondary to the T cell abnormalities. The latter were first proposed to be related to a defect in AICD, that would allow the accumulation of activated T cells in the peripheral pools (Van Parijs et al., 1997; Willerford et al., 1995). Later studies, however, have shown that failure of AICD may not be the major cause of the non-homeostasis status of the IL2Rα$^{-/-}$ mice and suggested that IL2Rα mediated signals were important in the control of bystander proliferation (Leung et al., 2000). We have investigated the possible causes for the phenotype of the IL2Rα$^{-/-}$ mice and provided an alternative possibility (see results section, article #2 and discussion herein and the general discussion section).

Thus, from the analysis of the different deficient mice for the different IL2R component chains, we see that the different cytokines signalling through the IL2R may have different roles. Next, the cytokines IL2, IL7 and IL15 and their role in homeostasis are discussed.

8.6.2- IL2

IL2 was first identified as a T cell growth factor *in vitro*. It was also shown that IL2R expression was induced after activation and that in the appropriate costimulatory conditions, TCR signalling also induces synthesis of IL2, providing an autocrine/paracrine loop. IL2 is involved in many immune and inflammatory responses and has a role in B and NK cell differentiation *in vitro*. Many of these proprieties were first described through *in vitro* studies and the interpretation of the enormous amount of information is sometimes difficult, taking into account the possible overlap with signals from other γ_c dependent cytokines. Here I will concentrate in what is known from the *in vivo* studies of IL2 function.

The description of the IL2$^{-/-}$ mouse (Schorle et al., 1991) provided no evidence for a suspected role of IL2 in T cell development. As the IL2Rα chain is expressed in some of the

immature stages of T cell development, a role for IL2 in T cell development had been expected. This was not confirmed, as T cell development in IL2$^{-/-}$ mice was normal (Schorle et al., 1991). The first report did not described any differences in the peripheral T and B cell pools of IL2$^{-/-}$ mice, but it confirmed limited T cell responses *in vitro*, unless exogenous IL2 was added (Schorle et al., 1991). These observations were rectified, as it was shown that if the *in vitro* responses were indeed affected, *in vivo* responses were within normal range (Kundig et al., 1993). In another study (Sadlack et al., 1993), the normality of the peripheral T cell pools of IL2$^{-/-}$ mice was proven wrong, as older (>4 weeks) mice developed a colitis-like disease and important disorders of peripheral homeostasis, with elevated proportions of activated T cells, splenomegaly and lymphoadenopathy, a phenotype similar to the one later found in the IL2R$\alpha^{-/-}$ mice (see above). Here again, CD4^{+} T cells were found responsible for the autoimmune disease (Sadlack et al., 1995; Kramer et al., 1995) and the latter study reported that the abnormal activation of IL-2$^{-/-}$ lymphocytes may be controlled by thymus-derived lymphocytes (Kramer et al., 1995).

IL2 is still considered to be an important growth and survival factor, but at the same time it is also described as having the ability to sensitise T cells to Fas-mediated AICD. This can be related to different signalling pathways dependent on theIL2Rβ. Thus, it has been suggested that IL2 signals can promote T cell proliferation and AICD through the STAT5 dependent signalling pathway (Van Parijs et al., 1999) and to promote T cell survival through activation of Akt and subsequent Bcl-2 expression (Kelly et al., 2002; Van Parijs et al., 1999). These independent pathways may be related to the different mechanisms that have been suggested for the control of hyperactivation of CD4^{+} T cells in IL2$^{-/-}$ mice (Wolf et al., 2001). The mechanisms and processes involved in IL2 deficiency syndrome and IL2Rα deficiency associated deregulation of peripheral T cell homeostasis were investigated and are discussed in the results section (article #2) and in the discussion section.

8.6.3- IL7

IL7 was first described as a factor produced by BM stromal cells, capable of supporting the growth and survival of immature B cells (Namen et al., 1988). The role of IL7 in T cell development was also demonstrated, as seen in IL7$^{-/-}$ or IL7R$^{-/-}$ mice, that displayed reduced thymic and peripheral T cell compartments (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). The role of IL7 in the peripheral compartments was seen, in IL7 transgenic mice (Mertsching et al., 1995) or in mice to which recombinant human IL7 was administrated (Komschlies et al., 1994), by an increase in T cell numbers (and also in other lineage cells), suggesting a role for IL7 in survival and/or expansion of peripheral T cells, as had been suggested by the peripheral phenotype of the IL7$^{-/-}$ or IL7R$^{-/-}$ mice (Peschon et al., 1994; von

Freeden-Jeffry et al., 1995). Indeed, $IL7R^{-/-}$ peripheral T cell were found to have impaired survival and proliferation capacities (Maraskovsky et al., 1996). Subsequent *in vitro* studies, using human cord blood $CD4^+$ T cells, provided evidence that suggested that IL7 was important for naïve T cell maintenance and even expansion, without conversion to a memory phenotype (Soares et al., 1998; Webb et al., 1999). This has been also suggested for mouse T cells as it has been shown that IL7R are expressed by naive and memory $CD8^+$ T cells (Schluns et al., 2000), interestingly, its expression was downregulated in activated $CD8^+$ T cells. As homeostatic proliferation of naïve $CD4^+$ and $CD8^+$ T cells was abrogated after transfer into lymphopenic $IL7^{-/-}$ hosts ($Rag^{-/-}IL7^{-/-}$) it was concluded that IL7 was relevant for homeostatic proliferation of naïve T cells (Schluns et al., 2000). The same study suggested that non-BM-derived cells were the source of IL7 used by naïve and memory T cells in homeostatic proliferation (Schluns et al., 2000). In another report IL7 was again identified as an important resource for naïve $CD4^+$ T cell survival (Vivien et al., 2001) or survival and homeostatic proliferation (Tan et al., 2001). In studies of peripheral T cell pool regeneration after BM transplantation, the IL7 was again identified as an important factor for both thymic dependent and independent regeneration pathways (Mackall et al., 2001). In this study, thymic emigrants and established peripheral T cells seemed to compete for IL7, thus IL7 availability was suggested to define the T cell "space", and thus, to determine the size of the peripheral T cell pool. Hence, IL7 seems to be one important resource, whose availability may become limiting and for which T cells may compete.

8.6.4- IL15

The last cytokine of this series is IL15. As determined by the analysis of the phenotype of both the $IL15^{-/-}$ (Kennedy et al., 2000) and the $IL15R\alpha^{-/-}$ mice (Lodolce et al., 1998), IL15 is essential for NK cell development, for the development of some populations of intestinal intraepithelial lymphocytes and for the maintenance or development of memory phenotype $CD8^+$ T cells (Kennedy et al., 2000; Lodolce et al., 1998). These latter are increased in IL15 transgenic mice (Marks-Konczalik et al., 2000). IL15 (with IL7) is also important for homeostatic proliferation of $CD8^+$ T cells (Goldrath et al., 2002; Kieper et al., 2002; Tan et al., 2002). Thus, IL15 seems to be an important cytokine for the $CD8^+$ T cell memory compartment. Interestingly, it does not seem to play a role in the $CD4^+$ memory T cell compartment (Tan et al., 2002).

In all, these studies place the γ_c using cytokines, namely IL7 and IL15 as important cytokines for peripheral T cell survival, and the differential usage by different subpopulations as an appealing mechanism for the establishment of the peripheral subpopulation structure. The

role of IL2 seems more complicated, due to the suggestion of the relevance of the same cytokine for both cell-maintenance and cell elimination processes. With our work, we hope to have provided evidence for the possible mechanisms involved.

9- THIS THESIS

In order to advance in the understanding of the mechanisms responsible for peripheral $CD4^+$ T cell homeostasis, I investigated the relevance of putative mechanisms contributing for peripheral T cell homeostasis. These were: thymic export, interactions between individuals and the role of the environment. Thus, The role of thymic export was evaluated by studying a situation where the thymic export could be modulated (section B article #1), the role of cellular interactions was investigated through the $CD4^+CD25^+$ regulatory T cell study (section B article #2 and additional results) and the role of putative resources in the establishment of the observed sub-population structure was evaluated, through the role of IL2 in the maintenance of the $CD4^+CD25^+$ regulatory T cell sub-population (section B article #2 and additional results). The significance of the results obtained is discussed in the final sections of this thesis, having in mind the aspects referred in the introduction section.

SECTION B

RESULTS

ARTICLE #1

" T Cell Homeostasis: Thymus regeneration and Peripheral T Cell Restoration in Mice with a Reduced Fraction of Competent Precursors"

Afonso R. M. Almeida, José A. M. Börghans, and Antonio A. Freitas

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T Cell Homeostasis: Thymus Regeneration and Peripheral T Cell Restoration in Mice with a Reduced Fraction of Competent Precursors

Afonso R.M. Almeida, José A.M. Borghans, and António A. Freitas

Lymphocyte Population Biology, Unité de Recherche Associée Centre National de la Recherche Scientifique 1961, Institut Pasteur, 75015 Paris, France

Abstract

We developed a novel experimental strategy to study T cell regeneration after bone marrow transplantation. We assessed the fraction of competent precursors required to repopulate the thymus and quantified the relationship between the size of the different T cell compartments during T cell maturation in the thymus. The contribution of the thymus to the establishment and maintenance of the peripheral T cell pools was also quantified. We found that the degree of thymus restoration is determined by the availability of competent precursors and that the number of double-positive thymus cells is not under homeostatic control. In contrast, the sizes of the peripheral CD4 and CD8 T cell pools are largely independent of the number of precursors and of the number of thymus cells. Peripheral "homeostatic" proliferation and increased export and/or survival of recent thymus emigrants compensate for reduced T cell production in the thymus. In spite of these reparatory processes, mice with a reduced number of mature T cells in the thymus have an increased probability of peripheral T cell deficiency, mainly in the naive compartment.

Key words: CD4 T cells • CD8 T cells • homeostasis • thymus regeneration • thymus export

Introduction

Regeneration of the immune system, in the adult, is one of the major challenges of today's cell therapy. T cell regeneration from hematopoietic stem cell precursors (HSCs)* is required after HIV infection and after bone marrow (BM) transplantation after aggressive cancer therapies (1–3). It can also be used in other clinical applications, such as gene therapy (4). In spite of major progresses in the use of HSCs for T cell reconstitution, we still lack important information. Contrary to other blood cell lineages developing from HSCs, T cell progenitors must first migrate to the thymus to mature. In the adult, this may pose a problem, as the thymus is atrophic and may no longer be able to generate T cells (5). We do not know what fraction of competent precursor cells is needed to restore complete thymus function, or what are the quantitative aspects of the regeneration of the double-positive (DP) and single-positive (SP) thymus compartments. The mechanisms that determine the number of T lymphocytes in the peripheral lymphoid system are also poorly understood. In young adult mice there is a continuous seeding of the periphery by newly formed thymus migrants (6). Nevertheless, the number of peripheral T cells remains even (7). This implies that either (a) the migrant cells are rapidly lost without ever colonizing the periphery, or (b) there is a continuous replacement of the peripheral cells by recent thymus migrants. Most studies indicate that a part of the peripheral T cell pool can be maintained independently of thymus export, but do not allow a precise evaluation of the role of thymus T cell production in physiological conditions (8). We developed a novel strategy that allows (a) a quantitative assessment of the fraction of competent pre-T cell precursors required to restore thymus function and (b) the evaluation of the contribution of the thymus to the peripheral T cell pools.

Address correspondence to Antonio A. Freitas, Lymphocyte Population Biology Unit, URA CNRS 1961, Institut Pasteur, 25 Rue du Dr. Roux, 75015 Paris, France. Phone: 33-1-45-68-8552; Fax: 33-1-45-68-8921; E-mail: afreitas@pasteur.fr

**Abbreviations used in this paper:* BM, bone marrow; DN, double negative; DP, double positive; HSC, hematopoietic stem cell precursor; Rag, recombination activating gene; SP, single positive.

Materials and Methods

Mice. B6.Rag2$^{-/-}$ (9), B6.CD3$\epsilon^{-/-}$ (10), B6.TCR$\alpha^{-/-}$(11), all Ly5^b, and C57Bl/6.Ly5^a mice were obtained from the Centre de Devélopment des Tecniques Avancées-Centre National de la Recherche Scientifique (CDTA-CNRS; Orléans, France).

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BM Chimeras. Host 8-wk-old recombination activating gene (Rag)$2^{-/-}$B6 mice were lethally irradiated (900 rad) with a ^{137}Ce source and received intravenously 2 to 4 × 10^6 T cell–depleted BM cells from different donor mice, mixed at different ratios. T cell depletion was done by 2–3 passages in a Dynal MPC6 or AutoMacs (Miltenyi Biotec) magnetic sorter after incubating the BM cells with anti-CD4, anti-CD8, and anti-CD3 biotinylated antibodies followed by anti–rat IgG1 or Streptavidin-coated Dynabeads. Purity was tested by flow cytometry and the injected BM cells were found to contain <0.1% T mature cells. By using donor and host mice who differ according to Ly5 allotype markers, we were able to discriminate between the T cells originating from the different donors. 10 to 20 wk after reconstitution mice were killed and BM, thymus, spleen, and LN cells suspensions were prepared as described (12). The total number of peripheral T cells represents the number of T cells in the spleen added to twice the number of T cells present in the mesenteric and inguinal LNs to account for the total LN mass.

Thymus Cell Export. Mice were anesthetized, the upper chest opened, and the thymus lobes exposed. One thymus lobe was injected with 10 μl of FITC (1 mg/ml) which resulted in the labeling of ~50–70% of all thymocytes (6). Mice were killed 24 h later and the recent thymus emigrants present in the spleen and LNs were identified by flow cytometry as live FITC$^+$ cells expressing Ly5^a, CD3, and CD4 or CD8.

Flow Cytometry Analysis. The following monoclonal antibodies were used: anti-CD8α (53–6.7), anti-CD3ε (145–2C11), anti-CD4 (L3T4/RM4–5), anti-CD25 (PC61), anti-CD45RB, anti-CD24/HSA (M1/69) from BD PharMingen, and anti-CD44 (IM781), anti-CD62L (MEL14) from Caltag. Cell surface four color staining was performed with the appropriate combinations of FITC, PE, TRI-Color, PerCP, biotin, and allophycocyanin (APC)-coupled antibodies. Biotin-coupled antibodies were secondary labeled with APC-, TRI-Color- (Caltag), or PerCP-coupled (Becton Dickinson) streptavidin. Dead cells were excluded during analysis according to their light-scattering characteristics. All acquisitions and data analyses were performed with a FACSCalibur™ (Becton Dickinson) interfaced to the Macintosh CELLQuest™ software.

Mathematical Analysis. The relationship between the number of competent T cells (or thymus cells) T in a given compartment and the number of competent cells N in a previous compartment was modeled by the following differential equation:

$$dT/dt = sN + p - mT, \quad (1)$$

where s denotes the rate at which cells transit from the N compartment into the T compartment, m represents the rate at which T cells exit from the T compartment due to mortality or differentiation into the next compartment, and p represents a homeostatic regulation term. As all compartments had reached steady-state levels at the times at which the mice were killed (similar T cell recoveries were obtained 8–20 wk after BM reconstitution), the experimental data were fitted to the steady-state level corresponding to Eq. 1: $T = sN/m + p/m$. The homeostatic regulation term was included only if it significantly ($\alpha = 0.005$) improved the fit to the data; in all other cases the data were fitted to the line $T = sN/m$. The optimal fits of the steady-state functions to the experimental data were determined using a generalized Gauss-Newton method to minimize the sum of the squared residuals (SSRs) between the logarithms of the data and the model. The logarithmic transformation was made because the experimental errors were likely to be proportional to the cell numbers measured. Note, however, that the model that was fitted (see above) is linear.

Results and Discussion

Thymus Regeneration. Thymus regeneration can be readily obtained by the injection of a very limited number of HSC precursor cells (13). The injected self-renewing pluripotential HSCs divide and completely restore the precursor cell pools in the BM and in the thymus. During clinical BM transplantation, however, newly injected competent precursors may be diluted among the host's incompetent cells. The quantitative relationship between the fraction of competent precursor cells able to colonize the thymus and the regeneration of DP and SP thymus cell compartments has never been studied in these conditions. Here, we evaluated the regeneration of the thymus by a limited fraction of competent precursor cells. Lethally irradiated lymphopenic B6.Rag$2^{-/-}$ mice were reconstituted with T cell–depleted BM cells from normal B6.Ly5^a donors alone or from normal B6.Ly5^a and T cell–deficient B6.Ly5^b mice mixed at several ratios. This strategy should reduce the number of competent precursors available for thymus colonization and regeneration, as normal Ly5^a competent precursor cells are diluted among Ly5^b incompetent precursors from the mutant donors (14, 15). 2 to 5 mo after BM reconstitution, when all T cell compartments had reached steady-state levels, we counted the number of cells from each donor type in the CD3$^-$CD4$^-$CD8$^-$ (double-negative [DN]), CD4$^+$CD8$^+$ (DP), and mature CD4$^+$ CD8$^-$/CD4$^-$CD8$^+$ (SP) compartments. We used three types of T cell–deficient BM donors: TCRα$^{-/-}$ mice with a block of T cell differentiation at the DP to SP transition, which have normal numbers of DP cells, but lack mature SP T cells (11), and CD3ε$^{-/-}$ or Rag$2^{-/-}$ mice with an earlier block of T cell differentiation at the DN to DP transition, which lack DP cells (9, 10). Studying thymus regeneration in the chimeras obtained with BM cells from these different mutants allows comparing the restoration of the DP and SP thymus compartments from a limited number of competent DN precursors. This could be done in the absence or in the presence of incompetent DP cells, i.e., in CD3ε$^{-/-}$ or Rag$2^{-/-}$ and in TCRα$^{-/-}$ mixed chimeras, respectively.

We found that the number of competent DP cells was proportional to the number of competent DN cells (15), i.e., a twofold lower number of competent DN cells resulted in a twofold reduction in the number of competent DP cells (Fig. 1, A and B). This proportionality was observed both in B6.Ly5^{b}TCRα$^{-/-}$/B6.Ly5^a chimeras (Fig. 1 A) whose TCRα$^{-/-}$ precursors can generate incompetent DP cells and in B6.Ly5^{b}CD3ε$^{-/-}$/B6.Ly5^a (Fig. 1 B) or B6.Ly5^{b}Rag$2^{-/-}$/B6.Ly5^a chimeras (not shown), which both lack incompetent DP cells. Thus, limiting numbers of DP precursor cells do not accumulate and restore the thymus DP compartment even in the absence of competitor incompetent DP cells. These findings indicate that, when the number of precursors is fewer than normal, the total number of DP cells is not regulated by homeostatic control mechanisms, i.e., there is no increase in the rate of division or survival of DP cells in mice with small DP compart-

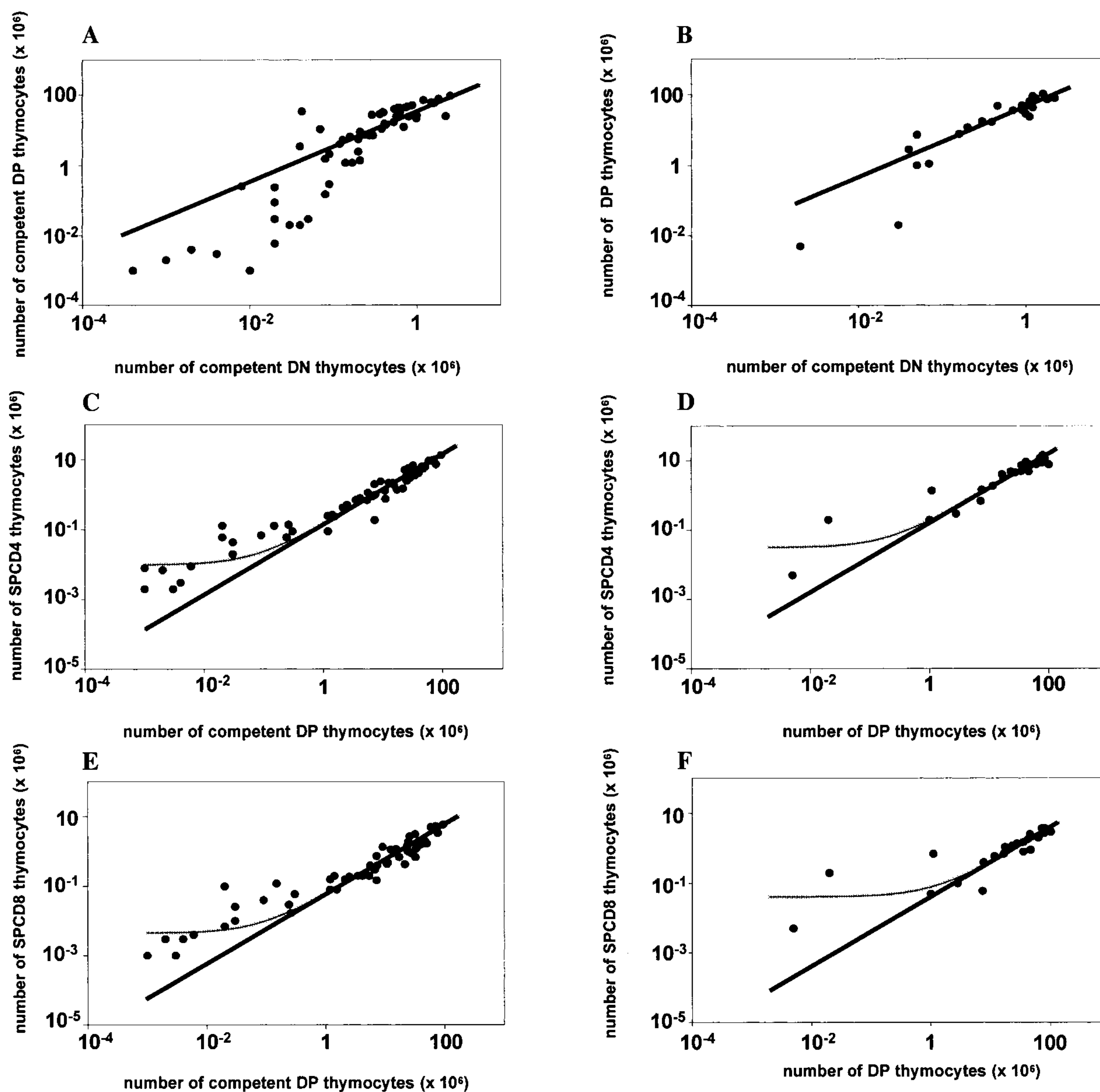

Figure 1. Thymus regeneration. Lethally irradiated $Rag2^{-/-}$ mice were reconstituted with BM cells from normal $B6.Ly5^a$ alone or diluted among incompetent BM cells from either $B6.Ly5^bTCR\alpha^{-/-}$ (A, C, and E) or $B6.Ly5^bCD3\epsilon^{-/-}$ (B, D, and F) donors. 8 to 20 wk after reconstitution the chimeras were killed and the number of competent $Ly5^a$ cells was evaluated in the different thymus cell compartments. For each chimera (●), the relationship between the number of competent cells in the $CD3^-CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP) compartments is shown in A and B, between the DP and the SPCD4 compartments in C and D, and in the DP and the SPCD8 compartments in E and F. The curves show the relationships between DN, DP, SPCD4, and SPCD8 cells as predicted by the mathematical model (see Mathematical Analysis). All datasets were fitted twice: once including all data points (thin lines), and once excluding the mice with very low DN ($<10^5$) or DP ($<10^6$) cell numbers (thick lines). In A and B, both of the fits could not be significantly improved by adding a homeostatic term. Moreover, both fits predicted a too high number of competent DP cells in mice with very few competent DN cells, suggesting that an additional mechanism (not included in the model) is involved. The model we used was sufficient, however, to conclude that in mice with at least 5% of the normal number of competent DN cells, the number of competent cells in the DP compartment was proportional to the size of the DN compartment (see thick lines). Likewise, in C–F we found a proportionality between the numbers of competent DP cells and SP cells (see thick lines) in all mice except the ones with very low numbers of competent DP cells ($<10^6$). The addition of a small homeostatic term only helped to describe the relatively high SP cells numbers in the latter mice (see thin lines), while it did not improve the fits between the model and the data from all other mice. Except in very poorly reconstituted mice, the size of each thymus compartment is thus proportional to the size of the compartment that precedes it. The parameter values (see Mathematical Analysis) that gave the best fits to the data are: s/m = (A) 35, (B) 47, (C) 0.14, (D) 0.17, (E) 0.06, (F) 0.04 (thick lines), and p/m = (C) 0.01, (D) 0.03, (E) 0.005, and (F) 0.04 (thin lines).

ments. In steady-state conditions, the number of competent DP cells was roughly 40-fold higher than the number of competent cells in the DN compartment; i.e., $\sim 10^6$ DN cells originated $\sim 40 \times 10^6$ DP cells. Interestingly, in mice with a very low number of competent DN cells ($<10^5$, i.e., $<5\%$ of normal) the number of competent DP cells was lower than expected from the otherwise proportional relationship between DN and DP cells (Fig. 1, A and B). This

may result from a limiting dilution effect due to the low frequency of competent precursors of which only 5/9 will make a productive TCRβ rearrangement and proceed in the T cell differentiation pathway. Alternatively, this could be due to a more efficient DP to SP transition at low cell numbers, which would cause depletion of the DP compartment (see below) (16). In conclusion, these findings indicate that in normal mice the number of competent DN precursor cells available strictly determines the number of DP cells.

In the thymus of the T cell–deficient/normal mixed BM chimeras the number of TCR^{high}SPCD4 (Fig. 1, C and D) or TCR^{high}SPCD8 cells (Fig. 1, E and F) was proportional to the number of competent DP cells. A twofold lower number of competent DP cells gave rise to a twofold lower number of TCR^{high} mature SP cells recovered from the thymus. The number of CD4 and CD8 cells in the SP compartment were ~15 and 5% of the number of competent DP cells, respectively, i.e., a DP compartment consisting of 10^7 cells gave rise to a SP compartment with 1.5×10^6 SPCD4 cells and 5×10^5 SPCD8 cells. At very low numbers of competent DP cells ($<10^6$) the number of SP cells was always higher than expected (see thick lines in Fig. 1). The data could therefore best be described by including a very small homeostatic term (see Mathematical Analysis). This increases the predicted number of SP cells in mice with very low numbers of competent DP cells, but does not affect the predicted number of SP cells in all other mice (see the thin lines in Fig. 1, C–F). One interpretation is that there is an increased efficiency of the DP to SP transition in poorly reconstituted mice (16), probably reflecting the higher stromal cell to thymocyte cell ratio. This explanation would be consistent with the relatively small number of DP cells found in mice with few competent DN cells. However, in mice with low numbers of thymus cells we could expect that the probability of generating the correct TCR is lower, decreasing the chances of positive selection. Alternatively, a homeostatic compensation mechanism may induce the proliferation or prolonged survival of the rare SP cells. Finally, reentry of mature peripheral T cells, which is negligible in normal conditions (<0.1% of PBL), may also contribute to biases the number of SP cells in the chimeras with low thymus cell numbers. In conclusion, these results indicate that in the range of 5–100% of the normal number of thymus cells the sizes of the DP and SP cellular compartments are fully determined by the input of competent DN cells. When the fraction of competent thymus cells is below 5% of normal there is a less efficient DN to DP transition and/or a more efficient generation of mature SP T cells.

Peripheral T Cell Pool Restoration. We showed that by decreasing the fraction of competent cells in the transplanted BM we were able to proportionally reduce the number of mature SP thymus cells. The experimental strategy employed thus allows for a quantitative correlation between T cell production in the thymus and the number of peripheral T cells. The relative contribution of the thymus to the maintenance of the peripheral T cell pool has been investigated either after thymus ablation (17) or by increasing the thymus mass with multiple ectopic transplants (18), procedures that strongly deviate from physiological conditions. Thymectomy in neonatal and adult mice results in a permanently reduced size of the peripheral T cell pool. In both cases, however, a significant number of T cells persist in absence of the thymus (19). Engraftment of multiple thymus lobes increases the functional thymus mass and the number of recent thymus emigrants. The peripheral T cell pool size, however, does not increase proportionally to the overall increase in thymus mass (20, 21).

To evaluate the impact of reduced thymus mature T cell numbers on the size of the peripheral T cell pool we first examined whether a reduction in the number of SP cells matched with a reduced rate of thymus cell output. It was previously shown that the fraction of recent thymus emigrants is constant at ~1–2% of thymocytes/day, independently of the number of thymus lobes and of an increase in the number of peripheral T cells (6, 22, 23). Thymus export in adult mice with diminished thymus T cell production and peripheral pools, however, has never been studied.

Table I. *Thymic Export in Mice with Reduced Thymus Function*

Fraction of competent C57Bl6 Ly5[a] BM cells injected	Recent thymus migrants ($\times 10^4$)[a]	
	$CD4^+$	$CD8^+$
100%	9.6	3.9
	9.0	2.6
	8.6	4.9
	8.0	3.0
	6.5	2.5
	4.1	2.0
	4.9	2.7
10%	3.2	1.1
	2.2	0.5
	1.2	0.4
	1.8	1.4
	1.4	0.8
	3.8	1.3
	2.3	0.9

Thymus cell export varies according to the fraction of competent precursor cells. $Rag2^{-/-}$B6 mice were lethally irradiated and reconstituted with BM cells from normal B6.$Ly5^a$ donors alone (100%) or from normal B6.$Ly5^a$ (10%) and T cell–deficient B6.$TCR\alpha^{-/-}Ly5^b$ (90%) mice. In these chimeras, the number of SP cells was proportional to the fraction of competent BM cells injected. Thymus export was evaluated 24 h after intrathymus injection of FITC.

[a]The number of recent thymus migrants was identified in the spleen and LN of the chimeras by flow cytometry as live $FITC^+$ cells expressing both CD4 (or CD8) and CD3.

We found here that, in chimeras with low numbers of SP cells, the sum of recent thymus emigrants (RTEs) in the peripheral lymphoid tissues was lower than in mice with normal numbers of SP cells. The relative thymus output in chimeras with low numbers of SP cells was, however, 3.2–3.4-fold higher than in control chimeras (Table I). Thus, the accessibility to thymus exit may be easier in the presence of reduced numbers of SP cells. Alternatively, RTEs may survive longer because of reduced competition in the periphery (24). These results indicate that the "efficiency" of thymus cell export increases with low numbers of thymus cells, but is insufficient to compensate for the reduced production of mature thymus cells. In conclusion, by using the mixed T cell–deficient/normal BM chimeras strategy we can reduce in a controlled fashion the production of mature SP T cells in the thymus and thereby the seeding of the peripheral tissues by thymus emigrants. Thus, this strategy indeed allows a quantitative assessment of the contribution of the thymus to the establishment and maintenance of the peripheral T cell pools.

We studied the total number of peripheral CD8 and CD4 T cells in mice with reduced thymus T cell production and export. In contrast to what we reported in the thymus, we found that the total number of CD4 and CD8 cells in the periphery was not proportional to the number of cells in the previous progenitor compartment, i.e., thymus SPCD4 (Fig. 2 A) and SPCD8 cells (Fig. 2 A). In most chimeras with reduced numbers of thymus SP cells the sizes of the peripheral T cell compartments were similar to those in the chimeras with normal numbers of thymus SP cells. Mathematical analysis of the data suggests that a compensatory homeostatic mechanism be involved, even in mice with a nearly normal thymus output. We estimate that in mice in which only 1% of the normal numbers of SPCD4 cells and SPCD8 cells were present, the peripheral CD4 and CD8 compartments still contained 25 and 12.5% of the normal, respectively. Thus, in the presence of reduced thymus output, T cell survival and/or proliferation are favored (8, 14, 25) as to attain normal peripheral T cell numbers. In concordance, we found that the lower was the number of peripheral $CD8^+$ or $CD4^+$ T cells, the higher was the fraction of activated $CD4^+CD45RB^{low}$ (Fig. 2 C) and $CD8^+CD44^+$ (Fig. 2 D) T cells. These findings demonstrate that the numbers of peripheral CD4 and CD8 T cells

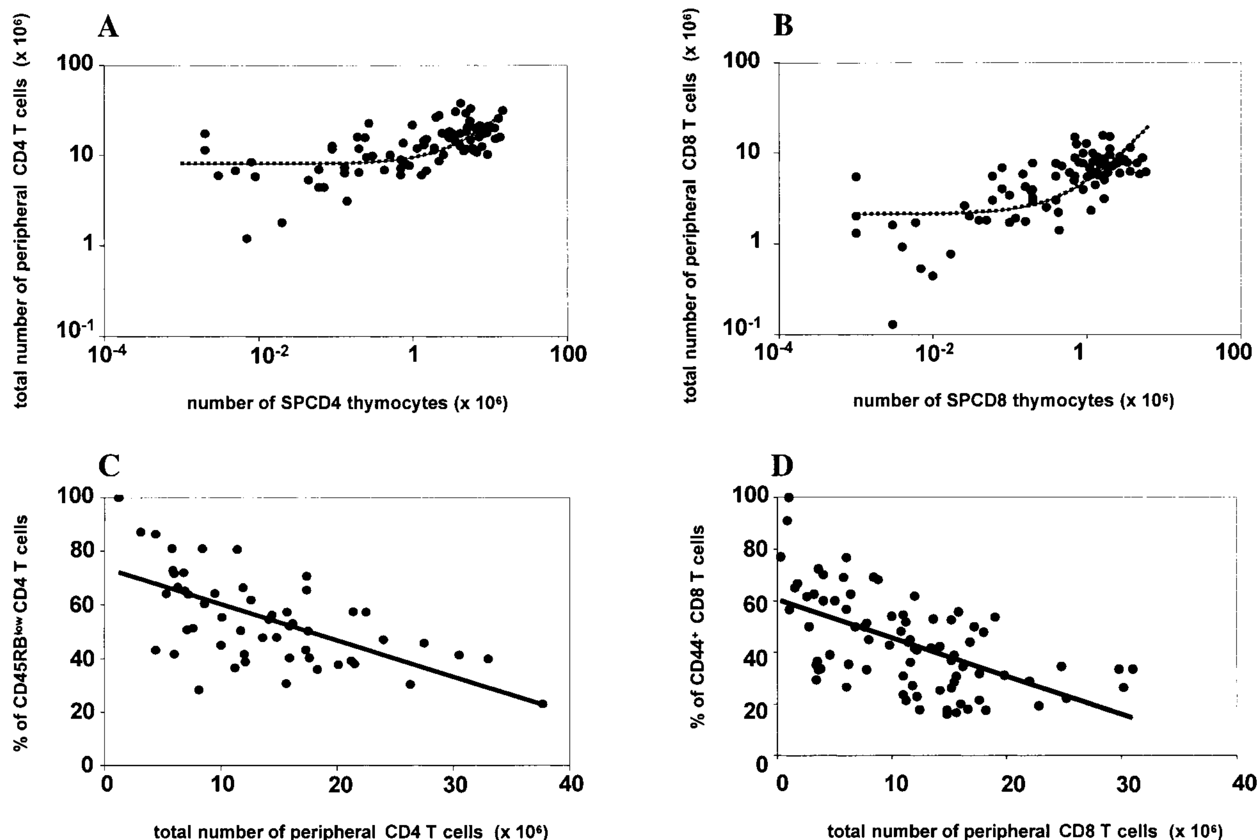

Figure 2. Restoration of the total peripheral T cell pools. Panel A shows the relationship between the number of peripheral $Ly5^a$ CD4 T cells (SPL+LN) and the number of competent $Ly5^a$ SPCD4 cells in the thymus of each individual $B6.Ly5^a/B6.Ly5^bTCR\alpha^{-/-}$ or $B6.Ly5^a/B6.CD3\epsilon^{-/-}Ly5^b$ chimera. (B) The same but for CD8 T cells. The data were fitted to the steady-state level corresponding to Eq. 1 including the homeostatic term (dashed lines), as this significantly improved the fits to the data, even if mice with very low SP numbers ($<10^5$) were not taken into account (not shown). Parameter results are: s/m = (A) 1.5, (B) 2.8, and p / m = (A) 8.0, (B) 2.1. Panel C shows the percentage of activated/memory $CD45RB^{low}CD4^+$ T cells as a function of the total number of peripheral CD4 T cells, while D shows the percentage of activated/memory $CD44^+CD8^+$ cells as a function of the total number of peripheral CD8 T cells. The lines in C and D are linear regression lines with $r = -0.6$ in both cases.

are only partly determined by the rates of thymus cell production and export. In summary, these results show that chimeras with reduced numbers of SP thymocytes can have normal peripheral T cell numbers, suggesting that in normal mice thymus T cell production exceeds the quantitative requirements to replenish the number of T cells in the peripheral pool. Chimeras with very low numbers of SP thymus cells do, however, have an increased probability of not being able to fully reconstitute the CD4 and the CD8 peripheral compartments.

Previous observations have lead to the definition of two cellular compartments in the peripheral T cell pool, with independent homeostatic regulation (26). There is a pool of naive T cells which is dependent on thymus T cell production comprising all recent thymus emigrants (21) and a pool of activated/memory T cells capable of persisting in

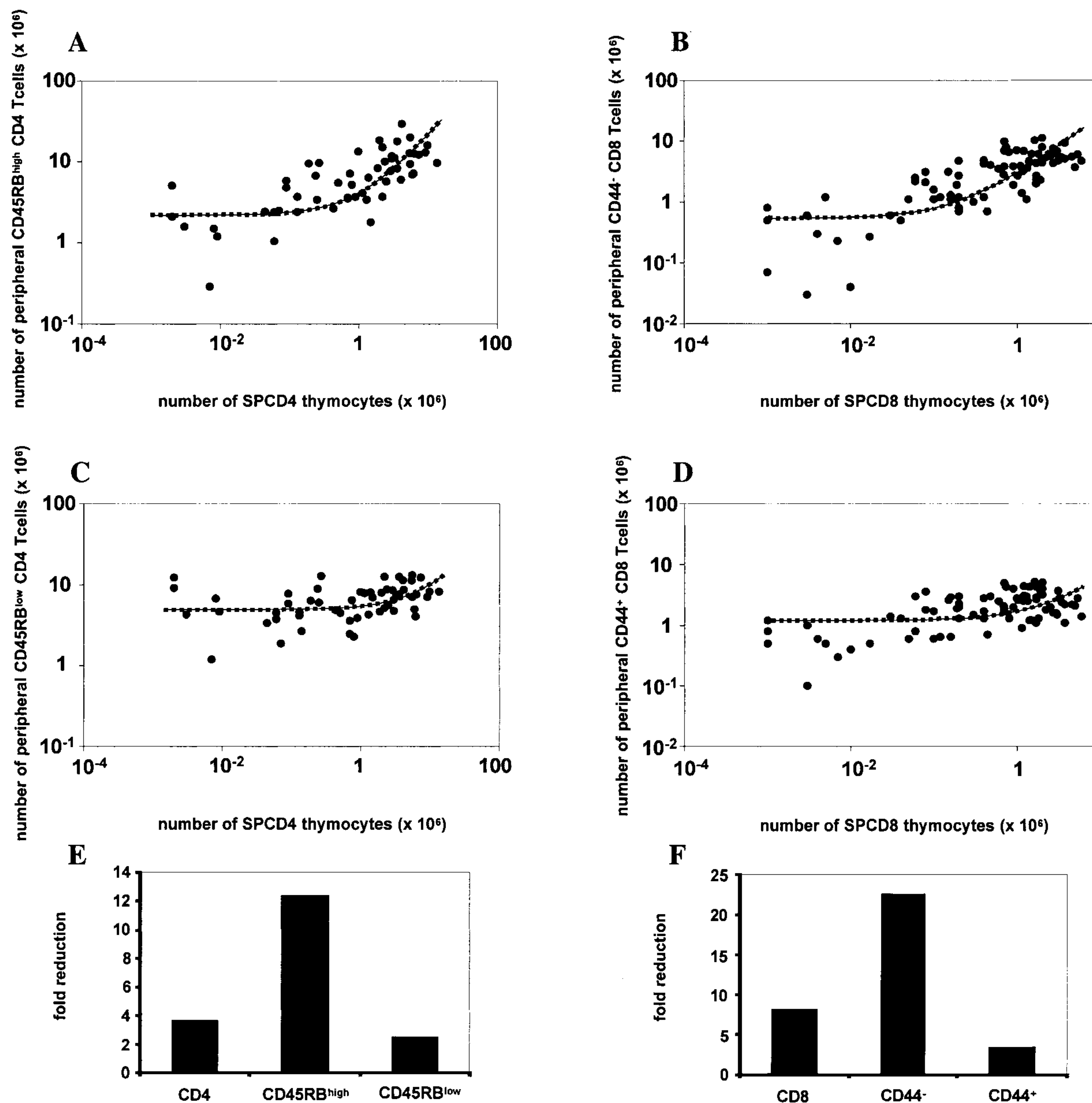

Figure 3. Restoration of the naive and activated/memory T cell pools. Panel A shows the relationship between the number of SPCD4 cells and the number of peripheral naive $CD45RB^{high}CD4^{+}$ T cells. (B) The relationship between the number of SPCD8 and the number of peripheral naive $CD44^{-}CD8^{+}$ T cells. (C) The relationship between the number of SPCD4 and the number of peripheral activated/memory $CD45RB^{low}CD4^{+}$ T cells. (D) The relationship between the number of SPCD8 and the number of peripheral activated/memory $CD44^{+}CD8^{+}$ T cells in all $B6.Ly5^{a}$/$B6.Ly5^{b}TCR\alpha^{-/-}$ and $B6.Ly5^{a}$/$B6.Ly5^{b}CD3\epsilon^{-/-}$ chimeras. The data were fitted to the steady-state level corresponding to Eq. 1 including the homeostatic term, as this significantly improved the fits to the data, even if mice with very low SP numbers ($<10^{5}$) were excluded (not shown). Parameter results are: s/m = (A) 2.0, (B) 2.6, (C) 0.5, (D) 0.5, and p / m = (A) 2.2, (B) 0.5, (C) 5.0, (D) 1.2. E and F show the fold reductions in the total, naive ($CD45RB^{high}CD44^{-}$) and activated/memory ($CD45RB^{low}CD44^{+}$) CD4 (E) and CD8 (F) peripheral compartments resulting from a 100-fold reduction (compared with fully reconstituted mice) in the thymus SPCD4 and SPCD8 compartments, respectively.

absence of thymus output (27). We define a naive T cell as a cell that does not express activation markers, i.e., in B6 mice, CD4 T cells that are $CD45RB^{high}$, and CD8 T cells that are $CD44^{-}$. We compared the effects of a reduced thymus output on the establishment of the naive ($CD4^{+}$ $CD45RB^{high}$ and $CD8^{+}CD44^{-}$; Fig. 3, A and B) and the memory/activated ($CD4^{+}CD45RB^{low}$ and $CD8^{+}CD44^{+}$; Fig. 3, C and D) peripheral T cell compartments. We found that the numbers of both naive and memory/activated T cells were not proportional to the number of thymus SP mature T cells. Upon a 100-fold reduction in the SP thymus cells, i.e., in mice with 1% of the normal number, there was a threefold reduction of the activated/memory cells, while the naive CD4 and CD8 compartments decreased 12- and 23-fold, respectively (Fig. 3, E and F). These results suggest the existence of a hierarchical organization that favors the replenishment of the activated/memory T cell pool in lymphopenic mice, as described previously for B cells (28). The size of the memory/activated compartment is thus indeed less dependent on thymus export than the size of the naive T cell pool. Still, the mice with very low thymus T cell production had an increased probability of not being able to fully reconstitute the peripheral memory/activated pools. Additionally, the diversity of the TCR repertoire in mice with very low T cell production was impaired. We studied the TCR Vβ chain expression by peripheral T cells in chimeras reconstituted with 100 or 1% competent BM cells. We found that while the patterns of Vβ chain usage in chimeras with normal thymus output were identical (Fig. 4 A), in mice with low thymus output they were unique in each individual mouse (Fig. 4 B). These findings suggest that in the presence of low thymus output the homeostatic proliferation of a few rare T cells lead to the establishment of an oligoclonal T cell repertoire (29). This may also explain the shift of peripheral T cell repertoires observed during aging, after thymus atrophy and reduced T cell production (5).

Concluding Remarks. We developed a novel experimental strategy to study T cell regeneration in mice with a limited fraction of competent precursor cells. The results obtained have major implications to the understanding of thymus regeneration after BM transplantation (1). We directly demonstrated that complete regeneration of the thymus DP and SP compartments is strictly determined by the availability of a sufficient fraction of competent DN precursors. This is due to the lack of compensatory homeostatic mechanisms that could increase the proliferation or survival of DP and SP thymus cells. Only when the number of thymus DN cell precursors is less than 5% of normal, reparatory mechanisms increase the efficiency of generation of mature SP T cells. These processes are nevertheless insufficient to overcome the deficit in precursor cell numbers. Our results suggest that complete thymus regeneration requires the complete elimination of incompetent precursor cells to prevent dilution of competent precursors and consequently the reduction of the fraction of competent DN cells present in the thymus.

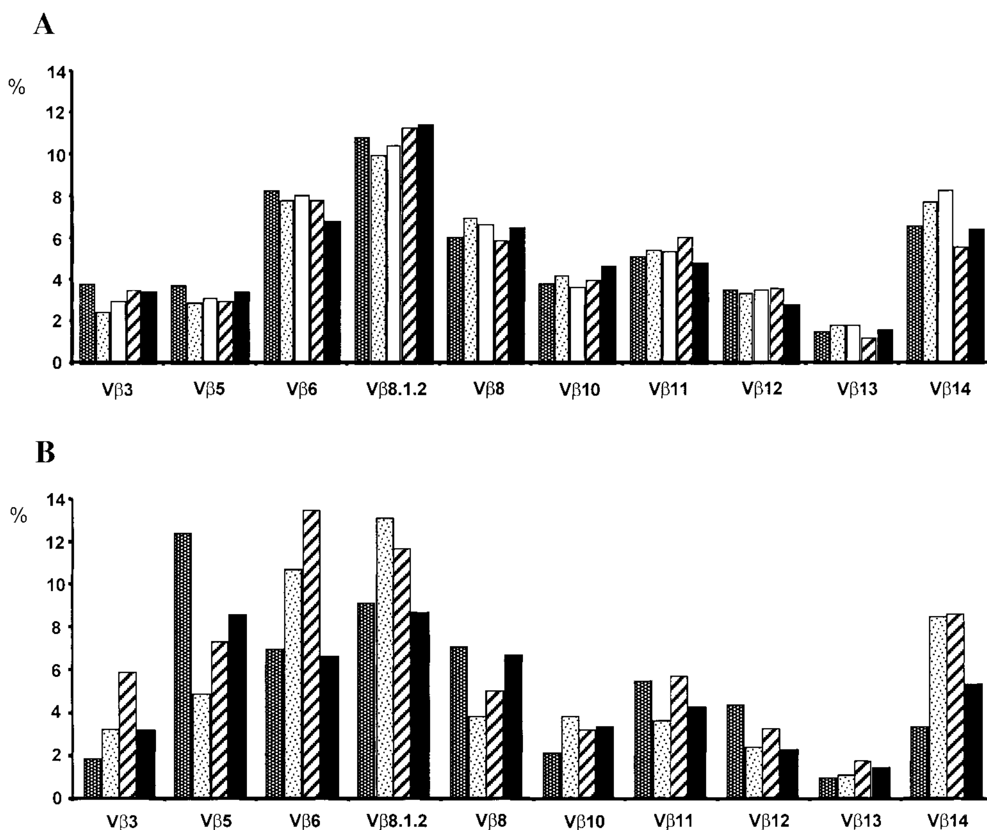

Figure 4. Vβ TCR repertoires in chimeras with normal and low T cell numbers. Representation of the different Vβ TCR families by the spleen T cells of different BM chimeras. Mice were reconstituted with either 100% BM cells from normal $B6.Ly5^{a}$ mice (A) or with a mixture of 1% BM cells from normal $B6.Ly5^{a}$ mice and 99% BM cells from T cell–deficient $B6.Ly5^{a}$/ $B6.Ly5^{b}TCR\alpha^{-/-}$ donors (B). Each bar represents the percentage of $CD3^{+}CD4^{+}$ T cells expressing each Vβ family in individual mice as assessed by flow cytometry. Similar results were obtained with $CD3^{+}CD8^{+}$ spleen T cells. Note that although the representation of each Vβ family is identical in all mice reconstituted with 100% BM cells from normal donors, it shows individual variations in mice reconstituted with a limited fraction of competent BM cells.

These results also shed light on the mechanisms of peripheral T cell restoration after tri-therapy of HIV infected individuals (30–32). By studying the peripheral T cell pools in mice with reduced thymus function we show that the size of the total peripheral T cell pool is regulated largely independently of thymus output. At the periphery, several compensatory mechanisms operate to bypass the reduced production of mature T cells in the thymus. We show, for the first time, that in mice with smaller thymus SP cell numbers and peripheral T cell pools the efficiency of thymus cell export improves. More importantly, homeostasis induces preferential activation of rare naive T cells and proliferation in the memory/activated pool. In spite of all these redeeming processes, insufficient production of mature T cells resulted in an increased probability of peripheral T cell deficiency, mainly in the naive compartment. Proliferation of peripheral T cells in lymphopenic mice ("homeostatic" proliferation) was previously described either in thymectomized mice during T cell recovery following T cell elimination (12) or following the fate of T cells adoptively transferred into T cell deficient hosts (33–39). Here we used a different approach to study peripheral T cell restoration that allowed us to establish a direct quantitative relationship between thymus function and peripheral T cell numbers. In summary, our studies demonstrate that complete peripheral T cell recovery requires a minimally functional thymus, which can only be ensured with a minimal number of competent DN precursors. Thus, the incomplete peripheral T cell restoration that is observed in most HIV patients after tri-therapy may reflect thymus compromise, which should be taken in consideration in the development of new therapeutic approaches (2, 3, 40).

Finally, these results also bear interest on the mechanisms of immune deficiency developing with aging. We found that mice with $<1\%$ of the normal number of thymus SP cells have reduced numbers of naive T cells and develop oligoclonal repertoires, a situation that mimics the evolution of the immune system in aged individuals.

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ARTICLE #2

"Homeostasis of peripheral CD4$^+$ T cells: IL-2Rα and IL-2 shape a population of regulatory T cells that controls CD4$^+$ T cell numbers"

Afonso R. M. Almeida, Nicolas Legrand, Martine Papiernick
& Antonio A. Freitas

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Homeostasis of Peripheral CD4$^+$ T Cells: IL-2Rα and IL-2 Shape a Population of Regulatory Cells That Controls CD4$^+$ T Cell Numbers[1]

Afonso R. M. Almeida,* Nicolas Legrand,* Martine Papiernik,† and António A. Freitas[2]*

We show that the lymphoid hyperplasia observed in IL-2Rα- and IL-2-deficient mice is due to the lack of a population of regulatory cells essential for CD4 T cell homeostasis. In chimeras reconstituted with bone marrow cells from IL-2Rα-deficient donors, restitution of a population of CD25$^+$CD4$^+$ T cells prevents the chaotic accumulation of lymphoid cells, and rescues the mice from autoimmune disease and death. The reintroduction of IL-2-producing cells in IL-2-deficient chimeras establishes a population of CD25$^+$CD4$^+$ T cells, and restores the peripheral lymphoid compartments to normal. The CD25$^+$CD4$^+$ T cells regulated selectively the number of naive CD4$^+$ T cells transferred into T cell-deficient hosts. The CD25$^+$CD4$^+$/naive CD4 T cell ratio and the sequence of cell transfer determines the homeostatic plateau of CD4$^+$ T cells. Overall, our findings demonstrate that IL-2Rα is an absolute requirement for the development of the regulatory CD25$^+$CD4$^+$ T cells that control peripheral CD4 T cell homeostasis, while IL-2 is required for establishing a sizeable population of these cells in the peripheral pools. ***The Journal of Immunology,*** **2002, 169: 4850–4860.**

Several different lines of evidence demonstrate that thymus T cell production does not determine the number of peripheral T cells. First, in the young adult mouse, T cell production in the thymus largely exceeds the number of cells required to replenish the peripheral T cell pools. Mice manipulated to have reduced rates of thymus T cell production can attain normal peripheral T cell numbers (1). Secondly, in mice grafted with multiple thymuses, the increased thymus mass and T lymphocyte production does not lead to the proportional increase of the peripheral T cell pool (2, 3). The number of T cells is also not limited by the peripheral T cell production capacity. Peripheral T cells in absence of the thymus in thymectomized hosts (4) or when transferred into T cell-deficient hosts are capable of considerable expansion (5–7). In a normal mouse, there are mechanisms that control both T cell survival and division in the peripheral pools and keep T cell numbers constant. It has been proposed that competition for resources or complex cell interactions play a role in lymphocyte homeostasis (8, 9). However, the mechanisms involved remain elusive.

Mutant IL-2R$\alpha^{-/-}$ mice represent a paradigm for perturbed lymphocyte homeostasis (10). They develop massive enlargement of peripheral lymphoid organs associated with T and B cell expansion and autoimmune disease (10), indicating that IL-2Rα is essential for the control of the size of the peripheral lymphoid compartment. It was generally believed that the defect in IL-2R$\alpha^{-/-}$ mice was cell autonomous and that IL-2Rα regulated the balance between clonal expansion and cell death following lymphocyte activation (10, 11). Thus, in the absence of the negative signals mediated by IL-2Rα, T cells would undergo uncontrolled expansion (10). However, it was recently shown that when placed in a normal environment, TCR transgenic (Tg)[3] IL-2R$\alpha^{-/-}$ cells exhibited normal clonal contraction after Ag-induced expansion (12), suggesting that activation-induced cell death (AICD) is kept and that IL-2Rα signals could also control bystander T cell activation (12). Alternatively, IL-2Rα could be required for the development and/or the function of a subpopulation of T cells capable of regulating peripheral T cell homeostasis. Different lines of evidence seem to support this latter alternative. First, recent findings showed that wild-type T cells could control the expansion of IL-2Rβ-deficient T cells in mixed bone marrow (BM) chimeras, a property attributed to a population of cytotoxic CD8 T cells (13). Secondly, previous studies have also shown that Ag-induced expansion of TCR Tg IL-2-deficient T cells could be controlled by CD25$^+$ T cells (14). Finally, results indicate that regulatory CD45RB^{low}CD25$^+$CD4$^+$ T cells limit naive CD4 T cell expansion and suggest that they may play a role in the control of peripheral T cell numbers (15).

We decided to investigate if populations of regulatory CD25$^+$CD4$^+$ T cells (16) could prevent the chaotic lymphocyte accumulation in IL-2R$\alpha^{-/-}$ mice and control the expansion of peripheral naive CD4 T cells. We found that CD25$^+$CD4$^+$ T cells could indeed control peripheral T cell accumulation and composition in mouse chimeras reconstituted with BM cells from IL-2R$\alpha^{-/-}$ mice and rescued these mice from death. Similarly, recombination-activating gene (Rag)2$^{-/-}$ chimeras reconstituted with a mixture of BM cells from IL-2Rα and IL-2-deficient donors

*Lymphocyte Population Biology, Unité de Recherche Associée Centre National de la Recherche Scientifique 1961, Institut Pasteur, and †Institut National de la Santé et de la Recherche Médicale Unité 345, Centre Hospitalo-Universitaire Necker, Paris, France

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[2] Address correspondence and reprint requests to Dr. Antonio A. Freitas, Lymphocyte Population Biology Unit, Unité de Recherche Associée Centre National de la Recherche Scientifique 1961, Institut Pasteur, 25 Rue du Dr. Roux, 75015 Paris, France. E-mail address: afreitas@pasteur.fr

[3] Abbreviations used in this paper: Tg, transgenic; AICD, activation-induced cell death; BM, bone marrow; Rag, recombination-activating gene; LN, lymph node; Treg, T regulatory; IBD, inflammatory bowel disease.

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remained healthy, and the number and distribution of $CD25^+$ and $CD25^-CD4$ T cells was as in normal mice. To relate these effects to homeostatic control of the CD4 peripheral T cell pool, we examined the selectivity and quantitative requirements ruling the expansion of naive $CD45RB^{high}CD25^-CD4^+$ and $CD45RB^{low}CD25^+CD4^+$ T cells transferred into $CD3\epsilon^{-/-}$ T cell-deficient host mice (17). We found that the two cell populations show different homeostatic plateaus and that $CD25^+CD4^+$ T cells can selectively inhibit the peripheral expansion of the naive CD4 T cells, but not CD8 T cells in a dose-dependent manner.

Materials and Methods

Mice

C57BL/6.Ly5.2 mice from Iffa-Credo (L'Arbresle, France), B6.$CD3\epsilon^{-/-}$ (17), B6.IL-$2R\alpha^{-/-}$ (10), B6.$TCR\alpha^{-/-}$ (18), and C57BL/6.Ly5.1 mice from the Centre de Development des Techniques Avancées-Centre National de la Recherche Scientifique (Orléans, France), B6.IL-$10^{-/-}$ (19) from The Jackson Laboratory (Bar Harbor, ME) and B6.IL-$2^{-/-}$ (20) from our breeding facilities or from Dr. A. Schimpl (Institute for Virology and Immunobiology, University of Wurzburg, Germany) were matched for age (6–12 wk) and sex.

Cell sorting and cell transfers

Lymph node (LN) cells from the Ly5.2 and Ly5.1 donor mice were first enriched for $CD4^+$ T cells by negative selection using a Dynal MPC6 MACS (Dynal Biotech, Great Neck, NY). Briefly, cells were incubated with a mixture of rat Abs directed to mouse B220 (RA3-6B2), Mac1 (CD11b), and CD8α (53–6.7), all from BD PharMingen (San Diego, CA), followed by sheep anti-rat Ig-coated Dynabeads (Dynal Biotech). After removing the positive fraction, >90% of the remaining population was $CD4^+$. These cells labeled with the appropriate combinations of anti-CD4 (L3T4/RM4-5), anti-CD45RB, and anti-CD25 (784), Abs were sorted on a $FACStar^{Plus}$ (BD Biosciences, Mountain View, CA). The purity of the sorted $CD45RB^{high}CD25^-CD4^+$ and $CD45RB^{low}CD25^+CD4^+$ populations varied from 96–99.9%.

Intact nonirradiated B6.$CD3\epsilon^{-/-}$ (17) hosts were injected i.v. with the purified CD4 T cell populations alone or mixed at different cell ratios. By using mice differing by Ly5 allotypes, we were able to discriminate the cells originating from the different donor mice. Host mice were sacrificed at different time intervals after cell transfer. Spleen, inguinal, and mesenteric LN cell suspensions were prepared and the number and phenotype of the cells from each donor population evaluated. The total peripheral T cells showed in the results represent the number of cells recovered in the host's spleen added to twice the number of cells recovered from the host's inguinal and mesenteric LNs.

Labeling with CFSE

Cells were labeled with CFSE as described (21). Briefly, sorted $CD4^+$ T cells (10^7/ml) were incubated for 10 min at 37°C with CFSE (10 μM).

BM chimeras

Host 8-wk-old $Rag2^{-/-}$ B6 mice were lethally irradiated (900 rad) with a ^{137}Ce source and received i.v. $2–4 \times 10^6$ T cell-depleted BM cells from different donor mice, mixed at different ratios. T cell depletion (<0.1%) was done in a Dynal MPC6 MACS after incubating the BM cells with anti-CD4, anti-CD8, and anti-CD3 biotinylated Abs followed by streptavidin-coated Dynabeads. By using donor and host mice that differ according to Ly5 allotype markers, we were able to discriminate between the T cells originating from the different donors.

Flow cytometry analysis

The following mAbs were used: anti-CD3ε (145-2C11), anti-CD4 (L3T4/RM4-5), anti-CD69 (H1.2F3), anti-CD25 (784), anti-CD45RB, anti-CD24/HSA (M1/69), and anti-TCRβ (H57) from BD PharMingen, and anti-CD44 (IM781) and anti-CD62L (MEL14) from Caltag Laboratories (San Francisco, CA). Cell surface four-color staining was preformed with the appropriate combinations of FITC, PE, tricolor, PerCP, biotin, and APC-coupled Abs. Biotin-coupled Abs were secondary labeled with APC-, tricolor-, (Caltag Laboratories) or PerCP-coupled (BD Biosciences) streptavidin. Dead cells were excluded during analysis according to their light-scattering characteristics. All acquisitions and data analysis were performed with a FACSCalibur (BD Biosciences) interfaced to the Macintosh CellQuest software.

Statistical analysis

Sample means were compared using the unpaired Student's t test. In case the variances of the two samples were considerably different, the data were log-transformed to see if the variances become more similar. If so, the unpaired t test was applied to the log-transformed data. Otherwise, Sattertwhaite's approximation was applied. Sample means were considered significantly different at $p < 0.05$.

Results

$CD25^+CD4^+$ T cells inhibit CD4 T cells from IL-$2R\alpha^{-/-}$ mice and prevent death of chimeras reconstituted with BM cells from IL-$2R\alpha^{-/-}$ mice

Mutant IL-$2R\alpha^{-/-}$ mice develop massive lymphocyte accumulation and autoimmune disease (10). It was proposed that in the absence of negative signals mediated through IL-2Rα, T cells would undergo uncontrolled expansion suggesting that the IL-$2R\alpha^{-/-}$ defect was cell autonomous (10). We asked if a population of normal T cells could control the chaotic accumulation of T cells in IL-$2R\alpha^{-/-}$ mice. We examined whether mature $CD25^+CD4^+$ T cells could alter the number and state of activation of CD4 T cells in B6.$Rag2^{-/-}$ chimeras reconstituted with 100% BM cells from B6.IL-$2R\alpha^{-/-}$ donors. Using these BM chimeras rather than intact B6.IL-$2R\alpha^{-/-}$ mice allowed us to increase the number of mice studied. We found that all chimeras injected exclusively with BM cells from B6.IL-$2R\alpha^{-/-}$ donors died within 40–50 days (Fig. 1*A*), with overt signs of runting, anemia, and in some mice, lymphoid hyperplasia (up to 300×10^6 lymphocytes). In contrast, the BM chimeras that received 10^5 $CD25^+CD4^+$ T cells from normal B6.Ly5.1 donors 2 wk after BM reconstitution were all alive 6 mo later (Fig. 1*A* and data not shown). The effects of the $CD25^+CD4^+$ T cells were time-dependent since only 50% of the chimeras survived if we delayed their transfer to 4 wk after BM injection (Fig. 1*A*). These differences suggest that either control of T cell numbers is more efficient earlier when the number of peripheral IL-$2R\alpha^{-/-}$ T cells is lower, or it may require the continuous presence of "regulatory" T cells at the onset of T cell production. The surviving chimeras remained healthy, the hematocrit levels were normal (40–45%), and the total number of T cells was similar (47.1 ± 7.7 and $81 \pm 9.7 \times 10^6$ for the two groups of BM chimeras injected with $CD25^+CD4^+$ T cells, respectively) to control mice ($40–60 \times 10^6$ CD4 T cells). The composition of the peripheral LN T cells was as in normal mice comprising 5–10% of $CD45RB^{low}CD25^+CD4^+$ T cells, all of B6.Ly5.1 origin, and 50–60% of nonactivated $CD45RB^{high}CD25^-CD4^+$ T cells all from IL-$2R\alpha^{-/-}$ origin (Fig. 1*B*). This is in contrast to donor IL-$2R\alpha^{-/-}$ mice, where most (>80%) T cells have an activated phenotype (Ref. 10; data not shown). We should point out that the transfer of up to 2×10^5 $CD25^-CD4^+$ T cells did not rescue the $CD25^{-/-}$ BM chimeras (data not shown).

To test if the production of regulatory T cells by the thymus could also control the chaotic peripheral accumulation of IL-$2R\alpha^{-/-}$ T cells, lethally irradiated lymphopenic B6.$Rag2^{-/-}$ mice were reconstituted with a mixture of T cell-depleted BM cells. In brief, 50% of the injected BM cells were from B6.Ly5.2IL-$2R\alpha^{-/-}$ donors and the remaining 50% from B6.Ly5.2.$TCR\alpha^{-/-}$ (unable to generate T cells) and normal B6.Ly5.1 donors mixed at different ratios. By keeping the fraction of cells from IL-$2R\alpha^{-/-}$ donors in the injected BM cohort at 50%, we fixed the rate of production of IL-$2R\alpha^{-/-}$ T cells in all chimeras studied. Thus, the resulting chimeras should all have the same number of peripheral IL-$2R\alpha^{-/-}$ T cells (1). By mixing BM cells from B6 normal and B6.$TCR\alpha^{-/-}$ donors, we reduced the number of competent precursors available for thymus colonization and regeneration, as the normal competent precursor cells are diluted among incompetent

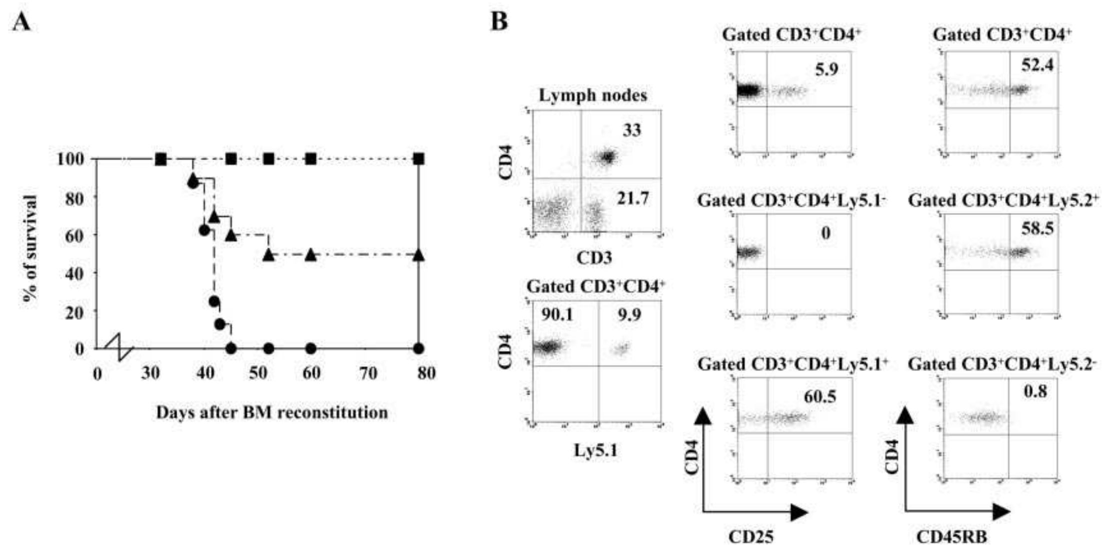

FIGURE 1. *A*, Lethally irradiated B6.Rag2$^{-/-}$ mice were reconstituted with 4×10^6 BM cells from B6.Ly5.2IL-2R$\alpha^{-/-}$ and were left alone (10 mice) or received 10^5 CD25$^+$CD4$^+$ T cells from normal B6.Ly5.1 donors 2 (9 mice) or 4 wk (8 mice) after BM reconstitution. Results show the time of survival of the chimeras reconstituted with BM cells from B6.Ly5.2IL-2R$\alpha^{-/-}$ alone (●), injected with CD25$^+$CD4$^+$ T cells from normal B6.Ly5.1 donors 2 (■) or 4 wk (▲) later. *B*, Phenotypic characterization of the peripheral LN CD4 T cells in a chimera reconstituted with BM cells from B6.Ly5.2IL-2R$\alpha^{-/-}$ that received 10^5 CD25$^+$CD4$^+$ T cells from normal B6.Ly5.1 donors 2 wk later. Chimeras were sacrificed for FACS analysis 16–20 wk after BM transfer. Similar results were obtained in the remaining eight mice from the same group.

precursors from the TCR$\alpha^{-/-}$ mutant donors (1). With this strategy, we could evaluate the role of different numbers of normal T cells in the control of a fixed number of IL-2R$\alpha^{-/-}$ T cells. We found that all the chimeras that could only generate IL-2R$\alpha^{-/-}$ T cells, i.e., which received 50% BM cells from B6.IL-2R$\alpha^{-/-}$ donors and 50% of BM cells from B6.TCR$\alpha^{-/-}$ donors, died 40–50 days after reconstitution (Fig. 2*A*). The presence of 5% cells from normal donors in the thymus of the chimeras was sufficient to rescue 80% of the mice (Fig. 2*A*). The presence of 10 or 50% of normal cells rescued 100% of the chimeras. In the 50/50 B6.Ly5.2IL-2R$\alpha^{-/-}$/B6.Ly5.1 chimeras, the representation of the two types of donor cells remained unchanged both in the thymus and in the peripheral T cell pools (data not shown). In the surviving BM chimeras containing a fraction of cells from normal donors, the total number of peripheral T cells and the relative distribution of the CD25$^+$ and CD25$^-$ CD4 T cell subsets were as in normal mice (Fig. 2*B*). Thus, a developing population of normal CD4 T cells controlled the accumulation and state of activation of the IL-2R$\alpha^{-/-}$ T cells. These results show that the lethal accumulation of peripheral T cells in IL-2R$\alpha^{-/-}$ mice is not cell autonomous, but due to the lack of a population of CD4$^+$ T cells essential for peripheral T cell homeostasis. We demonstrated that expression of the IL-2Rα chain is required for the generation of this population of regulatory CD4 T cells.

Control of T cell numbers in IL-2$^{-/-}$/IL-2R$\alpha^{-/-}$ BM chimeras

With age, IL-2$^{-/-}$ mice develop fatal inflammatory bowel disease (IBD) and lymphocyte proliferation (20). The peripheral T cell compartments of these mice show overt signs of T cell activation and lack a well-defined population of CD25$^+$, which never exceeds 1–2% of the CD4$^+$ T cells (14, 22). We investigated whether the CD25$^+$CD4$^+$ T cells from IL-2$^{-/-}$ mice could rescue the defects of CD4 T cell homeostasis observed in IL-2R$\alpha^{-/-}$ mice. We reconstituted lethally irradiated lymphopenic B6.Rag2$^{-/-}$ mice with a 50/50 mixture of T cell-depleted BM cells from B6.IL-2R$\alpha^{-/-}$ and B6.IL-2$^{-/-}$ donors. Control mice received BM cells from either B6.IL-2R$\alpha^{-/-}$ or B6.IL-2$^{-/-}$ mice equally mixed at 50/50 with BM cells from B6.TCR$\alpha^{-/-}$ donors. The presence of 50% of BM cells from IL-2$^{-/-}$ donors rescued the totality of the IL-2R$\alpha^{-/-}$ BM chimeras (Fig. 3*A*) and restored the CD4 T cell populations to normal. In the peripheral T cell pools of these chimeras, the number and the distribution of CD25$^+$ and CD25$^-$ CD4 T cells was as in normal mice (Fig. 3*B*). Thus, in presence of IL-2, the hemopoietic precursors from IL-2-deficient donor mice generated a stable population of mature peripheral CD25$^+$CD4$^+$ regulatory T cells able to control the homeostasis of the CD4 T cell compartment. Upon secondary transfer, this population of IL-2$^{-/-}$CD25$^+$CD4$^+$ cells was able to rescue B6.IL-2R$\alpha^{-/-}$ chimeras (data not shown). Chimeras injected with BM cells from B6.IL-2$^{-/-}$ donors alone show a normal number of CD25$^+$ and CD25$^-$ CD4 T cells (data not shown). This finding indicates that in Rag2$^{-/-}$ hosts, resident non-T cells can provide a source of endogenous IL-2 (23) sufficient to compensate for the lack of its production by the IL-2$^{-/-}$ hemopoietic cells. Overall, these results demonstrate that IL-2 is required for the establishment of a stable population of CD25$^+$CD4$^+$ regulatory T cells in the peripheral pools. In absence of this, population control of CD4 T cell numbers is lost and the mice develop lymphoid hyperplasia and autoimmune diseases. Overall, these findings indicate that populations of CD25$^+$ and CD25$^-$CD4 T cells may have different homeostatic properties and that they may regulate each other. We decided to investigate this possibility using a cell transfer strategy.

Fate of naive CD4$^+$ and CD25$^+$CD4$^+$ T cells transferred into T cell deficient hosts: different homeostatic plateaus

Peripheral T cells, when transferred into T cell-deficient hosts, are capable of considerable expansion (5–7), but their number is controlled at a homeostatic plateau. To investigate the homeostasis of peripheral CD4$^+$ T cell subpopulations, different numbers of purified CD4$^+$ cells, that is, CD45RB^{high}CD25$^-$, CD45RB^{low}CD25$^-$, and CD45RB^{low}CD25$^+$ cells were i.v. transferred into syngeneic CD3$\epsilon^{-/-}$ T cell-deficient hosts. In hosts receiving as few as 5×10^3 and as many as 10^5 cells, CD45RB^{high}CD25$^-$CD4$^+$ T cells (from now on referred to as naive CD4) expanded to reach stable equilibrium at

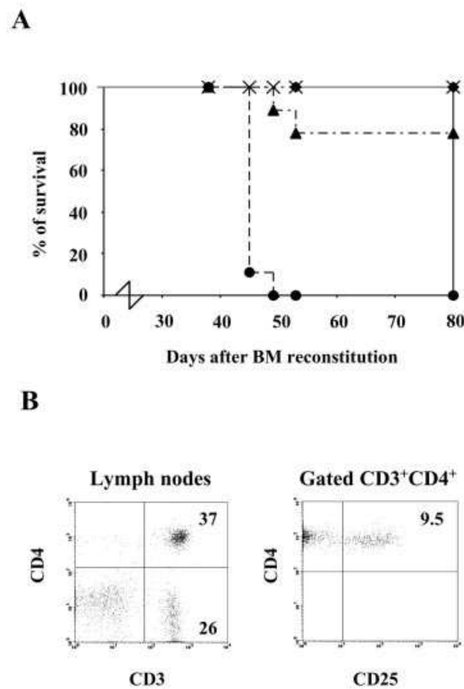

FIGURE 2. *A*, Lethally irradiated B6.Rag2$^{-/-}$ mice were reconstituted with 4 × 10^6 cells from a mixture of 50% BM cells from B6.Ly5.2IL-2R$\alpha^{-/-}$ and 50% of BM cells from B6.Ly5.2.TCR$\alpha^{-/-}$ and normal B6.Ly5.1 donors, the latter mixed at different ratios. Results show the time of survival of the chimeras reconstituted with 50% BM cells from B6.Ly5.2IL-2R$\alpha^{-/-}$ and 50% of BM cells from B6.Ly5.2. TCR$\alpha^{-/-}$ (●), 50% BM from B6.Ly5.2IL-2R$\alpha^{-/-}$, 45% BM from B6.Ly5.2.TCR$\alpha^{-/-}$, and 5% BM from normal B6.Ly5.1 (▲), 50% BM from B6.Ly5.2IL-2R$\alpha^{-/-}$, 40% BM from B6.Ly5.2.TCR$\alpha^{-/-}$, and 10% BM from normal B6.Ly5.1 (×), 50% BM from B6.Ly5.2IL-2R$\alpha^{-/-}$, and 50% BM from normal B6.Ly5.1 (■). Number of mice per group: nine. *B*, Dot plot shows the frequency of CD25$^+$CD4$^+$ T cells in the LNs of chimeras reconstituted with 50% BM from B6.Ly5.2IL-2R$\alpha^{-/-}$ and 50% BM from normal B6.Ly5.1. Similar results were obtained in the other chimeras.

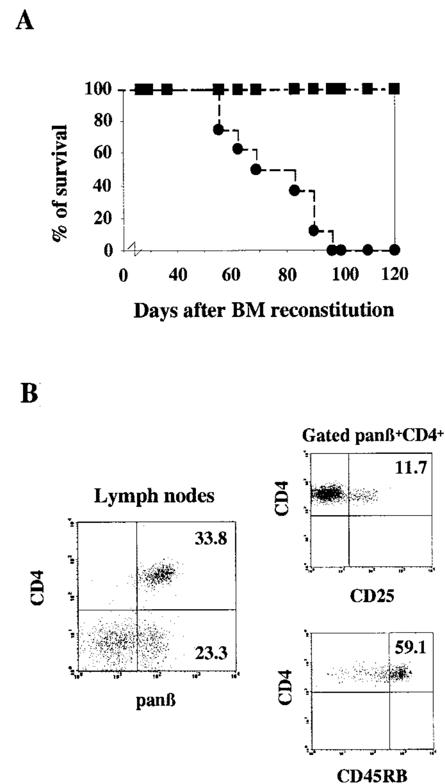

FIGURE 3. Lethally irradiated B6.Rag2$^{-/-}$ mice were reconstituted with ~1 × 10^6 cells from a mixture of 50% BM cells from B6.IL-2R$\alpha^{-/-}$ and 50% of BM cells from B6.IL-2$^{-/-}$. Control chimeras received a mixture of 50% BM cells from B6.IL-2R$\alpha^{-/-}$ or B6.IL-2$^{-/-}$ and 50% of BM cells from B6.Ly5.2.TCR$\alpha^{-/-}$. *A*, Results show the time of survival of the chimeras reconstituted with 50% BM cells from B6.IL-2R$\alpha^{-/-}$ and 50% of BM cells from B6.IL-2$^{-/-}$ (■), 50% BM cells from B6.IL-2R$\alpha^{-/-}$ and 50% of BM cells from B6.TCR$\alpha^{-/-}$ (●), 50% BM cells from B6.IL-2$^{-/-}$ and 50% of BM cells from B6.TCR$\alpha^{-/-}$ (data not shown). Note that the transfer of lower numbers of precursor cells delayed lymphoid reconstitution and death (compare to Fig. 2*A*) of the host mice injected with BM cells from B6.IL-2R$\alpha^{-/-}$ and from B6.TCR$\alpha^{-/-}$ donors. Number of mice/group: nine. *B*, Phenotypic characterization of the peripheral LN CD4 T cells in a chimera reconstituted with BM cells from B6.IL-2R$\alpha^{-/-}$ and B6.IL-2$^{-/-}$ donors. Similar results were obtained in the remaining mice from the same group.

~1–2 × 10^7 cells, 10–12 wk after transfer (Fig. 4). In mice injected with the same number of CD45RB^{low}CD25$^+$CD4$^+$ T cells (from now on referred to as CD25$^+$CD4$^+$), these cells also expanded but reached equilibrium at 10-fold lower values, i.e., at 1–2 × 10^6 cells/hosts (Fig. 4). Transfer of increasing numbers (>10^5) of cells did not modify the final cell recovery (data not shown). These results indicate that naive CD4$^+$ and CD25$^+$CD4$^+$ T cells are both able to expand and accumulate at the periphery, but their final number is regulated at different homeostatic plateau levels. It should be noted that at later times after transfer, mice injected with naive CD4$^+$ T cells developed a wasting autoimmune disease and eventually died (>15 wk), while hosts of CD25$^+$CD4$^+$ T cells remained healthy (data not shown). Total nonseparated LN CD4$^+$ T cells containing 10% CD25$^+$ cells expanded to a plateau of ~1–2 × 10^7 cells, but fail to develop signs of wasting disease (data not shown). Activated CD45RB^{low}CD25$^-$CD4$^+$ T cells expanded to similar plateaus as naive CD4$^+$ T cells (data not shown).

Sequential and secondary cell transfers

Cellular competition and the presence or absence of resident T cell populations can alter the peripheral fate of newly arriving thymus emigrants (8, 9, 24). To truly establish the homeostasis of the transferred peripheral T cells, we asked if the presence of a resident T cell population could interfere with the expansion of a second newly injected cell population or whether the injection of a new population could modify the fate of a resident population. We "parked" 5 × 10^4 Ly5.1 naive CD4$^+$ T cells in different hosts. Seven weeks later, each host received the same number of a second population of Ly5.2 naive CD4$^+$ or CD25$^+$CD4$^+$ T cells. Age-matched control mice received either the first or the second population alone. We sacrificed the mice at 7 (before the second injection) or 14 wk after the first injection. After transfer, naive T cells acquired a CD45RB^{low} activated/memory phenotype, but only a few (1–2%) became CD25^{low} (data not shown). In mice injected sequentially with two populations of naive CD4$^+$ T cells, the expansion of both populations was limited through competition and they shared the peripheral compartment of the host (Fig. 5*A*). The total T cell recovery was the same as in mice injected with either population alone (~2 × 10^7). The transfer of 5 × 10^4 CD45RB^{low}CD25$^+$CD4$^+$ cells into mice injected 7 wk before with CD45RB^{high}CD25$^-$CD4$^+$ cells suppressed significantly ($p < 0.001$) further expansion of the established 5 × 10^6 CD45RB^{high}CD25$^-$CD4$^+$-derived T cell population (Fig. 5*B*). The total T cell recovery diminishes accordingly. The number of

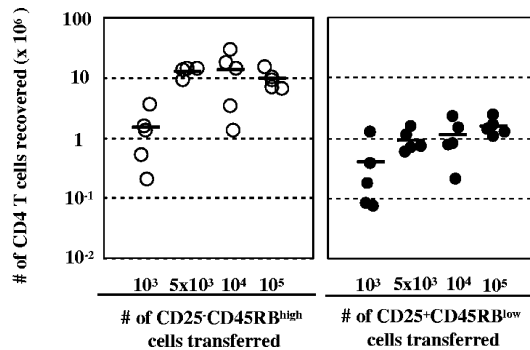

FIGURE 4. Expansion capacity of CD4 T cell populations of C57BL/6 mice. Different numbers of purified naive $CD4^+$ and $CD25^+CD4^+$ T cells (1,000–100,000) were transferred into $CD3\epsilon^{-/-}$ mice. The results show the number of CD4 T cells recovered 10–11 wk after transfer in the spleen and LN of each individual host (the mean value is also shown).

cells recovered from the second population of $CD45RB^{low}$ $CD25^+CD4^+$ T cells did not change. These results show that a limited number (5×10^4) of newly transferred $CD45RB^{low}CD25^+$ $CD4^+$ T can suppress the expansion of an abundant (5×10^6) population of resident $CD4^+$ T cells.

We also parked 5×10^4 Ly5.1 $CD25^+CD4^+$ T cells. Seven weeks later, each host received 5×10^4 Ly5.2 naive $CD4^+$ or $CD25^+CD4^+$ T cells. The transfer of a second population of $CD45RB^{low}CD25^+CD4^+$ T cells did not significantly modify the number of the established cells. The resident cells were able to persist and the new cells were able to accumulate as in noninjected hosts (Fig. 5*C*). In the mice hosting the first population of $CD25^+CD4^+$ T cells, newly transferred naive $CD4^+$ T cells expanded and induced a 3- to 4-fold increase ($p < 0.01$) in the number of resident CD4 T cells from $CD25^+CD4^+$ origin (Fig. 5*D*). These results show that newly injected naive $CD4^+$ T cells helped the growth of the progeny of $CD25^+CD4^+$ T cells ($p < 0.01$). In contrast, the resident $CD25^+CD4^+$ T cell progeny do not significantly ($p = 0.5$) inhibit the growth of newly transferred naive $CD4^+$ T cells. In hosts that received $CD25^+CD4^+$ cells, only ~30% of the recovered T cells remained $CD25^+$ (data not shown). This could represent true phenotypic changes or the expansion of a few contaminant CD4 T cells in the injected $CD25^+$ population. We investigated the suppressive capacities of the resident cells that express or not CD25. For this purpose, $CD25^+CD4^+$ T cells were parked for 7 wk in host mice. At the end of this time period, $CD25^+CD4^+$ T cells and $CD45RB^{low}CD25^-CD4^+$ T cells derived from the parked population were injected alone or coinjected with naive $CD4^+$ T cells from different Ly5 donors into secondary $CD3\epsilon^{-/-}$ hosts. Although the resorted $CD25^+$ cells retained the capacity to suppress the growth of naive $CD4^+$ T cells ($p < 0.001$), the suppressive capacity of the resorted $CD25^-$ cells was absent or reduced ($p = 0.21$; Fig. 6). These results suggest that the suppressor effects correlate with the surface expression of CD25.

$CD25^+CD4^+$ T cells inhibit peripheral expansion of naive CD4 T cells

We showed that $CD25^+CD4^+$ T cells could control chaotic accumulation of IL-$2R\alpha^{-/-}$ T cells. We decided to investigate whether they could also control the homeostatic plateau of naive CD4 T cells transferred to T cell-deficient hosts. We quantified both the numbers of cells involved and the selectivity of the interactions. We transferred 10^4 purified naive Ly5.1 $CD4^+$ T cells or CD8 T cells alone or coinjected with variable numbers of Ly5.2 $CD25^+CD4^+$ T cells, ranging from 3×10^3–10^5, into $CD3\epsilon^{-/-}$ hosts (Fig. 7, *A* and *B*). We found that the $CD25^+CD4^+$ T cells limited the accumulation of naive $CD4^+$ T cells (15) and that their suppressive effects were dose-dependent (Fig. 7*A*). Increasing

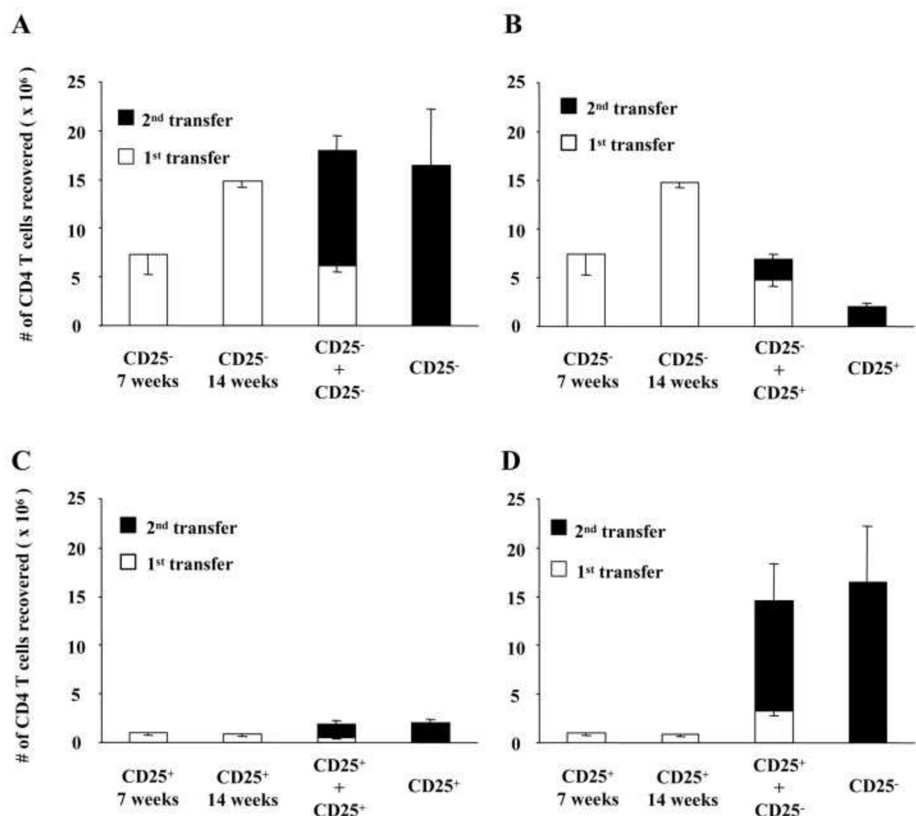

FIGURE 5. Sequential cell transfers. *A*, T cell-deficient mice were injected with 5×10^4 naive $CD4^+$, and 7 wk later they received 5×10^4 naive $CD4^+$ T cells, which differ in the Ly5 allotype. All mice were sacrificed 14 wk after the first injection. Control mice received either the first (*left column* in each quadrant) or the second (*right column* in each quadrant) population alone and were killed 7 wk after transfer. Note that the hosts were age matched, i.e., recipients were injected with the first population at 7 wk of age and received the second population at 14 wk of age, a difference which may explain the greater growth of the second cell population when transferred alone. *B*, As for *A*, except that the mice were injected first with 5×10^4 naive $CD4^+$ and 7 wk later they received 5×10^4 $CD25^+CD4^+$ T cells. Differences between $CD25^-$-derived cells at 14 wk in absence or in presence of V cells were highly significant ($p < 0.001$). *C*, As for *A*, except that the mice were injected first with 5×10^4 $CD25^+CD4^+$ and later $CD25^+CD4^+$ T cells. Results show the mean + SEM (four to five mice per group) of the number of cells recovered from the first (□) or the second (■) injected population. *D*, As for *A*, except that the mice were injected first with 5×10^4 $CD25^+CD4^+$ and later 5×10^4 naive $CD4^+$ T cells. The use of Ly5 different T cells allows the easy identification of T cells from each donor population.

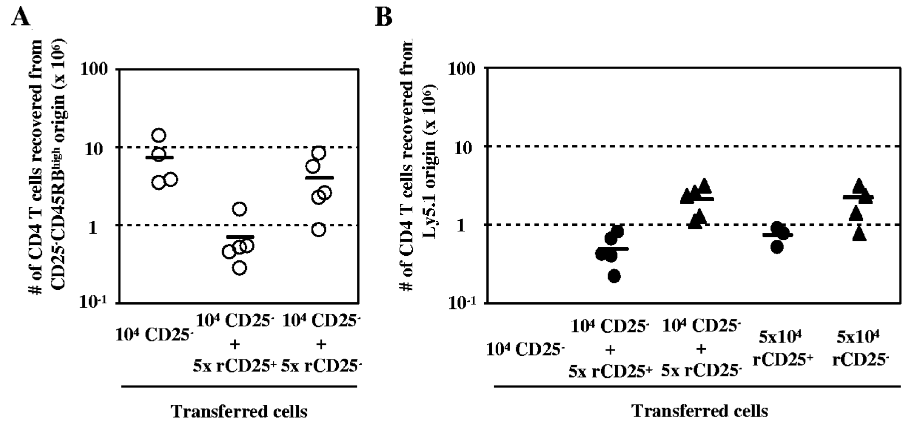

FIGURE 6. Secondary cell transfers: 10^4 naive CD4$^+$ Ly5.2 T cells were transferred alone or coinjected with 5×10^4 of either CD25$^+$ and CD25$^-$ Ly5.1 T cells recovered from mice injected 7 wk before with 3×10^4 CD25$^+$CD4$^+$ Ly5.1 T cells. Control mice were injected with CD25$^+$ and CD25$^-$ Ly5.1 T cells alone. *A*, Results show the total number of CD4 T cells from naive CD4$^+$Ly5.2 origin. The mean value is also shown. *B*, Shows the total number of Ly5.1 T cells recovered in mice injected with CD25$^+$ (●) and CD25$^-$ (▲) cells recovered from mice injected 7 wk before with 10^4 CD25$^+$CD4$^+$Ly5.1 T cells alone or coinjected with naive CD4$^+$Ly5.2 (*A*). The mean value is also shown.

numbers of CD25$^+$CD4$^+$ T cells progressively suppressed the expansion of the cotransferred naive CD4$^+$ T cells, and at a 10:1 cell ratio, we recovered 10-fold less T cells from naive CD4$^+$ origin ($p < 0.005$). Total T cell recovery diminished according to the level of suppression, that is, overgrowth of the coinjected CD25$^+$CD4$^+$ T cells did not compensate for the lack of expansion of the naive T cells (p-NS) (Fig. 7*B*). We also found that CD25$^+$CD4$^+$ T cells did not affect the growth of coinjected total LN CD8 T cells ($p = 0.4$; Fig. 7*C*), indicating that their inhibitory effects are lineage-specific. By varying either the number of T cells injected, or the ratio CD25$^+$/CD25$^-$ T cells, we found that the number of T cells from naive CD25$^-$CD4 origin recovered was not dependent on the number of cells transferred, but determined by the CD25$^+$/CD25$^-$ ratio present in the inoculum (Fig. 7*D*). These results raised the possibility that the CD25$^+$CD4$^+$ T cells might have blocked division of the naive CD4$^+$ T cells. To test this possibility, we compared the fate of CFSE-labeled naive T cells transferred alone (Fig. 7*E*, *top panel*) or in the presence of an excess of CD25$^+$ cells (Fig. 7*E*, *bottom panel*). Three days after transfer (Fig. 7*E*), the patterns of dilution of the CFSE labeling were similar in both groups of mice, and we recovered an identical number of cells in the two groups of host mice (data not shown). At day 10, the majority of the transferred cells were CFSE$^-$, indicating that these cells underwent several rounds of division. However, the fraction of CFSE$^-$ cells was higher, and we recovered 27-fold more CD4 T cells in the mice injected with naive T cells in absence of CD25$^+$ cells (Fig. 7*E*). The differences in total cell recovery could be due to either an increase in cell survival or to an increase in the rate of cell division of the CD4$^+$CD25$^-$ naive T cells when transferred alone. Thus, the present results do not allow discriminating between these two possibilities or if the increase of the number of cells corresponds to an increased fraction of cells that enter cell cycle or to a reduced cell cycle time. Studies on the annexin V labeling of the transferred populations were not conclusive (data not shown). On the whole, these results indicate that the suppressive effects are not obtained through complete block of proliferation, but do not allow us to distinguish whether they affect the rate of cell expansion or the survival (accumulation) of the newly generated T cells. In contrast, we found that activated CD45RB^{low}CD25$^-$CD4$^+$ T cells did not control expansion of naive CD4 T cells (data not shown).

CD25$^+$CD4$^+$ T cells inhibit peripheral expansion of IL2Rα$^{-/-}$ CD4 T cells

Finally, we studied whether the same forms of interaction also applied to populations of CD4 T cells from IL-2Rα$^{-/-}$ and IL-2$^{-/-}$ mice. We found that CD25$^+$CD4$^+$ T cells from normal donors inhibited the expansion of the CD4$^+$ T cells from IL-2Rα$^{-/-}$ mice transferred into CD3ε$^{-/-}$ hosts ($p < 0.05$; Fig. 8*A*). Similarly, naive CD4$^+$ T cells from IL-2$^{-/-}$ origin expanded and were suppressed ($p < 0.01$) while CD25$^+$CD4$^+$ T cells from IL-2$^{-/-}$ mice slightly suppressed the expansion of naive CD4$^+$ T cells from normal donors as well as CD25$^+$CD4$^+$ T cells from normal donors at a 1:1 cell ratio (Fig. 8*B*). Altogether, these findings suggest that the control exerted by the CD25$^+$CD4$^+$ T cells on the accumulation of peripheral CD4 T cells in the IL-2Rα$^{-/-}$ is due to their ability to regulate peripheral CD4 T cell homeostasis.

Discussion

Peripheral T cells, in absence of a thymus (4, 25) or when transferred to T cell-deficient hosts (5, 7, 26), are capable of considerable expansion. The sequential transfer of a T cell population into successive hosts has shown that one T cell can generate up to 10^{15} cells (7). This indicates that in a normal mouse, peripheral T cell division is limited by mechanisms that probably include resource competition and complex cell interactions (9). We studied the role of T cell interactions in the control of the number of peripheral CD4$^+$ T cells. In particular, we investigated if CD25$^+$CD4$^+$ T cells, which exert regulatory functions (27–32), could also govern peripheral CD4$^+$ T cell homeostasis.

IL-2Rα$^{-/-}$ mutant mice are reported as a paradigm for perturbed lymphocyte homeostasis (10). The lack of the IL-2Rα was believed to impair AICD in vivo (10), to modify the balance between clonal expansion and cell death, resulting in the deregulation of both the size and content of the peripheral lymphoid compartments. The primary uncontrolled T cell activation lead subsequently to secondary policlonal B cell activation and autoantibody production. However, recent findings have shown that when placed in a normal environment, TCR Tg CD25$^{-/-}$ T cells exhibited a significant reduction in Ag-induced expansion due to normal AICD (12). This observation was interpreted as indicating that the regulatory role of IL-2Rα signals was mediated through the control of bystander T cell activation (12). We now demonstrate that the chaotic lymphocyte accumulation developed in adult IL-2Rα$^{-/-}$ mice is not cell autonomous: it is due to the lack of a T cell population essential for the homeostasis of peripheral T cell numbers. Two main lines of evidence support this conclusion. First, the presence of a limited number of CD25$^+$CD4$^+$ T cells rescues mouse chimeras reconstituted with BM from IL-2Rα$^{-/-}$ mice from chaotic lymphocyte accumulation, polyclonal B and T cell activation, and death, and restores the peripheral lymphoid compartment to normal. Second, we show that CD25$^+$CD4$^+$ T cells inhibit peripheral expansion of CD4 T cells from IL-2Rα$^{-/-}$ mice transferred into T cell-deficient adoptive hosts. Moreover, by

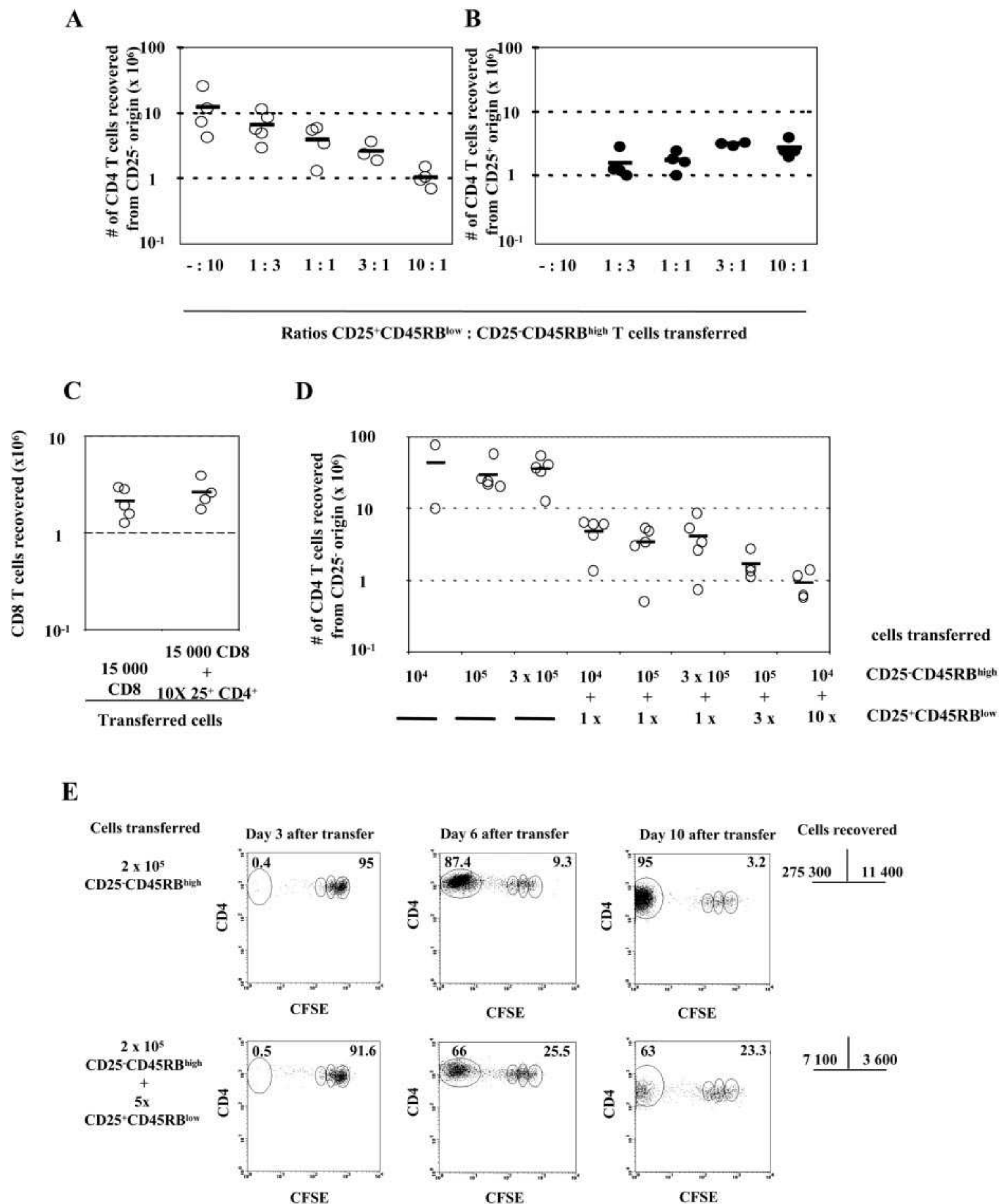

FIGURE 7. *A*, A population of 10,000 naive $CD4^+$ cells was transferred alone or mixed with increasing numbers of $CD25^+CD4^+$ cells from different Ly5 allotype congenic donors into $CD3\epsilon^{-/-}$ hosts. Results show the number of CD4 T cells from naive $CD4^+$ origin recovered 8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). *B*, The total number of CD4 T cells from $CD25^+CD4^+$ origin recovered in the same hosts. *C*, A population of 15,000 purified $CD8^+$ LN cells was transferred alone or mixed 150,000 $CD25^+CD4^+$ cells from different Ly5 allotype congenic donors into $CD3\epsilon^{-/-}$ hosts. Results show the number of CD8 T cells recovered 8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). *D*, Different numbers of purified naive $CD4^+$ were cotransferred with $CD25^+CD4^+$ T cells at different cell ratios. The results show the number of CD4 T cells from naive $CD4^+$ origin recovered 8–9 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). *E*, A total of 2×10^5 CFSE-labeled $CD45RB^{high}CD25^-CD4^+$ T cells from B6.Ly5.1 donors were transferred alone (*top panels*) or in the presence (*bottom panels*) of 1×10^6 $CD45RB^{low}CD25^+CD4^+Ly5.2$ T cells into irradiated (400 rad) $CD3\epsilon^{-/-}$ hosts. At days 3, 6, and 10, posttransfer mice were sacrificed and the expression of CFSE analyzed in gated $Ly5.1^+CD4^+$ T cells. The figures show the relative fraction (percentage) of cells that have divided >8 times and in the first three rounds of division as well as the total number of $CFSE^-$ and $CFSE^+$ cells at day 10.

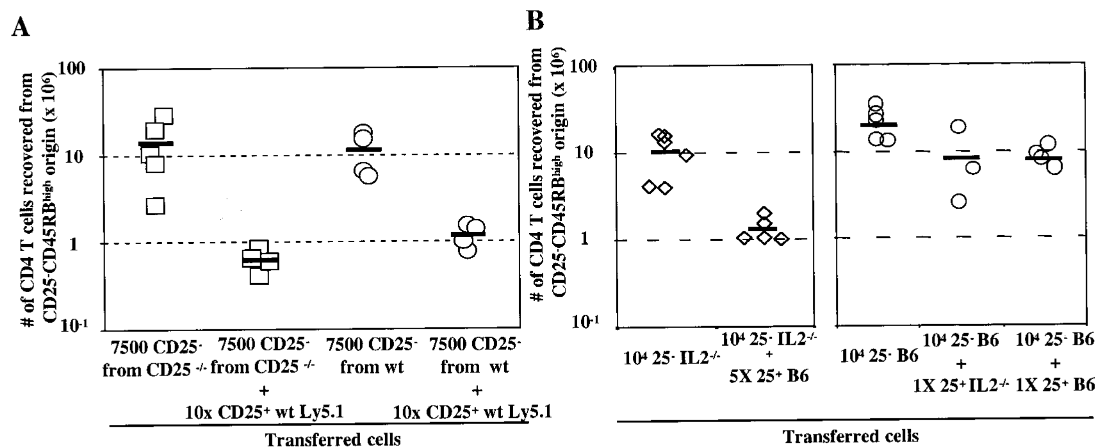

FIGURE 8. *A*, $CD4^+$ ($CD25^-$) T cells (7,500) from IL-2R$\alpha^{-/-}$ or normal B6 donors were transferred alone or coinjected with 75,000 $CD25^+CD4^+$ from normal mice. The results show the number of CD4 T cells recovered 7–8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). *B*, Naive $CD4^+$ ($CD25^-$) T cells (10,000) from IL-$2^{-/-}$ mice were transferred alone or coinjected with 10,000 $CD25^+CD4^+$ from B6.Ly5.1 mice, and naive $CD4^+$ ($CD25^-$) T cells (10,000) from normal B6.Ly5.1 donors were transferred alone or coinjected with 10,000 $CD25^+CD4^+$ from normal B6.Ly5.2 or IL-$2^{-/-}$ mice. The results show the number of CD4 T cells from $CD25^-$ origin recovered 7–8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). Note that cells from IL-$2^{-/-}$ mice inhibit expansion of naive T cells from normal donors and that the $CD25^+CD4^+$ T cells from normal B6 donors inhibited the expansion of the cells from IL-$2^{-/-}$ mice.

using IL-2-deficient mice, which also develop lymphoid hyperplasia and autoimmunity late in life (20), we show that IL-2 is required for the establishment of a stable and sizeable population of peripheral $CD25^+CD4^+$ regulatory cells. Thus, IL2-Rα/IL-2 signals are not involved in control of bystander activation, but are instead required for the generation and peripheral expansion/survival of a population of regulatory CD4 T cells essential for peripheral CD4 T cell homeostasis.

We further dissected and quantified the type of cell interactions involved in peripheral homeostasis by following the fate of separated populations of CD4 T cells transferred into immune-deficient hosts (7). We found that after transfer into CD3$\epsilon^{-/-}$ T cell-deficient hosts, purified naive $CD4^+$ T cells expanded to reach a stable plateau at $\sim$1–2 $\times$ 10^7 cells, independently of the number of injected cells. When a second population of naive $CD4^+$ cells was transferred into the same hosts, the growth of each population was limited and the total T cell recovery was the same as in mice injected with only one population. We did not observe an overt advantage of either the tenant or the newcomer cells. These findings confirmed that cellular rivalry could alter the fate of T cells at the periphery (8, 9, 24), and attested that the expansion of the transferred naive $CD4^+$ T cells is under homeostatic control (7). We found that the accumulation of the $CD25^+CD4^+$ T cells in T cell-deficient hosts is limited by a homeostatic plateau which singularly operates at values 10-fold lower than for total $CD4^+$ or naive $CD4^+$ T cells, i.e., at 1–2 $\times$ 10^6 cells/host. We confirmed by the cotransfer of these two T cell populations that the presence of $CD25^+CD4^+$ T cells limited the expansion of the naive $CD4^+$ T cells (15). Total T cell recovery diminished accordingly to the levels of suppression thus, excluding the presence of competition between the two populations.

We expanded these observations and showed that the inhibitory effects were dose-dependent and lineage-specific, as they did not affect naive $CD8^+$ T cell expansion in vivo. However, lineage specificity seems dependent on the experimental conditions, as $CD25^+CD4^+$ T cells were shown to control memory but not naive $CD8^+$ T cells (33), and to suppress both $CD4^+$ and $CD8^+$ T cell activation in vitro (34, 35). Suppression of naive $CD4^+$ T cell growth was obvious when the number of $CD25^+CD4^+$ T cells exceeded the number of the naive $CD4^+$ T cells by a factor of 10, less noticeable and variable when the two populations were present at similar numbers. Thus, the physiological relevance of the $CD25^+CD4^+$ T cells could be arguable since in normal mice their number rarely exceeds 10% of the total $CD4^+$ T cell pool. However, upon sequential cell transfers, we found that the injection of a limited number (5 $\times$ 10^4) of $CD25^+CD4^+$ T cells arrests the growth of an expanding population of 5 $\times$ 10^6 resident naive $CD4^+$-derived T cells. More importantly, a limited number of $CD25^+CD4^+$ T cells rescues mice reconstituted with BM cells from IL-2R$\alpha^{-/-}$ donors, controls the chaotic T cell accumulation, and reestablishes a peripheral T cell pool with a normal subpopulation composition. Thus, the suppressive effects mediated by the $CD25^+CD4^+$ T cells have physiological relevance since they were also obtained at physiological ratios of $CD25^+$ to $CD25^-$. The changes in effectiveness observed between the different experimental protocols and schedules of injection may be due to the different fraction/number of activated cells to be suppressed. Immediately following transfer, most naive CD4 T cells are activated, while later, near steady state equilibrium, only a fraction proliferates (7). Alternatively, they could be due to the different capacity of the regulatory cells to suppress homeostatic proliferation, occurring in the cell transfer experiments, and natural T cell proliferation and reconstitution occurring in the BM chimeras. Finally, the homing of the $CD25^+CD4^+$ T cells could also differ between the different experimental protocols used. In experiments where the $CD25^+CD4^+$ T cells were transferred at the same time as the naive CD4 T cells, differential homing abilities of the two populations could explain the apparently too high $CD25^+$:$CD25^-$ ratio needed in the cotransfer experiments. The homing of the $CD25^+CD4^+$ T cells could be favored by the presence of activated T cells in the periphery of the host mice as it could happen in the sequential transfer experiments.

How do the $CD25^+CD4^+$ T cells regulate homeostasis of naive $CD4^+$ T cells? To investigate the possible effects of $CD25^+CD4^+$ T cells in blocking the proliferation of $CD25^-$ cells, we have transferred CFSE-labeled $CD25^-CD4^+$ T cells alone or with an excess of $CD25^+$CD4 regulatory T cells (Fig. 7*E*). The dilution of CFSE labeling observed in both situations is similar, but the accumulation of cells observed in the CFSE-negative fraction of cells accounts for the differences in the number of cells recovered. Thus, homeostatic proliferation could be said to occur in both situations, but its extent could

be limited only when $CD25^+CD4^+$ regulatory T cells are present. The possibility that $CD25^+$CD4 regulatory T cells are capable of inhibiting the extent of homeostatic proliferation suggests the interesting possibility that the observed regulation of self-reactive responses is just a side effect of a broader function of these cells in the control of peripheral T cell numbers. If these cells control the magnitude of expansion of all naive $CD25^-CD4^+$ T cells, this may also include expansion of self-reactive clones present within that population. However, the opposite can also be true, and the control of self-reactive responses may result in the control of total cell numbers recovered. In this study, we show that the presence of T regulatory (Treg) cells prevents the activation of CD4 T cells from $CD25^{-/-}$ origin, including self-reactive clones, and allows the establishment of a normal size naive peripheral T cell compartment; sequential cell transfer, the $CD25^+CD4^+$ regulatory T cells suppress the expansion of activated T cells engaged in homeostatic proliferation, reducing the number of cells recovered.

It has been shown that IL-10 mediates the regulatory functions of the $CD25^+CD4^+$ T cells (28, 36–39), but in vitro studies have excluded the role of IL-10 in $CD25^+$ T cell-mediated suppression (40). We examined the role of IL-10 in the suppression of T cell proliferation in vivo; in contrast to a previous report (15), we found that $CD25^+CD4^+$ T cells from IL-$10^{-/-}$ mice inhibit the expansion of naive T cells as effectively as $CD25^+$ cells form wild-type mice (data not shown). This indicates that the effects of the $CD25^+CD4^+$ T cells on T cell expansion are IL-10-independent. However, we confirmed that $CD25^+$ cells from IL-$10^{-/-}$ mice failed to protect against the wasting disease induced by the naive $CD4^+$ T cells (data not shown). TGFβ has also been implicated in IBD protection, and recent claims suggest that it may play a role in T cell homeostasis (41–43). However, we found that both naive and activated CD4 T cells expressed similar levels of mRNAs for the three subforms of TGFβ (data not shown). Moreover, $CD25^+$ T cells from TGFβ-deficient mice are referred to be suppressors (44). We also found that $CD25^+CD4^+$ cells from TNF-$\alpha^{-/-}$ and LT$\alpha^{-/-}$ mice inhibit expansion of naive T cells (data not shown), excluding their role in this process. By using *lpr* and *gld* mutant mice, we excluded a possible role of Fas/Fas ligand interactions in these processes (data not shown). The possible involvement of CTLA-4 in T cell homeostasis is also unlikely, as it has also been shown that $CD25^+CD4^+$ T cells from CTLA-4-deficient mice exhibit suppressor activity (45). Recent results implicating the glucocorticoid-induced TNFR (TNFRS18) in the regulatory activity of the $CD25^+$ T cells (46, 47) are not conclusive (44). Other possibilities are under investigation.

The suppressive capacity of the $CD25^+CD4^+$ T cells, while maximal upon injection, was virtually lost when these cells were parked for 2 mo in the hosts. It is possible that regulation of existing and newly transferred cells differs, and/or that "parked" cells may evolve functionally. Upon secondary transfer, we showed that the parked $CD25^+CD4^+$ T cell retained their suppressive abilities. Our in vivo observations contrast with recent in vitro data showing that T cells having lost CD25 expression suppress expansion of naive CD4 T cells (48). This apparent discrepancy may simply reflect differences in the in vitro and in vivo behavior of the Treg cells. Other reports have shown that the $CD25^+$ cells progeny of in vivo activated naive $CD4^+$ T cells were not able to confer effective protection of disease (49) or to control in vitro T cell proliferation (40). Moreover, $CD25^-CD4^+$ thymocytes, when transferred to immune-deficient hosts, cannot generate a Treg cell population and induce autoimmune disease (50). In addition, the autoimmune manifestations that occur in neonatal thymectomized mice (day 3) correlate with the absence of $CD25^+CD4^+$ T cells, and the reintroduction of $CD25^+$ cells generated in 3-day-old Tx mice was unable to prevent disease (40), which could be avoided by $CD25^+CD4^+$ T cells from normal donors (28). These observations indicate that the regulatory functions may be a property of a specific cell subpopulation, but that inside this subpopulation these functions correlate with the expression of the CD25 marker and may require continuous T cell stimulation (27, 32). The inhibitory effects may require direct T-T cell interactions (40) or act via a third party presenting cell. We found that the initial $CD25^+$:$CD25^-$ ratio strictly determined the final number of CD4 T cells, suggesting a direct relationship between the two populations. Inhibition does not seem to require Ag specificity or mutual cognate recognition by the interacting cells, since in vitro the two populations do not need to recognize the same ligand (51). However, maintenance of the Ag-specific regulatory cells seems to require the continuous presence of the Ag (32).

To the question of whether the regulatory cells may represent a separate $CD4^+$ T cell lineage (52), the answer is yes. The IL-2R$\alpha^{-/-}$ mutant mice lack these cells. The transfer of a limited number of $CD25^+CD4^+$ T cells in mouse chimeras reconstituted with BM cells from IL-2R$\alpha^{-/-}$ mice prevents lethal lymphocyte accumulation. However, delayed transfer of the $CD25^+CD4^+$ cells was less effective in protection, stressing the importance of regulatory/naive cell ratios at the time of the initial peripheral seeding. The presence of as few as 5% of cells from a normal donor developing in the thymus of the chimeras suffices to reestablish full control of the number and state of activation of the peripheral CD4 IL-2R$\alpha^{-/-}$ T cells. It has been shown that $CD25^+CD4^+$ T cells are generated in the thymus (38, 50, 53). Our findings support this claim. We also found that transferred $CD25^+CD4^+$ T cells can persist for prolonged periods in absence of the thymus, as observed in the IL-2R$\alpha^{-/-}$ BM chimeras. Thus, these cells can be either long-lived or capable of self renewal at the periphery. Interestingly, in the different mixed IL-2R$\alpha^{-/-}$/normal BM chimeras, the number of peripheral $CD25^+CD4^+$ T cells was the same independently of the fraction of normal cells present in the BM inoculum. IL-2-deficient mice lack a significant number of $CD25^+CD4^+$ cells at the periphery (38) and develop lymphoid hyperplasia and IBD (20). Recent studies suggested that disturbed peripheral homeostasis in IL-2-deficient mice resulted from either an IL-2-dependent AICD defect and/or the lack of $CD25^+CD4^+$ regulatory cells (14). We found that IL-$2^{-/-}$-derived cells rescue the IL-2R$\alpha^{-/-}$ BM chimeras from death, and that in these chimeras, the number and the distribution of $CD25^+$ and $CD25^-$CD4 T cells in the peripheral T cell compartments was as in normal mice. Thus, in the presence of IL-2, the BM precursors from IL-2-deficient donor mice generated a well-defined population of $CD25^+CD4^+$ regulatory T cells capable to control the number of CD4 T cells in the peripheral compartments. These findings suggest that IL-2 is required for the peripheral survival and maintenance of the subset of the $CD25^+$ regulatory cells produced by the thymus. Production of IL-2 by proliferating CD4 T cells may also contribute to the survival of the regulatory cells. Thus, we may envisage a feedback loop in which expanding naive CD4 T cells contribute to their own regulation. The role of IL-2/IL-2R interactions in T cell homeostasis is further supported by results showing that IL-2R$\beta^{-/-}$ T cells in mice reconstituted with a mixture of IL-2R$\beta^{-/-}$ T and IL-2Rβ^+ BM cells did not expand or develop into an abnormally activated stage (13). However, in this last study, the cells responsible for the homeostatic control were believed to be CD8 T cells (13) and not the $CD25^+CD4^+$ T cells that we now identified as capable of controlling IL-2Rα-deficient T cells. It is possible that other cell populations contribute to regulate peripheral T cell pools.

In summary, we demonstrate the role of T cell interactions in the control of the size of the peripheral $CD4^+$ T cell pool. We show that homeostasis of peripheral $CD4^+$ T cells follows subpopulation structure, $CD25^+CD4^+$ T cells limiting the accumulation of dividing naive $CD4^+$ T cells. We found that IL-2Rα-deficient mice lack a subpopulation of regulatory cells essential for CD4 T cell homeostasis. Adoptive replacement of the $CD25^+CD4^+$ T cell population prevents the chaotic accumulation of lymphoid cells in the peripheral compartments of IL-2Rα-deficient mice. It also prevents the subsequent polyclonal T and B cell activation, autoimmune hemolytic anemia, and IBD observed in IL-2R$\alpha^{-/-}$ mice (10). We also show that IL-2-deficient mice also lack a sizeable population of $CD25^+CD4^+$ T cells that expands in presence of IL-2 to control autoimmunity and lymphoid hyperplasia in IL-2Rα chimeras. To conclude, we show that the mechanism by which IL-2Rα and IL-2 play an essential role on T cell homeostasis is by shaping a population of $CD25^+CD4^+$ regulatory T cells that control peripheral CD4 T cell numbers. We demonstrate that IL-2Rα is an absolute requirement for the generation of the regulatory cells. These cells generate in the thymus in the absence of IL-2, but require IL-2 to establish a stable functional population in the peripheral compartments.

Acknowledgments

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ADDITIONAL RESULTS

"Searching for the Mechanisms responsible for *in vivo* $CD4^{+}CD25^{+}$ regulatory T cell mediated suppression"

Introduction

In a recent article (Almeida et al., 2002) we have identified a population of $CD4^+CD25^+$ regulatory T cells (reviewed in Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2000) as an important player in T cell homeostasis and the expression of CD25 as a requirement for their generation. We have shown that the absence of this population of regulatory T cells is responsible for the perturbed homeostasis observed in both the $CD25^{-/-}$ (Willerford et al., 1995) and the $IL2^{-/-}$ (Sadlack et al., 1993; Schorle et al., 1991) mice. In the absence of this regulatory T cell population, the $CD4^+$ T cells present are constitutively activated and expand in an uncontrolled way (Sadlack et al., 1993; Willerford et al., 1995) but this can be reversed by the presence of $CD4^+CD25^+$ regulatory T cells (Almeida et al., 2002).

The mechanism of regulation by $CD4^+CD25^+CD45RB^{low}$ regulatory T cells has been the subject of numerous studies, reporting on the ability of these cells to prevent the development of autoimmune or autoimmune-like diseases provoked by $CD4^+CD25^-$ $CD45RB^{high}$ T cells *in vivo* (Asseman et al., 1999; Powrie et al., 1996; Read et al., 2000; Seddon and Mason, 1999; Takahashi et al., 2000) or on the ability of the $CD4^+CD25^+$ regulatory T cells to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro* (Cederbom et al., 2000; Itoh et al., 1999; McHugh et al., 2002; Nakamura et al., 2001; Shimizu et al., 2002; Stephens et al., 2001; Takahashi et al., 1998; Takahashi et al., 2000; Thornton and Shevach, 1998; Thornton and Shevach, 2000). When put together, the conclusions drawn from these studies are far from being conclusive, as different molecules and mechanisms are suggested. Interestingly, the two major categories of regulatory mechanisms described so far segregate with the two major categories of experimental systems used: in studies performed *in vivo*, the most commonly suggested mechanisms are related to the secretion of suppressive cytokines, like IL10 (Asseman et al., 1999), TGFβ (Powrie et al., 1996) or IL4 (Seddon and Mason, 1999) while *in vitro* studies point for a cell-contact dependent mechanism (Nakamura et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 2000). Importantly, the readout for the regulatory activity is not necessarily the same for *in vitro* and *in vivo* studies: the usual readout for *in vitro* regulatory activity is suppression of proliferation of other populations while the readout for *in vivo* regulatory activity is more complex but usually involves protection from disease induced by other populations (reviewed in Maloy and Powrie, 2001; Shevach, 2000).

Based on our previous observations on the role of $CD4^+CD25^+CD45RB^{low}$ regulatory T cells in the control of T cell numbers *in vivo* (Almeida et al., 2002) we investigated the mechanism by which $CD4^+CD25^+CD45RB^{low}$ regulatory T cells exert their suppressive activity *in vivo*, and report in this additional results chapter, results that rule out an essential

role for a variety of putative effector mechanisms of both cell-contact dependent and soluble molecule dependent categories, namely Fas-FasL interactions and secretion of TNFα, TNFβ (LTα), and IL10. We also show that the $CD4^{+}CD25^{-}CD45RB^{low}$ T cells are not able to mediate suppression, but that these cells compete with the $CD4^{+}CD25^{-}CD45RB^{high}$ originated cells for the occupancy of the same pool. Our results confirm some previous conclusions on the role of soluble molecules in the ability to prevent disease (Asseman et al., 1999) but not others on the ability of these molecules in the control of peripheral expansion (Annacker et al., 2001) and represent evidence favouring the notion of the $CD4^{+}CD25^{+}$ regulatory T cells as an important component of the peripheral organization of the mature pools, but with a possible redundancy of effector mechanisms.

Materials and Methods

Mice. C57Bl/6.Ly5.2 mice were obtained from Iffa-Credo (France), B6.CD3$\varepsilon^{-/-}$ (Malissen et al., 1995), B6 *lpr*, B6 *gld*, and C57BL/6.Ly5.1 mice from the CDTA-CNRS (Orléans, France), B6.IL-10$^{-/-}$ (Kuhn et al., 1993). B6 TNF$\alpha^{-/-}$ and B6 LT$\alpha^{-/-}$ (De Togni et al., 1994) from the Jackson Laboratories. All mice were matched for age (6-12 weeks) and sex.

Cell sorting and cell transfers. LN cells from the Ly5.2 and Ly5.1 donor mice were first enriched for CD4$^+$ T cells by negative selection using a Dynal MPC6 magnetic cell sorting. Briefly cells were incubated with a cocktail of rat antibodies directed to mouse B220 (RA3-6B2), Mac1 (CD11b) and CD8α (53-6.7), all from Pharmingen (San Diego, CA, USA), followed by Sheep anti rat Ig coated Dynabeads (Dynal). After removing the positive fraction the remaining population was >90% CD4$^+$. These cells were labeled with the appropriate combinations of anti-CD4 (L3T4/RM4-5), anti-CD45RB and anti-CD25 (7D4) antibodies and sorted on a FACStar^{Plus} (Becton Dickinson, San Jose, CA USA). The purity of the sorted CD4$^+$CD25$^-$CD45RB^{high} and CD4$^+$CD25$^+$CD45RB^{low} populations varied from 95-99.9%.
Intact non-irradiated B6.CD3$\varepsilon^{-/-}$ (Malissen et al., 1995) hosts were injected *i.v.* with the purified CD4$^+$ T cell populations alone or mixed at different cell ratios. By using mice differing by Ly5 allotypes we were able to discriminate the cells originating from the different donor mice. Host mice were sacrificed at different time intervals after cell transfer. Spleen, inguinal and mesenteric lymph nodes cell suspensions were prepared and the number and phenotype of the cells from each donor population evaluated. The total peripheral T cells showed in the results represent the number of cells recovered in the host's spleen added to twice the number of cells recovered from the host's inguinal and mesenteric LNs.

Bone Marrow chimeras. Host 8-week-old Rag$_2^{-/-}$B6 mice were lethally irradiated (900rad) with a ^{137}Ce source and received intravenously 2 to 4x10^6 T cell depleted BM cells from different donor mice. T cell depletion (<0.1%) was done in a Dynal MPC6 magnetic sorter after incubating the BM cells with anti-CD4, anti-CD8 and anti-CD3 biotinylated antibodies followed by Streptavidin coated Dynabeads.

Flow cytometry analysis. The following monoclonal antibodies were used: anti-CD3e (145-2C11), anti-CD4 (L3T4/RM4-5), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti CD45RB, anti-CD24/HSA (M1/69) and anti-TCRβ (H57) from Pharmingen (San Diego, CA, USA), and anti-

CD44 (IM781), anti-CD62L (MEL14) from Caltag (San Francisco, CA, USA). Cell surface four color staining was preformed with the appropriate combinations of FITC, PE, TRI-Color, PerCP, Biotin and APC-coupled antibodies. Biotin-coupled antibodies were secondary labeled with APC-, TRI-Color- (Caltag, San Francisco, CA, USA) or PerCP-coupled (Becton Dickinson, San Jose, CA, USA) streptavidin. Dead cells were excluded during analysis according to their light-scattering characteristics. All acquisitions and data analysis was performed with a FACScalibur (Becton Dickinson, San Jose, CA USA) interfaced to a Macintosh CellQuest software.

Ribonuclease protection assay. The synthesis and quantification of specific mRNA's was studied using the Multi-Probe RNase protection assay system RiboQuant (Pharmingen, San Diego, CA, USA), following closely the instructions of the manufacturer. Equal numbers ($1x10^6$) of Facs sorted (>95% pure) $CD4^+CD45RB^{high}CD25^-$ and $CD4^+CD45RB^{low}CD25^+$ T cells were resuspended in Trizol reagent (Life technologies) for RNA extraction. Each population RNA was tested for the presence of mRNA for IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, γIFN, TNFβ, LTβ, TNFα, INFβ, TGFβ1, TGFβ2, TGFβ3, and MIF, using the Multi-probe template sets mCK-1 and mCK-3b. Each high-specific-activity [^{32}P]-labelled anti-sense RNA probe set was hybridised with sample RNA in excess in solution. After free probe and other single-stranded RNA are digested with Rnases. The remaining protected probes are purified, resolved in denaturing polyacacrylamide gels, and were quantified by phsphorimaging. The quantity of each mRNA species in the original RNA sample was then determined based on the intensity of the appropriately-sized, protected probe segment. Note that each probe band (left lane in the gels) migrates slower than its protected band ($CD25^+$ and $CD25^-$ lanes); this is due to flanking sequences in the probe that are not protected by mRNA. (the probe is directly loaded into the gel, without Rnase treatment).

Results

Fas-FasL interactions are not relevant for $CD4^+CD25^+$ regulatory T cell mediated *in vivo* suppression of expansion.

One possibility for the interaction between the two populations would be direct interaction via the Fas-FasL system. The Fas-FasL interaction could be a tempting way to explain cell-contact dependent suppression *in vivo*, by a process of cell "fratricide" (reviewed in Sabelko-Downes and Russell, 2000). The $CD4^+CD25^+$ T cells could express FasL upon activation, and act on the Fas expressing $CD45RB^{high}CD25^-CD4^+$ T cells, inducing AICD of these latter. An active role of the Fas-FasL system in $CD4^+CD25^+$ T cell mediated suppression could be part of the explanation for the phenotypes of the *lpr* ($Fas^{-/-}$) and *gld* ($FasL^{-/-}$) mice (Andrews et al., 1978; Roths et al., 1984), which present a lymphoproliferative disorder characterized by the presence of autoreactive $CD4^+$ T cell clones and accumulation of a DN $CD3^+$ population (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992).

In order to investigate the role of the Fas-FasL system in the $CD4^+CD25^+$ mediated suppression of expansion of $CD4^+CD25^-CD45RB^{high}$ T cells *in vivo*, we transferred purified $CD4^+CD25^-CD45RB^{high}$T cells from *lpr* mice into syngeneic $CD3\varepsilon^{-/-}$ T cell deficient hosts, alone or in presence of different ratios of $CD4^+CD25^+CD45RB^{low}$ regulatory T cells from B6Ly5.1 mice (fig.1A). As a control, $CD4^+CD25^-CD45RB^{high}$ T from C57Bl6 origin were also transferred alone or with co-transferred $CD4^+CD25^+CD45RB^{low}$ regulatory T cells from B6Ly5.1 mice at different ratios. Mice were sacrificed 8 weeks after transfer and the recovery from each origin determined by flow cytometry, through the Ly5 marker. The results obtained indicate that $CD4^+CD25^-CD45RB^{high}$ T cells from *lpr* donors expand in the immunodeficient $CD3\varepsilon^{-/-}$ hosts as normal B6 when transferred alone and this expansion is suppressed in a dose-dependent manner by cotransferred $CD4^+CD25^+CD45RB^{low}$ regulatory T cells, as happens with the control $CD4^+CD25^-CD45RB^{high}$ T cells from B6 origin (fig.1A). This result strongly suggests that the Fas-FasL system is not involved in the observed suppression by $CD4^+CD25^+CD45RB^{low}$ regulatory T cells of $CD4^+CD25^-CD45RB^{high}$ T cell expansion.

To confirm the non-involvement of the Fas-FasL system in the $CD4^+CD25^+CD45RB^{low}$ regulatory T cell mediated suppression, we used the same cell transfer system, using as suppressor population $CD4^+CD25^+CD45RB^{low}$ T cells from *gld* mice, that carry a mutation in the FasL protein that renders it non-functional (Takahashi et al., 1994). As shown (fig.1B), $CD4^+CD25^+CD45RB^{low}$ regulatory T cells from *gld* mice were as good suppressors as cells

from C57Bl6 mice *in vivo*. Altogether, these results rule out a role for the Fas-FasL system in the suppression of CD4^{+}CD25^{-}CD45RB^{high} T cell expansion *in vivo*.

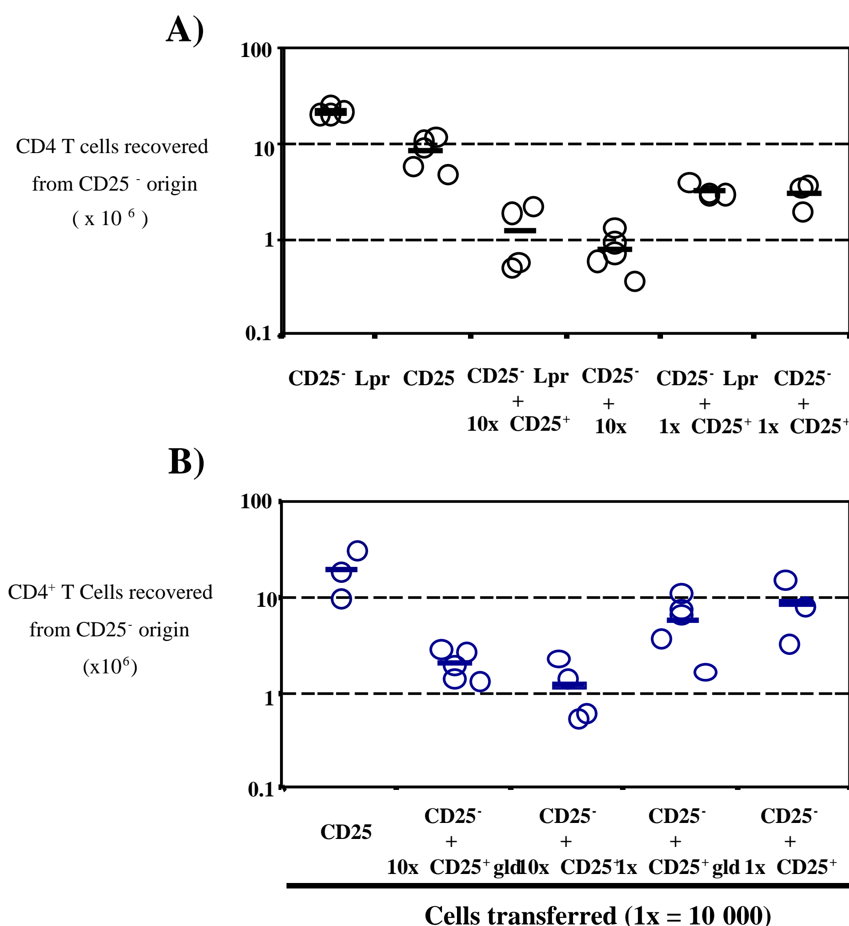

Figure 1: Fas/FasL experiments

A) CD4^{+}CD45RB^{high}CD25^{-} (CD25^{-}) T cells (10000) from normal or *lpr* donors were transferred alone or co-injected with 100000 CD4^{+}CD25^{+}CD45RB^{low} from normal B6 mice into CD3ε$^{-/-}$ hosts. The results show the number of CD4 T cells recovered 7-8 weeks after transfer in the spleen and LN of each individual host. Note that the CD4^{+}CD25^{+}CD45RB^{low} T cells from normal B6 donors inhibit expansion of naive CD4 T cells from lpr mice.

B) CD4^{+}CD25^{-}CD45RB^{high} (CD25^{-}) T cells (10000) from normal B6 donors were transferred alone or co-injected with 100000 CD4^{+}CD25^{+}CD45RB^{low} from normal or *gld* mice. The results show the number of CD4 T cells From CD25^{-} origin recovered 7-8 weeks after transfer in the spleen and LN of each individual host. Note that cells from *gld* mice inhibit expansion of naive T cells from normal donors.

TNFα is not involved in the mechanism of suppression by CD4^{+}CD25^{+}CD45RB^{low} regulatory T cells.

TNF was first identified as a molecule able to mediate cell killing (reviewed in Wallach et al., 1999). In order to investigate the role of TNFα as a possible soluble mediator of CD4^{+}CD25^{+} regulatory T cell suppression, we used the same cell transfer system this time using CD4^{+}CD25^{+}CD45RB^{low} regulatory T cells from TNFα$^{-/-}$ donors. We tested their ability to suppress the expansion of CD4^{+}CD45RB^{high}CD25^{-} T cells from B6Ly5.1 donors after transfer into CD3ε$^{-/-}$ hosts. As shown (fig.2), TNFα$^{-/-}$ CD4^{+}CD25^{+}CD45RB^{low} regulatory T cells were good suppressors *in vivo*, suggesting that TNFα cannot be responsible alone for suppression by CD4^{+}CD25^{+} regulatory T cells.

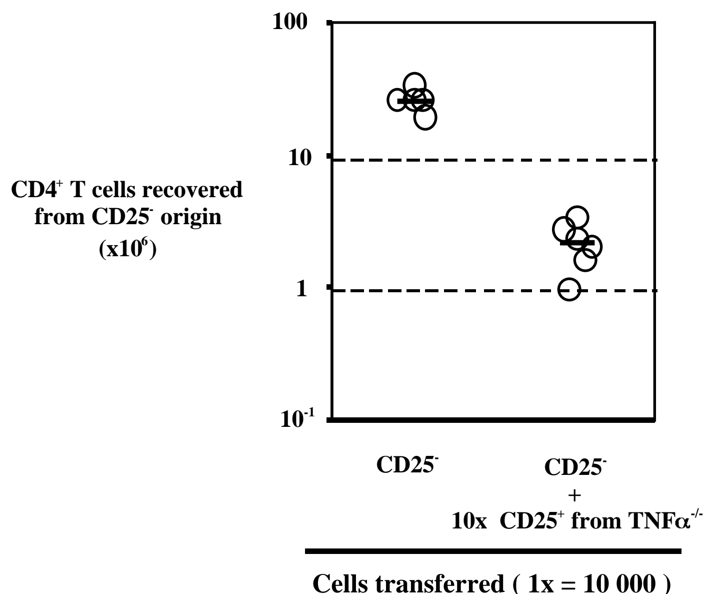

Figure 2: TNFα experiments

$CD4^+CD25^-CD45RB^{high}$ ($CD25^-$) T cells (10000) from normal B6 donors were transferred alone or co-injected with 100000 $CD4^+CD25^+CD45RB^{low}$ from normal or $TNF\alpha^{-/-}$ mice. The results show the number of CD4 T cells recovered 7-8 weeks after transfer in the spleen and LN of each individual host. Note that $CD4^+CD25^+$ T cells from $TNF\alpha^{-/-}$ mice inhibit expansion of naive T cells from normal donors.

LTα is not involved in the mechanism of suppression by $CD4^+CD25^+CD45RB^{low}$ regulatory T cells.

In an attempt to unveil cytokines produced by $CD4^+CD25^+CD45RB^{low}$ regulatory T cells we tested by RNAse Protection Assay the presence of mRNA for a variety of cytokines in purified $CD4^+CD25^+CD45RB^{low}$ or $CD4^+CD25^-CD45RB^{high}$ T cells (fig.3A). Both naive and activated cells expressed similar levels of mRNAs for the 3 different isoforms of TGFβ (fig.3A), what suggested that this cytokine would not be involved in the suppressor activity of the $CD4^+CD25^+$ regulatory T cells. Interestingly, differential expression was observed regarding LTα message (fig.3A). LTα is a cytokine with an important role in LN and splenic organization, as LTα deficient mice do not have Lymph Nodes and have a severely disturbed splenic organization (De Togni et al., 1994). This phenotype does not correlate with the phenotype of the $CD25^{-/-}$ (Willerford et al., 1995) or the $IL2^{-/-}$ (Sadlack et al., 1993; Schorle et al., 1991) mice, that display severe lymphoproliferative disorders (Sadlack et al., 1993; Willerford et al., 1995) secondary to their deficiency in $CD4^+CD25^+$ regulatory T cells (Almeida et al., 2002). This fact could be either due to the irrelevance of LTα production by $CD4^+CD25^+$ regulatory T cells for their suppressive function or to the impossibility to develop lymphoproliferation disorders in the absence of lymph nodes and of normal splenic architecture.

In order to investigate this issue, we reconstituted irradiated syngeneic $Rag_2^{-/-}$ hosts with BM cells from LTα deficient donors and studied the survival and the peripheral

composition of the lymphocyte pools of the chimeras (figs.3B and C). In this situation, the architecture of the secondary lymphoid organs is normal and we can study the effects of LTα deficiency in peripheral T cell homeostasis. Two observations deserve attention. First, the survival of these chimeras is much better than the survival of $Rag_2^{-/-}$ hosts reconstituted with $CD25^{-/-}$BM cells (fig.3B and Almeida et al., 2002) and second that some of these chimeras eventually do get sick and die, with signs of wasting disease and IBD (fig.3B and not shown). These results were in accordance with a partial role for LTα in the function of regulatory T cells, maybe in a similar way as IL10 and TGF-β, that have been shown to be essential for prevention of IBD in cell transfer models of IBD induction by naïve type $CD4^+$ T cells (Asseman et al., 1999; Powrie et al., 1996). In order to test this we have tested the ability of $CD4^+CD25^+$ cells from LT$\alpha^{-/-}$ origin in the suppression of expansion of $CD4^+CD25^-$ $CD45RB^{high}$ T cells after transfer into CD3$\varepsilon^{-/-}$ hosts (fig.3D). In order to obtain $CD4^+CD25^+$ cells from LT$\alpha^{-/-}$ origin we sacrificed the surviving chimeras (fig.3B) and collected $CD4^+CD25^+$ cells from the lymph nodes (fig.3C). These LT$\alpha^{-/-}$ cells proved to be good suppressors *in vivo*, suppressing as well as C57BL/6 origin $CD4^+CD25^+$ T cells (fig.3D). No signs of IBD were observed in these host mice what suggests that LTα is also not important for protection from disease induced by the transferred $CD4^+CD25^-CD45RB^{high}$ T cells.

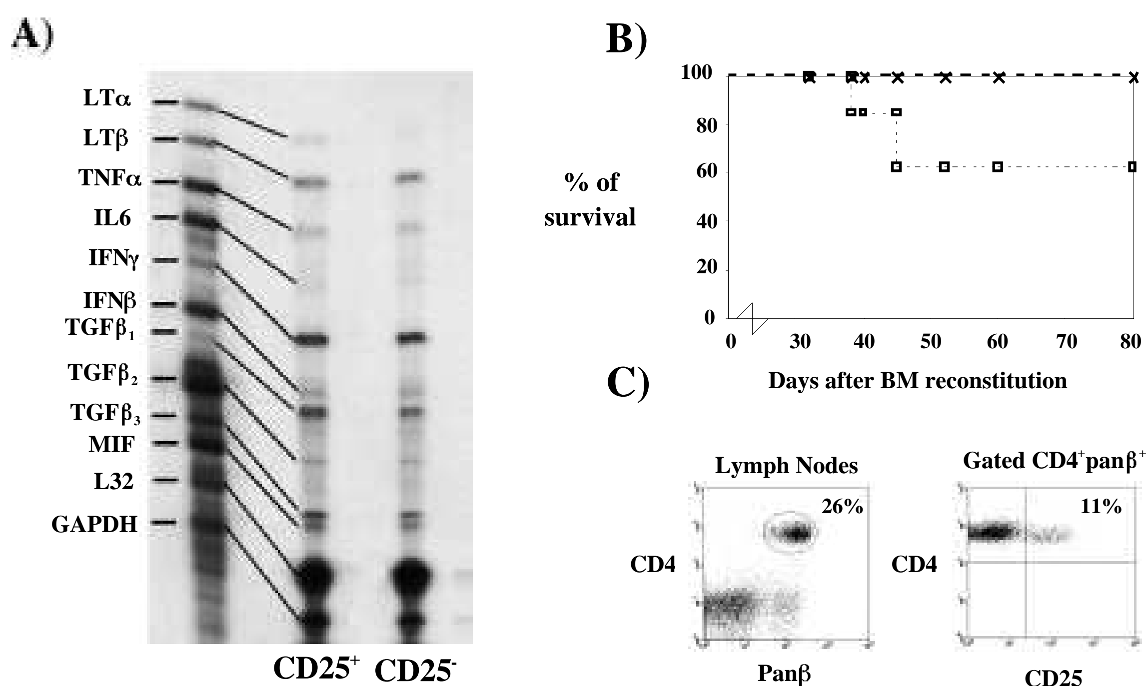

Figure 3: LTα experiments – **A)** RNase protection assay for interleukins production by $CD4^+CD25^+CD45RB^{low}$ ($CD25^+$) and $CD4^+CD25^-$ $CD45RB^{high}$ ($CD25^-$) T cells. Note that the only cytokine displaying differential expression, is LTα (top band), with some expression in the $CD25^+$ lane and not in the $CD25^-$ lane. Note also that each probe band (left lane) migrates slower than its protected band ($CD25^+$ and $CD25^-$ lanes); this is due to flanking sequences in the probe that are not protected by mRNA. **B)** Lethally irradiated B6.Rag2$^{-/-}$ mice were reconstituted with $2x10^6$ cells from B6.LT$\alpha^{-/-}$ donors. Control chimeras received $2x10^6$ cells from B6. Ly5.1 mice. Results show the time of survival of the chimeras reconstituted with BM cells from B6.LT$\alpha^{-/-}$ donors (❑) or with BM cells from B6.Ly5.1 donors (×). Number of mice/group: 8. **C)** Phenotypic characterization of the peripheral LN CD4 T cells in a chimera reconstituted with BM cells from B6.LT$\alpha^{-/-}$ donors. Similar results were obtained in the remaining mice from the same group.

D)

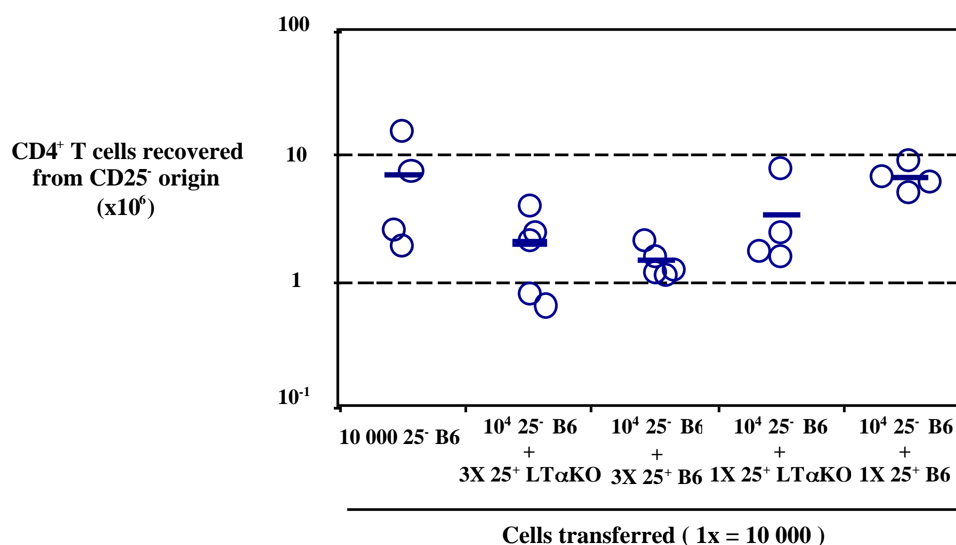

Figure 3: LTα experiments (Cont.)– D) $CD4^+CD25^-CD45RB^{high}$ ($CD25^-$) T cells (10000) from normal B6 donors were transferred alone or co-injected with 30000 or 10000 $CD4^+CD25^+CD45RB^{low}$ from normal or from the surviving $Rag2^{-/-}$ reconstituted with BM from $LT\alpha^{-/-}$ mice (**B** and **C**). The results show the number of CD4 T cells from $CD25^-$ origin recovered 7-8 weeks after transfer in the spleen and LN of each individual host. Note that $CD4^+CD25^+$ T cells from $LT\alpha^{-/-}$ origin inhibit expansion of naive T cells from normal donors.

The inhibition of expansion of the $CD45RB^{high}CD25^-CD4^+$ T cells by $CD45RB^{low}CD25^+CD4^+$ T cells is not mediated through IL-10.

The Transfer of $CD4^+CD25^+CD45RB^{low}$ T cells protects host mice from organ specific autoimmunity (Asano et al., 1996; Sakaguchi et al., 1995) or IBD (Powrie et al., 1994a; Powrie et al., 1994b) induced by $CD4^+CD25^-CD45RB^{high}$ T cells. These protective effects are believed to be mediated by TGF-β or IL-10 (Asano et al., 1996; Asseman et al., 1999; Groux et al., 1997; Papiernik et al., 1998; Powrie et al., 1996). By RNase protection assay we confirmed that $CD4^+CD25^+$ cells produce constitutively mRNA for IL-10, in contrast to naive $CD4^+$ T cells (fig.4A). We asked whether the inhibitory effects of the $CD4^+CD25^+CD45RB^{low}$ T cells on the expansion of $CD4^+CD25^-CD45RB^{high}$ T cells were mediated by IL-10. To test this hypothesis we used $IL\text{-}10^{-/-}$ mice (Kuhn et al., 1993). At 12 weeks of age the total $CD4^+$ T cell number was slightly increased in these mice but, more importantly, the fraction and the number of $CD4^+CD25^+CD45RB^{low}$ T cells was as in normal mice (fig.4B). After transfer into $CD3\varepsilon^{-/-}$ hosts purified $CD4^+CD25^-CD45RB^{high}$ and $CD4^+CD25^+CD45RB^{low}$ T cells from $IL\text{-}10^{-/-}$ donors behave similarly to the cells from a normal donor (not shown). Upon co-transfer the $CD4^+CD25^+CD45RB^{low}$ T cells from $IL\text{-}10^{-/-}$ mice suppressed expansion of $CD4^+CD25^-$

CD45RB^{high} T cells from normal mice as efficiently as CD4^{+}CD25^{+}CD45RB^{low} T cells from normal IL-10^{+} donors (fig.4C). We concluded that control of peripheral T cell numbers by CD4^{+}CD25^{+}CD45RB^{low} T cells is not mediated by IL-10. Importantly, these mice developed signs of IBD, confirming the requirement for this cytokine in the control of disease (not shown).

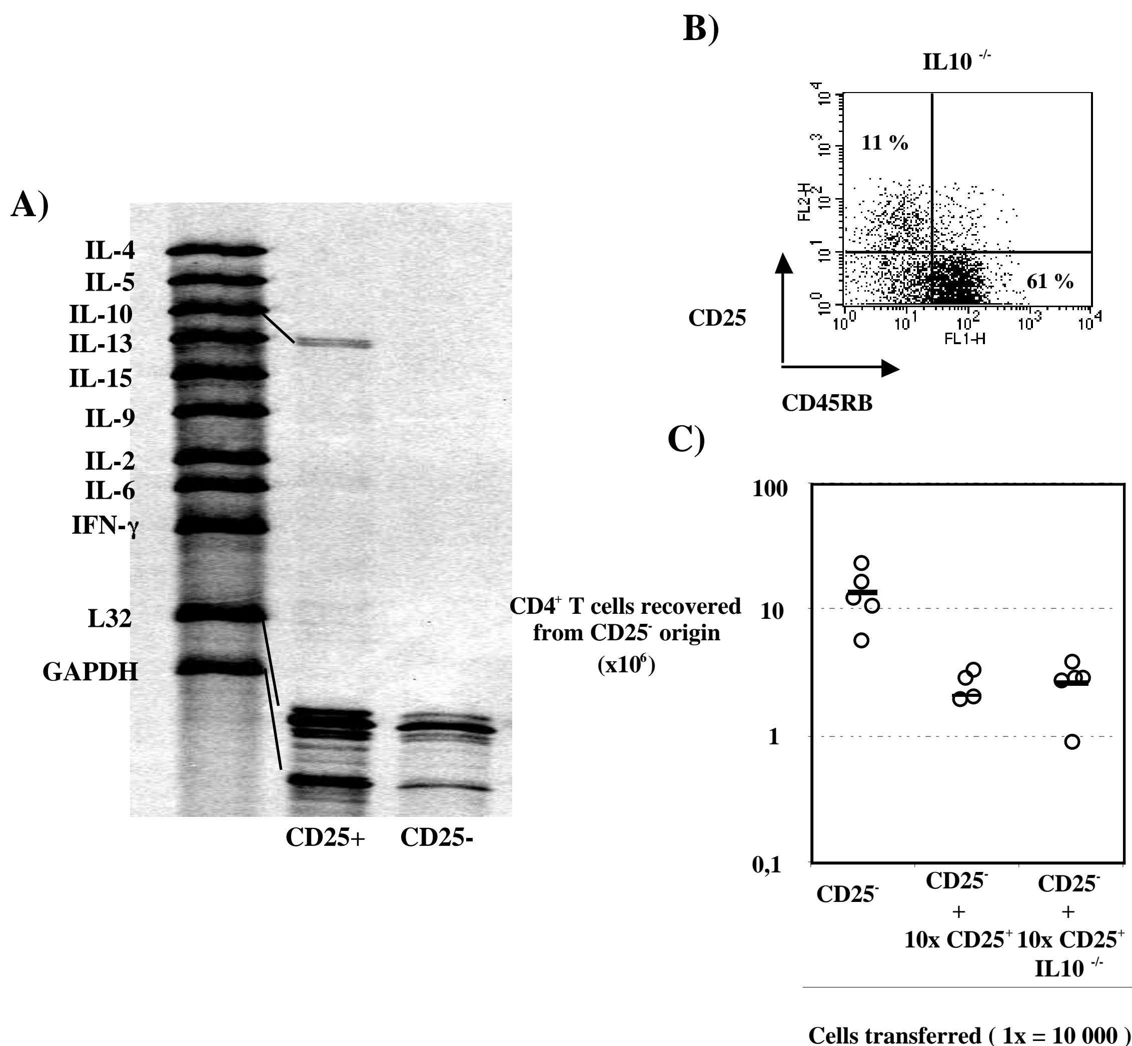

Figure 4: IL-10 experiments – A) RNase protection assay for interleukin production by CD4^{+}CD25^{+}CD45RB^{low} (CD25^{+}) and CD4^{+}CD25^{-}CD45RB^{high} (CD25^{-}) T cells. Note that the only cytokine displaying differential expression, is IL10 (visible band), with some expression in the CD25^{+} lane and not in the CD25^{-} lane. Note also that each probe band (left lane) migrates slower than its protected band (CD25^{+} and CD25^{-} lanes); this is due to flanking sequences in the probe that are not protected by mRNA. **B)** Pattern of CD45RB and CD25 expression by gated CD4 LN T cells from IL-10$^{-/-}$ LN cells. The % of CD4^{+}CD25^{+}CD45RB^{low} and CD4^{+}CD25^{-}CD45RB^{high} cells is shown. **C)** CD4^{+}CD25^{-}CD45RB^{high} (CD25^{-}) T cells (10000) from normal B6 donors were transferred alone or co-injected with 100000 CD4^{+}CD25^{+}CD45RB^{low} from normal or IL-10$^{-/-}$ mice into CD3ε$^{-/-}$ hosts. The results show the number of CD4 T cells from CD25^{-} recovered 7-8 weeks after transfer in the spleen and LN of each individual host. Note that cells from IL-10$^{-/-}$ mice inhibit expansion of naive T cells from normal donors.

$CD4^+CD25^-CD45RB^{low}$ T cells do not suppress $CD4^+CD25^-CD45RB^{high}$T cell expansion, but compete for the same peripheral niche.

Another unsolved question relates to the suppressive ability of the $CD25^-$ fraction of the $CD4^+CD45RB^{low}$ T cell subset in the prevention of expansion of $CD4^+CD25^-CD45RB^{high}$ T cells upon transfer into immunodeficient hosts. The role of the $CD25^-$ fraction of the $CD4^+CD45RB^{low}$ peripheral pool is controversial, though it is considered that regulatory T cells do exist in the $CD25^-$ fraction. Thus, it was reported that regulatory T cells able to prevent autoimmune diabetes in rats can be found in the $CD25^-$ fraction of peripheral $CD4^+$ T cells (Stephens and Mason, 2000) and $CD4^+CD25^-CD45RB^{low}$ T cells from mice were reported to prevent wasting disease induced by $CD4^+CD25^-CD45RB^{high}$ T cells upon transfer into $Rag_2^{-/-}$ hosts (Annacker et al., 2001). We addressed this issue using the same cell transfer system and evaluating the ability of $CD4^+CD25^-CD45RB^{low}$ T cells to suppress expansion of $CD4^+CD25^-CD45RB^{high}$ T cells and the ability of $CD4^+CD25^+CD45RB^{low}$ T cells to suppress expansion of $CD4^+CD25^-CD45RB^{low}$ T cells (see table on figure 5). The results obtained shown that $CD4^+CD25^-CD45RB^{low}$ T cells expand as $CD4^+CD25^-CD45RB^{high}$ T cells when transferred alone into immunodeficient $CD3\varepsilon^{-/-}$ hosts (fig.5A). In contrast to what observed when $CD4^+CD25^-CD45RB^{high}$ T cells are co-transferred with $CD4^+CD25^+CD45RB^{low}$ T cells (figs. 2, 4, 5 and Almeida et al., 2002), the expansion of $CD4^+CD25^-CD45RB^{low}$ T cells is not suppressed by co-transfer of $CD25^+$ regulatory T cells. When the $CD4^+CD25^-CD45RB^{low}$ T cells are co-transferred with naïve $CD4^+CD25^-CD45RB^{high}$ T cells, the latter do not expand as much as they do when transferred alone (compare in figure 5A, group III with group IV, left), but the absolute number of $CD4^+$ T cells recovered in these mice is not reduced (fig.5B), as the $CD4^+CD25^-CD45RB^{low}$ T cells expand in the hosts. Thus, the apparent inhibition of expansion is due to competition for the same peripheral niche.

I	**10 000 25^-45RB^{low}**	**–**
II	**10 000 25^-45RB^{low}**	**100 000 25^+45RB^{low}**
III	**10 000 25^-45RB^{high}**	**–**
IV	**10 000 25^-45RB^{high}**	**100 000 25^-45RB^{low}**

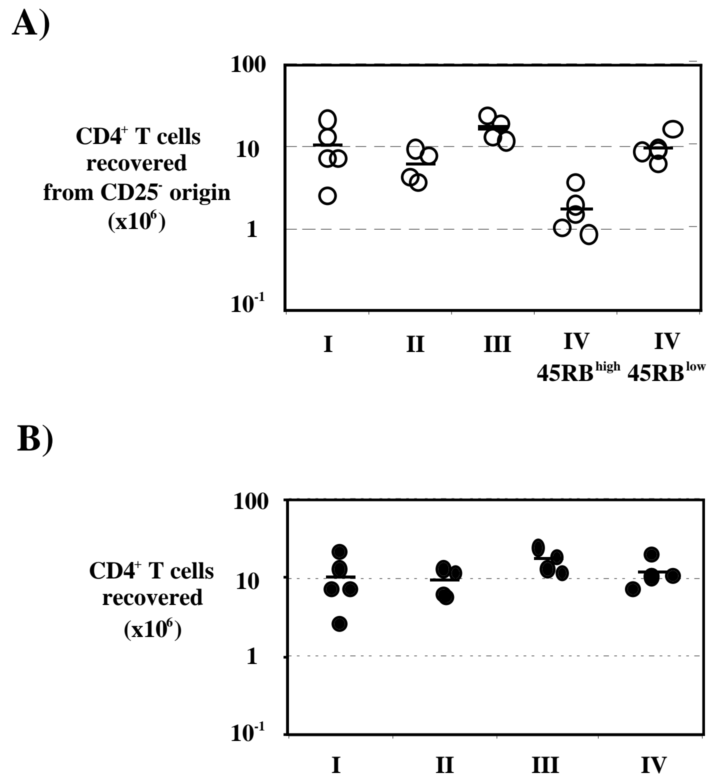

Figure 5: $CD4^+CD25^-CD45RB^{low}$ studies– **A)** $CD4^+CD25^-CD45RB^{low}$ T cells (10000) from normal B6.Ly5.1donors were transferred alone or co-injected with 100000 $CD4^+CD25^+CD45RB^{low}$ from normal B6 mice and $CD4^+CD25^-CD45RB^{high}$ T cells (10000) from normal B6.Ly5.1donors were transferred alone or co-injected with 100000 $CD4^+CD25^+CD45RB^{low}$from normal B6 mice (see table) into $CD3\varepsilon^{-/-}$ hosts. The results show the number of CD4 T cells from $CD25^-$ origin recovered 7-8 weeks after transfer in the spleen and LN of each individual host. **B)** The results show the total number of $CD4^+$ T cells recovered 7-8 weeks after transfer in the spleen and LN of each individual host, the same as in **A**). Note that the total $CD4^+$ T cell numbers recovered do not differ between groups, arguing against a suppressive role of the $CD4^+CD25^+CD45RB^{low}$on $CD4^+CD25^-CD45RB^{low}$ T cell expansion or of the $CD4^+CD25^-CD45RB^{low}$ T cells on $CD4^+CD25^-CD45RB^{high}$ T cell expansion.

Discussion

The $CD4^+CD25^+$ T cells are important components of the peripheral T cell pools, exerting a regulatory role of certain immune responses, regulating autoimmune responses and controlling peripheral T cell numbers. In order to understand their mode of action, the mechanism of immune response regulation and of suppression of expansion must be unveiled. Attempts to identify the mechanism responsible for $CD4^+$ regulatory T cell function have not been successful so far, though a number of candidate mechanisms and molecules have been identified (reviewed in Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2000). Thus, regulatory T cell function in the control of autoimmune disease by $CD4^+CD25^+CD45RB^{low}$ T cells has been reported to be dependent on IL10 in mice (Asseman et al., 1999) and humans (Groux et al., 1997) or TGF-β (Powrie et al., 1996) and in the rat tiroiditis model, TGF-β and IL4 have been identified as mediators of regulatory function by regulatory $CD4^+$ T cells (Seddon and Mason, 1999). These *in vivo* models of regulatory function have been paralleled by *in vitro* studies of regulatory T cell mediated suppression. These latter suggested that a cell-contact dependent mechanism was responsible for the regulatory mediated suppression, as suppression was not observed across a membrane and required cell contact (Takahashi et al., 1998; Thornton and Shevach, 1998). More, the addition of neutralizing anti-IL10, anti-IL4 and anti-TGF-β antibodies to the cultures would not have any effect in the suppression (Takahashi et al., 1998; Thornton and Shevach, 1998) and *in vitro* suppression was obtained when regulatory $CD4^+$ T cells were obtained from mice deficient in IL4 or IL10 (Thornton and Shevach, 1998). These conclusions are conflicting with the *in vivo* studies but differences may arise from the different experimental systems and from the different proprieties of the $CD4^+$ regulatory T cells assessed (regulation *versus* suppression). The same for the suggested role of CTLA4 in these processes. Here again, the reports of an essential role of CTLA4 in $CD4^+CD25^+$ mediated regulation (Read et al., 2000; Takahashi et al., 2000) are not supported by *in vitro* data (Jonuleit et al., 2001; Levings et al., 2001; Thornton and Shevach, 1998) and the fact that $CD4^+CD25^+$ T cells from $CTLA4^{-/-}$ mice still present suppressive activity (Takahashi et al., 2000) also argues against a mandatory role for CTLA4 in $CD4^+CD25^+$ mediated regulation.

In this study, we took advantage of our own experimental system (Almeida et al., 2002), relying in the cell transfer of purified populations of $CD4^+$ T cells into immunodeficient mice. We have previously shown that $CD4^+CD25^+$ regulatory T cells suppress the expansion of co-transferred $CD4^+CD25^-CD45RB^{high}$ naïve T cells in a dose-dependent manner,

providing suppression readout for $CD4^+CD25^+$ regulatory T cell action *in vivo*. In this additional results section we report on the role of putative effector molecules and mechanisms in $CD4^+CD25^+$ regulatory T cell mediated suppression of expansion *in vivo*.

Though it had been suggested that Fas-FasL interactions were not involved in suppression *in vitro* (Takahashi et al., 1998; Thornton and Shevach, 1998), we have investigated their role for suppression *in vivo* and found it to be neglectable or none. TNFα had also been shown *in vitro* to be dispensable for $CD4^+CD25^+$ mediated suppression (Takahashi et al., 1998), an observation that we confirm in our *in vivo* experimental system. Our observation that unstimulated sorted $CD4^+CD25^+$ T cells express higher amounts of mRNA for LTα led us to investigate the role of this cytokine in $CD4^+CD25^+$ suppressor activity. Our results leave also this molecule out of the $CD4^+CD25^+$ mediated suppression. The observation that some of the $Rag^{-/-}$ mice reconstituted with BM cells from $LT\alpha^{-/-}$ mice developed signs of IBD can be indicating a role for this molecule in the regulatory activity of these cells, and not in their suppressor activity, but our cell transfer experiments do not seem to support this idea. This will be investigated in future studies. Our results obtained with $IL10^{-/-}$ $CD4^+CD25^+$ regulatory T cells seem to contradict previously published results (Annacker et al., 2001), ascribing the suppressor activity of these cells to IL10 production. As referred, it has been shown that IL-10 or TGFβ mediates the regulatory functions of the $CD4^+CD25^+CD45RB^{low}$ T cells (Asano et al., 1996; Asseman et al., 1999; Groux et al., 1997; Papiernik et al., 1998; Powrie et al., 1996). We confirmed that the $CD25^+$ cells from $IL\text{-}10^{-/-}$ mice failed to protect against the wasting disease induced by the $CD4^+CD25^-CD45RB^{high}$ T cells (not shown), but our results show that these cells are not impaired in their suppressor activity. Thus, the capacity of the $CD4^+CD25^+CD45RB^{low}$ T cells to control IBD is likely a tissue-specific effect preventing intestinal inflammation through the local production of IL-10 (Asseman et al., 1999). Several other lines of evidence seclude disease protection from the control of peripheral T cell numbers: 1) Adult $IL\text{-}10^{-/-}$ mice fail to develop massive $CD4^+$ T cell proliferation before development of IBD (Kuhn et al., 1993); 2) $CD25^+CD4^+$ T cells from $IL\text{-}10^{-/-}$ mice suppress T cell proliferation after *in vitro* stimulation with anti-CD3 (Thornton and Shevach, 1998); 3) $CD25^-CD4^+CD45RB^{low}$ cells protect from IBD, but do not prevent peripheral expansion of naive $CD4^+$ T cells (Annacker et al., 2001 and fig.5). We suggest that two important regulatory functions of the $CD4^+CD25^+CD45RB^{low}$ T cell population are protection from IBD that has been shown to be IL-10 dependent (Asseman et al., 1999) and suppression of T cell expansion, which is IL-10 independent. Although TGFβ has been implicated in IBD protection, and recent claims suggest that it may play a role in T cell homeostasis (Gorelik and Flavell, 2000; Lucas et al., 2000; Nakamura et al., 2001) we found

that both naive and activated $CD4^+$ T cells expressed similar levels of mRNAs for the 3 different sub-forms of TGFβ.

The identification of the mechanism responsible for $CD4^+CD25^+$ regulation is still an open question. If some mechanisms have been suggested, they do not seem responsible for all of the proprieties of $CD4^+$ regulatory T cells, as seen with cytokines or as found with CTLA4. This could be due to differences in the regulatory T cell population studied or to some redundancy in the effector mechanisms for regulation and suppression. The data here reported could be dependent on redundancy of the different mechanisms operating in $CD4^+CD25^+$ mediated suppression. Alternatively, the difficulty in the identification of a consensual mechanism for these regulatory T cells could be due to the different proprieties investigated. In this study, we present data on the suppressive ability of the $CD4^+CD25^+$ regulatory T cell population *in vivo*, building a bridge between the *in vivo* and *in vitro* described proprieties of these cells. It can be that soluble molecules mediate a regulatory action of these regulatory T cells but that the suppressive proprieties are dependent on a cell-contact dependent mechanism. A segregation between the suppressive and the regulatory proprieties of these cells is what we have found for the role of IL10. Further studies should contribute to elucidate these issues. We provide here information on some putative mechanisms. Others are being investigated.

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SECTION C

DISCUSSION

How are peripheral T cell numbers maintained stable? Which are the basic components of peripheral T cell homeostasis? How is peripheral homeostasis conciliated with the basic function of the immune system and of its components?

The mechanisms and processes increasing or decreasing peripheral T cell numbers are many times both source of stability and source of perturbation, as these mechanisms and processes act towards to or away from the equilibrium point. One of the purposes of the study of homeostasis is to understand the mechanisms responsible for the control of peripheral T cell number in order to restore equilibrium. However, to restore homeostasis may be a too vague notion. Homeostasis is a characteristic of a given system, and it can be achieved at different levels of equilibrium. Thus, an immunodeficient individual may have peripheral T cell numbers under homeostatic control, but at too low values to attain immunocompetence levels. The same way, immunocompetence does not depend only on the quantitative composition of the peripheral T cell pools; it is also dependent on the presence of different classes and sub-populations of T cells. Thus, homeostasis has not only a quantitative component. It has also a qualitative component. Another aspect, of T cell homeostasis in particular, is the differential contribution of the thymus in discrete phases of the life of an organism. We know that the thymus does not contribute the same way in the neonatal, young and adult ages.

To make advances in the ambitious task of answering the questions posed above, the peripheral T cell homeostasis problem must be divided into smaller and manageable problems. In this section, I will discuss the implications of the results described in section B for peripheral T cell homeostasis, and in particular, peripheral $CD4^{+}$ T cell homeostasis, having in mind quantitative, qualitative and age-dependent aspects of peripheral T cell homeostasis.

The discussion will be divided in five independent units:

Chapter 10-Homeostasis within the thymus.

Chapter 11-The role of the thymus and thymic export for peripheral T cell homeostasis.

Chapter 12- Homeostasis through sub-population structure: The role of $CD4^{+}CD25^{+}$ T cells.

Chapter 13-The role of cytokines in the establishment of the observed sub-population structure; IL2 and $CD4^{+}CD25^{+}$ regulatory T cells.

In chapter 14, a summary of the general conclusions of this study will try to connect the dots and give a general view of peripheral T cell homeostasis.

In the final chapter of this discussion (chapter 15) implications for the human case will be briefly discussed.

10- HOMEOSTASIS WITHIN THE THYMUS

As seen in the introduction section (chapter 4), the existence of homeostasis within the thymus had not been investigated in detail. We have investigated this, as the extensive proliferation that occurs at several steps during the thymic developmental processes (see chapter 3) could be adjusted by homeostatic restraints or drives.

10.1- Thymic colonization

Thymic colonization by BM precursors is limited by a requirement for minimal numbers of precursors and also by the existence of finite niches for colonization by BM precursors. It has been shown that there is a minimal requirement for BM precursors in order to reconstitute a normal thymic size, as i.v. reconstitution of irradiated hosts by BM cells was not attained below a given number of BM cells (Scollay et al., 1986). However, the minimal numbers of BM cells required are not the definitive information, as the proportion of T cell precursors available in the BM cell suspensions inoculated may vary and the commitment to the T cell lineages may occur in the thymus (reviewed in Akashi et al., 2000). It has also been suggested that the thymic colonization by T cell precursors occurs in waves and that a feedback control operates, reducing the numbers of precursors in situations where the thymus is refractory to precursor seeding (Foss et al., 2001). If the possibility of niche occupancy by precursors and consequent difficulty to integrate the thymic developmental pathway by new precursors is arguable, the feedback control of production of putative T cell precursors seems unlikely. If that would be the case, the ability to reconstitute the thymus of an irradiated host mouse should be dependent to some degree on the precise age of the donor mouse. There are no reports of such situation and my own experience does not

support this notion. In any case, the thymus does need a life long supply of precursors from BM origin, as shown by the transient reconstitution by thymus-derived precursors or by BM precursors injected intra-thymically (Scollay et al., 1986).

As for the existence of an upper limit for thymic colonization, this seems to be supported by the studies performed in hyperthymic mice. It has been shown that young or old mice grafted with several thymic lobes under the kidney capsule are capable to generate a thymic mass that largely exceeds the thymic mass of these host mice before grafting (Metcalf, 1965b). This suggests that the precursors available are in excess to the ability of the thymic tissue to incorporate or to expand them. Thus, the incorporation of BM derived precursors in the thymic developmental pathway seems to be under control, with lower and upper limits. However, this does not mean that the thymus cellularity is under homeostatic control, as the initial stages of thymic development are a very small proportion of the total thymocyte number.

10.2- Homeostasis during thymic development

To investigate the existence of compensatory mechanisms regulating thymocyte numbers, we have developed an experimental system (Almeida et al., 2001) (article #1), relying on dilution of competent precursors in precursors from donors with developmental blocks at discrete stages of T cell development, namely $Rag_2^{-/-}$ or $CD3\varepsilon^{-/-}$, whose precursors cannot proceed further than the DN3 stage (Malissen et al., 1995; Shinkai et al., 1992) or $TCR\alpha^{-/-}$ mice, whose precursors do not proceed from the DP to the SP thymic compartment (Mombaerts et al., 1992a). In this situation, we could observe whether the thymic cellularity was a function of the number of precursors colonizing it or whether other factors would control thymic cellularity, revealing homeostatic mechanisms that could operate to maintain thymocyte numbers in situations where the number of precursors was limited. Diluting B6Ly5.1 competent BM cells in $TCR\alpha^{-/-}$ BM cells allowed us to evaluate the possibility of the existence of homeostatic mechanisms controlling SP thymocyte number, as in this situation, the SP thymic compartment is only constituted by cells originated in the competent fraction of the total BM precursors given. When we performed BM chimeras reconstituting $Rag_2^{-/-}$ hosts with mixtures of BM precursors from competent B6Ly5.1 donors diluted in BM precursors from $CD3\varepsilon^{-/-}$ or $Rag_2^{-/-}$ donors, we could investigate the possibility of compensation mechanisms operating after the DN3 stage, when extensive proliferation occurs (Penit et al., 1995; Shortman et al., 1990).

Our results indicate that there is no homeostatic compensation in these intrathymic stages of development, as the number of DP thymocytes was found to be correlated linearly

with the number of DN cells whether in presence or absence of competitor cells (B6Ly5.1 vs. TCR$\alpha^{-/-}$ or B6Ly5.1vs CD3$\varepsilon^{-/-}$, respectively) (Almeida et al., 2001) and the situation was similar for the SP compartment, as the number of SP thymocytes was found to linearly correlated with the number of DP thymocytes in chimeras (B6Ly5.1 vs. TCR$\alpha^{-/-}$) where the SP thymic compartment is constituted only by thymocytes from competent B6Ly5.1 BM origin. These general conclusions confirm partially conclusions of another report (van Meerwijk et al., 1998), on the inexistence of homeostatic mechanisms at the DN to DP transition, though our study includes a larger spectre of competent precursor numbers. This larger spectre of the fraction of competent precursors, and the finer mathematical analysis performed, allowed us, at a detailed level, to distinguish at very low levels of competent precursors, a less than expected value for DP numbers. This could be due to a faster transition of DP to the next stage (SP), or could be due to limiting dilution effects, at very low levels of competent precursors.

Our results do not confirm the suggested homeostatic compensation for the CD8$^+$ SP thymocyte compartment (van Meerwijk et al., 1998). However, the suggested 1.5 fold compensation observed in the proportion of CD8$^+$ SP thymocytes may not represent a very significative increase and it is not clear how it correlates with absolute cell numbers in the study. The observed faster *de novo* generation of CD8$^+$ SP thymocytes in these chimeras post irradiation (van Meerwijk et al., 1998) could also be reflecting irradiation-derived phenomena. In our study we found a linear correlation between the number of DP and the number of SP thymocytes, both CD4$^+$ and CD8$^+$. When a detailed analysis was performed, we observed a higher efficiency of SP thymocyte generation (both CD4$^+$ and CD8$^+$) when the competent BM fraction was very low. The possible faster generation of SP thymocytes in this situation could also explain the lower than expected values for DP thymocyte numbers observed. When analysed as a whole, our data do not support the existence of homeostasis or homeostasis driven processes during thymic development. Our observation that the DP thymocyte number is 40 fold the number of DN thymocytes fits with the known proportions of DP (80%) compared to the known proportion (2%) of DN (or TN, numbers were calculated for CD4$^-$CD8$^-$CD3$^-$ thymocytes) and the same for the SP compartments. If this situation does not seem surprising in view of the known short duration of the DP thymocyte stage (Egerton et al., 1990; Huesmann et al., 1991), it is relevant that all the selection processes occurring during these stages (see chapter 3) are not object of homeostasis driven processes. It is also relevant the suggestion inferred from our data, that the premigrant expansion phase (Ernst et al., 1995; Hare et al., 1998; Penit and Vasseur, 1997) is also not the target for homeostasis driven phenomenon.

The availability of selection “niches” for positive selection (Huesmann et al., 1991; Merkenschlager, 1996; Merkenschlager et al., 1994), can impose limits for the number of

thymocytes selected. As referred in chapter 4, when most of the DP thymocytes express a selectable transgenic TCR, the formation of mature SP cells is 10 to 20 times more efficient as observed in normal mice. However, this means that only 20% of the DP thymocytes become mature (Huesmann et al., 1991). This is due to the limited availability of stromal cells (Merkenschlager, 1996; Merkenschlager et al., 1994) capable of mediating positive selection, as most DP thymocytes with a selectable transgenic TCR will undergo maturation when they represent only 5% or less of the total DP pool (Huesmann et al., 1991). If this is a mechanism that could be responsible for maintenance of thymocyte numbers (or of SP thymocyte numbers) is not clear, as the transgenic mice situation may be too far from the physiological condition, and this kind of competition can be extremely rare in the physiological situation. In our experimental system, we are not evaluating the mechanisms responsible for the existence of an upper limit for thymocyte level; we are investigating the existence of homeostasis seen as mechanisms able to counter a thymocyte deficiency. The existing data clearly suggest that limited niche availability is a mechanism that limits maximal thymocyte numbers. We would suggest that this mechanism is responsible for the upper limit of thymocyte numbers but that at most of thymocyte number range the number of thymocytes is determined by the number of precursors, possibly by the number of precursors that enter the thymic T cell developmental pathways.

10.3- Homeostasis in the aging thymus

As seen (chapter 5) the thymus involutes with age, what is reflected in thymocyte number and also in the numbers of thymic emigrants (Scollay et al., 1980). As referred above, this situation does not seem to be reflecting a reduced ability of the aged BM, as thymic lobes grafts under the kidney capsule of aged mice were repopulated (Metcalf, 1965b), but seems to be due to differences in thymic tissue. Thus, old recipients of BM transplantation from young origin were not able to regenerate thymocyte numbers to the same levels as young recipients of the same BM cells (Mackall et al., 1998). Importantly, when the thymocyte composition of old thymi was analysed, the thymocyte distribution in the DN, DP and SP $CD4^+$ and SP $CD8^+$ compartments was normal (Jamieson et al., 1999; Mackall et al., 1998). This suggests that the relationships that we observed may be maintained in the aged thymus.

10.4- Conclusion

To conclude, I would suggest that the thymocyte numbers are not under homeostatic control, and may be determined by the number of colonizing T cell precursors or by the numbers of BM derived precursors that are driven into the T cell developmental pathway already in the thymus. If the latter is the case, then the thymic tissue itself may be the major player determining thymocyte numbers. This seems to be supported by the studies concerning thymic aging.

The inexistence of homeostatic compensation mechanisms within the thymus, and the linear relationship observed between the DP and SP thymocyte numbers, allowed us to use this system to study the impact of a reduced thymic export in the composition of the peripheral compartments of mice of the same age.

11- THE ROLE OF THE THYMUS AND THYMIC EXPORT IN PERIPHERAL T CELL HOMEOSTASIS

Thymic export provides the colonizing cells of the peripheral T cell pools, but as the peripheral T cells are themselves able to proliferate and repopulate the periphery, the relative contribution of these two components of peripheral T cell homeostasis is an important issue for the understanding of the mechanims leading to the maintenance of peripheral T cell numbers.

Relying in our experimental system (Almeida et al., 2001), that allowed us to control the size of the SP thymocyte compartment, we have evaluated the dependency of the peripheral T cell pools on thymic export.

11.1- Quantitative aspects

The maintenance of peripheral T cell numbers throughout adult life and in aged individuals, when thymic mass is decreased and thymic export is reduced (Scollay et al., 1980) could be interpreted as an indication that thymic export is not relevant for the size of the peripheral T cell pools beyond an initial colonization phase. However, in the aging situation, the time factor may be determining an artificially constant size of the total peripheral T cell pools, masking a deficiency in some sub-populations of lymphocytes by an unrelated increase in the size of other sub-populations. In order to evaluate the impact of thymic export in the size

of the peripheral T cell pools, one must be able to control the amount of exported T cells into peripheral compartments of mice of the same age. This has been performed in the two extreme situations. The evaluation of the size of the peripheral T cell pools after thymectomy (Metcalf, 1965a; Miller, 1965; Rocha et al., 1983; Taylor, 1965), (thus thymic output is null) has shown that the peripheral T cell pool is 40% reduced following thymectomy (Rocha et al., 1983). The other extreme situation was assessed in the hyperthymic mice studies (thymus >1), following the impact of the augment of the thymic mass (by grafting thymic lobes under the thymic capsule) in the size of the peripheral T cell pools (Berzins et al., 1998; Berzins et al., 1999; Leuchars et al., 1978; Metcalf, 1965b). Three main conclusions can be drawn from these studies, first, that the size of the peripheral T cell pools is not a function of the number of thymuses present in the mouse (Berzins et al., 1998; Berzins et al., 1999; Leuchars et al., 1978); second, that the rates of thymic export by individual grafted lobes were independent of the number of thymuses grafted and may be independent of the degree of replenishment of the peripheral T cell pool (Berzins et al., 1998; Leuchars et al., 1978). Thus, there is no negative feedback control of the peripheral T cell pool over thymic export (Berzins et al., 1998; Leuchars et al., 1978; Tanchot and Rocha, 1997); third, that the peripheral T cell pool may increase in size, an increase that is proportional to the number of thymic lobes grafted (Berzins et al., 1998; Berzins et al., 1999; Leuchars et al., 1978).

We investigated (Almeida et al., 2001) how thymic export contributes to the peripheral T cell pools throughout a range of values of thymic export, in between the two extreme situations referred. First, we evaluated the number of RTEs found in the peripheral pools of mice reconstituted with 100% of BM precursors from competent B6Ly5.1 origin or with only a 10% fraction of competent BM. We found that thymic export was reduced when the size of the thymic SP compartment was reduced, but the reduction was not proportional. Thus, the efficiency of thymic export seemed to be increased or, alternatively, the incorporation of thymic emigrants could be more successful in mice with less competitor cells. This result seems to be conflicting with the conclusions drawn after antibody-mediated peripheral T cell depletion (Gabor et al., 1997). In this study, it was found that thymic export was not affected by a 70% reduction in peripheral T cell numbers. This reduction may not be enough to cause an increase in the rate of thymic export or in the incorporation of thymic emigrants in the peripheral T cell pools. We do not know also the subset distribution of the remaining 30% of peripheral T cell numbers; if these were constituted mainly by naïve T lymphocytes it is then normal that no differences were perceived. Also, the thymus of these antibody-treated mice could be at its maximal capacity for T cell generation and could be incapable of an increase in the efficiency of thymic export.

When we investigated the existence of a linear correlation between the numbers of SP thymocytes and the numbers of total $CD4^+$ or $CD8^+$ peripheral T cells we were unable to

fit the data to this assumption. Thus, the peripheral T cell numbers were similar in most of the chimeras, confirming the existence of homeostatic mechanisms contributing to the maintenance of peripheral CD4$^+$ or CD8$^+$ T cell numbers, others than thymic export. Even if the peripheral T cell numbers were found to be similar in most of the chimeras, an increased probability to find chimeras with reduced peripheral T cell numbers was found in the mice reconstituted with the lowest fraction of competent BM precursors, suggesting that the thymus does have a role, even if a limited one, in the maintenance of peripheral T cell numbers of mice with the same age. With the help of a simple mathematical model, we were able to measure the importance of these homeostatic mechanisms in the maintenance of peripheral T cell numbers; this allowed us to compare the relevance of homeostasis driven mechanisms for the size of different peripheral T cell compartments. Thus, we can state that the peripheral CD4$^+$ T cell pool is less dependent on thymic output than the peripheral CD8$^+$ T cell pool (article #1, figs. 3E and 3F).

Our experimental model does not allow us to identify the exact mechanisms involved. Besides the observed increase in the efficiency of thymic export, candidate mechanisms include an increase in survival, increased proliferation or both. Homeostatic proliferation (Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999) (see 8.4) is a good candidate, as in these chimeras the reduced thymic export should result in lymphopenia, even if transient. In accordance with a role of homeostatic proliferation, we found that mice with reduced SP thymic compartments had a higher proportion of activated/memory phenotype cells, suggesting previous activation or homeostasis driven proliferation of these cells (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999). This leads us to the qualitative role of thymic output.

11.2- Qualitative aspects

As seen in chapter 6 (6.2), the role of the thymus is better appreciated when the peripheral T cell compartment is considered not as a single pool of T cells but as being composed of different sub-populations of T cells, with different contributions for the immunocompetence of the individual. As we have seen in the introduction (chapter 7), the peripheral T cell pools seem to be organized in such a way that the naïve and activated/memory compartments have independent homeostatic regulation (Tanchot and Rocha, 1995; Tanchot and Rocha, 1998), a division that allows the maintenance of both pools. We have evaluated the thymic export dependency of these two separate compartments, for both the CD4$^+$ and the CD8$^+$ peripheral T cell compartments. Our results provided definitive evidence that the naïve T cell

pool is more dependent on thymic export than the activated/memory compartment (Almeida et al., 2001). This is observed as a more important contribution of the activated/memory compartment in any situation where thymic export is reduced, notoriously seen when the peripheral T cell pools are immunodeficient. Thus, the homeostatic compensation mechanisms that operate when thymic export is reduced tend to act primarily in the replenishment of the activated/memory T cell pool. This suggests a hierarchical organization of the peripheral T cell pools, favouring the activated/memory pool. Nonetheless, it is important to note that the naïve T cell pool was also shown to be the target for homeostasis driven mechanisms. Thus, it is the ability of these mechanisms to compensate the lack of thymus export that is impaired, in such a way that the probability of immunodeficiency is higher for the peripheral naïve T cell pool. This seems to be in accordance with a role for homeostatic proliferation in the maintenance of T cell numbers in the absence or reduction of thymic export, as homeostatic proliferation is associated with an upregulation of activation markers (Bender et al., 1999; Ernst et al., 1999; Kieper and Jameson, 1999), and has been described in the naïve compartment as well (Seddon et al., 2000), though it is not clear if homeostatic proliferation of naïve phenotype T cells represents a lag time for acquisition of activation markers. However, the inhibition of homeostatic proliferation by the presence of other cells imposes a limit for the relevance of homeostatic proliferation. Also, our data show that homeostatic proliferation is not sufficient to replenish the peripheral T cell pools of mice with a very reduced thymic export.

In some of the studies of hyperthymic mice (Berzins et al., 1998; Berzins et al., 1999), it was proposed that the RTEs were "exempt" of peripheral T cell homeostasis, for a period of 3 weeks (Berzins et al., 1999). Our own results suggest that rather than being exempt from peripheral homeostasis mechanisms, recent thymic migrants are likely to be strongly affected by mechanisms acting to counter lymphopenic conditions, engaging in homeostatic proliferation and losing their naïve phenotype as a result of it. This fact can be one of the major difficulties when restoring the peripheral T cell pools after T cell depletion. If the regeneration of the activated/memory pool may be possible, then the regeneration of the naïve T cell pool may be obstructed by homeostatic proliferation and reveal to be too dependent on the regeneration of a normal thymic function. The suggested escape from homeostasis mechanisms for a period of three weeks (Berzins et al., 1999) may represent the experimental system explored, an experimental system where the homeostatic equilibrium is attained at higher values of naïve T cells. Confirming the requirement for thymic output for the maintenance of peripheral diversity, we have shown in the chimeras with <1% of normal BM fraction that the repertoires resulting from the homeostatically driven mechanisms are probably restricted (Almeida et al., 2001), as it occurs as a result of peripheral expansion of T cells after transfer into immunodeficient mice (La Gruta et al.,

2000). Our observations on the naïve to activated/memory phenotype shift and on repertoire restriction seem to mimic the observations in aged individuals (Barrat et al., 1997; Mackall and Gress, 1997) suggesting that this experimental system may be used to explore the causes and consequences of aging in the immune system.

11.3- Conclusion

Thymic export is essential for the colonization of the peripheral T cell pools but it also plays an important role throughout life, supplying the naïve T cell pool with new T cells, and, consequently, with new specificities. Thus, the role of thymic export may not be essential for the avoidance of immunodeficiency (as seen by lymphocyte counts) but it may be important for immunocompetence, in particular after peripheral T cell depletion. In normal conditions, the hierarchical organization of the peripheral T cell pools and the separation of the homeostatic control of the naïve and the activated/memory T cells seem to provide the means to assure immunocompetence throughout most of the life span of the organism.

12- HOMEOSTASIS THROUGH SUB-POPULATION STRUCTURE: THE ROLE OF $CD4^+CD25^+$ T CELLS

The peripheral T cell pools contain smaller sub-populations of T cells, distinguishable by their cell-surface phenotype and by their functional characteristics (see 7.2). This distinguishes $CD4^+$ and $CD8^+$ T cell pools and, inside each of these, naïve, effector and memory compartments (see 7.2). However, the large amount of cell-surface markers available would allow us to define an extremely larger number of sub-populations. We have shown (Almeida et al., 2002) that the $CD4^+CD25^+$ regulatory T cells are an essential sub-population for the maintenance of peripheral T cell numbers and for the maintenance of the normal proportion of the naïve and activated/memory T cell sub-populations. Thus, I would suggest the inclusion of another basic compartment in the peripheral $CD4^+$ T cell pool: the $CD4^+CD25^+$ T cell sub-population, comprising approximately 10% of the peripheral $CD4^+$ T cells (fig. 12).

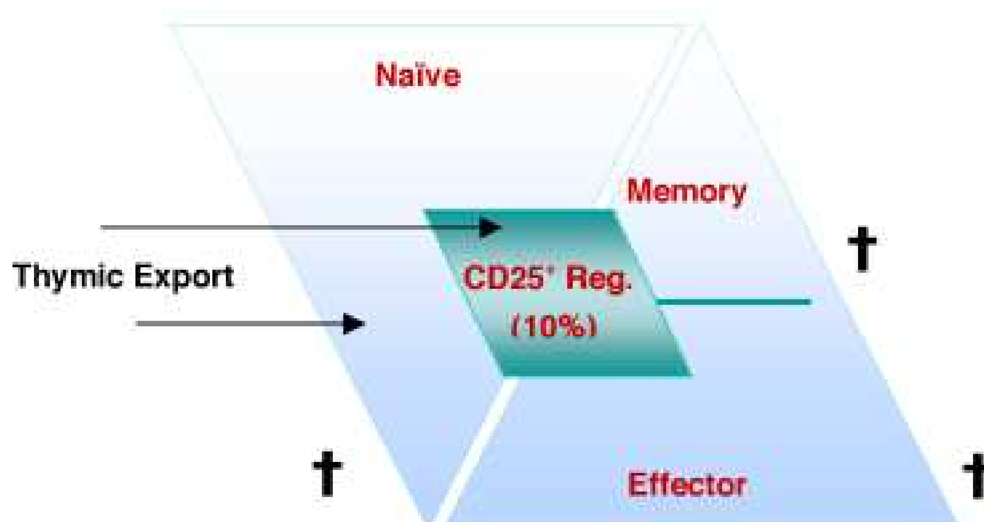

Figure 12: The peripheral CD4+ T cell pool. The figure represents a suggestion for the representation of peripheral CD4+ T cell sub-population structure. The CD4+CD25+ regulatory T cells occupy a central position, influencing the size of the other sub-populations. It is still not clear if naïve and activated pools should be considered inside the regulatory CD4+CD25+ T cell pool. †: Output from the respective T cell pools due to cell death

12.1- CD4+CD25+ regulatory T cells are a specific lineage of CD4+ T cells

The CD25 marker (cluster designation for the IL2Rα chain) (Nelson and Willerford, 1998) defines a sub-population of CD4+ T cells that represents 5-10% of the peripheral CD4+ T cell pool, and this proportion is conserved from mice (Papiernik et al., 1998; Sakaguchi et al., 1995) to humans (Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Taams et al., 2001). We have shown (Almeida et al., 2002) that the presence or absence of this subpopulation has crucial relevance for peripheral homeostasis. Thus, in the CD25-/- (Willerford et al., 1995) and in the IL2-/- (Schorle et al., 1991) the CD4+CD25+ T cells are absent (Willerford et al., 1995) or severely reduced (Papiernik et al., 1998) and, as a result of this (Almeida et al., 2002), the peripheral T cell pools display an uncontrolled activation, resulting in the absence of homeostasis and in a large number of autoimmune manifestations, often with a fatal outcome (Sadlack et al., 1995; Sadlack et al., 1993; Willerford et al., 1995). This had been first explained by an intrinsic defect of the CD25-/- or IL2-/- cells, as IL2 and IL2 signalling are involved in AICD processes (Van Parijs et al., 1997; Van Parijs et al., 1999). Later studies have suggested that AICD mechanisms could be intact in CD25-/- cells and that IL2R mediated signals were involved in the control of bystander

proliferation (Leung et al., 2000), while the phenotype of the IL2$^{-/-}$ CD4^{+} T cells could be abrogated by the presence of normal BM derived cells (Kramer et al., 1995). Our study suggests that the most striking features of the IL2$^{-/-}$ and the IL2R$\alpha^{-/-}$ mice are not directly derived from a role of IL2 and IL2R mediated signals in the control of the magnitude of responses, but rather derived from the requirement by a specific lineage of regulatory T cells of IL2R expression and IL2 signalling for generation and survival (Almeida et al., 2002; Wolf et al., 2001). Thus, the expression of the IL2Rα chain (CD25) is particularly relevant as it correlates specifically with responsiveness to IL2 (Nelson and Willerford, 1998). This places the IL2-IL2Rα axis in the center of these phenomena and rules out the influence of the other γc dependent cytokines. The lineage specificity of the CD4^{+}CD25^{+} regulatory T cells is also suggested by the fact that induction of CD25 expression by *in vitro* activation of CD4^{+}CD25^{-} T cells does not generate cells with suppressive activity (Suri-Payer et al., 1998) and by our own observation that only cells retaining CD25 expression after transfer into immunodeficient hosts maintain suppressive abilities *in vivo* (Almeida et al., 2002). Finally, the lineage specificity of the CD4^{+}CD25^{+} regulatory T cell sub-population is further suggested by data suggesting that these cells are generated as such in the thymus (Bensinger et al., 2001; Itoh et al., 1999; Jordan et al., 2001; Papiernik et al., 1998). This may reflect an independent developmental pathway for CD4^{+}CD25^{+} regulatory T cells, which may not be generated from the DP thymocyte subset (Papiernik et al., 1998). After reaching the periphery, however, these cells seem to be long-lived or to have self-renewing abilities, as we show by rescuing for extremely long periods (>1 year) CD25$^{-/-}$ BM chimeras with a single transfer of 10^{5} CD4^{+}CD25^{+} regulatory T cells (Almeida et al., 2002). The major objection to the CD4^{+}CD25^{+} regulatory T cell as a specific lineage of CD4^{+} T cells lies on the for long known association of CD25 expression with T cell activation (Nelson and Willerford, 1998). This implies that in the referred 10% of peripheral CD4^{+} T cell that express this marker, a contaminating population of activated non-regulatory CD4^{+} T cells is always present (Maloy and Powrie, 2001). Our observation that whenever the CD4^{+}CD25^{+} regulatory T cells are present in the transferred inoculums, the expression of the CD25^{+} by the CD4^{+}CD25^{-} originated T cells is from residual to null (1-2%), though all cells display an activated phenotype (as seen by downregulation of the CD45RB marker) seems to suggest that these contaminating CD25^{+} non-regulatory T cells do not represent an important proportion of the total CD4^{+}CD25^{+} T cells usually present in the peripheral CD4^{+} T cell pools. Thus, the observed 10% of peripheral CD4^{+}CD25^{+} T cells may closely indicate the proportion of the CD4^{+} T cells from the regulatory lineage essential for the maintenance of peripheral T cell homeostasis at the levels observed.

12.2- $CD4^+CD25^+$ regulatory T cells may maintain peripheral T cell numbers at the established levels

The proprieties of the $CD4^+CD25^+$ regulatory T cells had been characterized in several experimental systems, relying in the ability to abrogate autoimmune disease developing after transfer of naïve $CD4^+$ T cells into immunodeficient hosts (Asano et al., 1996; Powrie et al., 1996; Powrie et al., 1994b; Sakaguchi et al., 1995) or on the ability of the $CD4^+CD25^+$ regulatory T cells to suppress expansion of $CD4^+CD25^-$ T cells *in vitro* (Takahashi et al., 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000). Thus, the consequences for peripheral T cell homeostasis had not been evaluated *in vivo*, but the readout for *in vitro* regulatory T cell activity was based on the ability to suppress expansion (proliferation) of naïve $CD4^+$ T cells. We have investigated the consequences for peripheral T cell homeostasis of the $CD25^+/CD25^-$ $CD4^+$ T cell interaction, relying on cell count readout for $CD4^+CD25^+$ regulatory T cell activity *in vivo* (Almeida et al., 2002). Thus, as seen above, the $CD4^+CD25^+$ regulatory T cells were able to regenerate normal T cell homeostasis in the two mutant mouse models referred; these cells are able to control peripheral T cell numbers. We have investigated the magnitude of the control of peripheral T cell expansion by $CD25^+$ regulatory $CD4^+$ T cells, performing a number of cell transfer experiments into immunodeficient $CD3\varepsilon^{-/-}$ mice. By varying the $25^+/25^-$ ratio in the transferred cells, we were able to verify that the control of $CD25^-$ T cell expansion by $CD4^+CD25^+$ regulatory T cells is not reflected in just a controlled *versus* uncontrolled outcome. We observed that the $CD4^+CD25^+$ regulatory T cells were able to control expansion in a dose-dependent manner. Also, by maintaining the ratios but varying the cell numbers transferred, we were able to show that the size of the controlled peripheral $CD4^+$ T cell pool thus obtained is a function of the initial ratios transferred (Almeida et al., 2002). Even if the highest suppression was observed at a regulatory to non-regulatory ratio of 10 to 1, that seems to be far from the physiological 1 to 9 ratio, the fact that the cell numbers recovered in our experimental system were a function of the ratios transferred is relevant and suggests that the size of the peripheral T cell pool is dependent on the $CD25^+$ regulatory $CD4^+$ T cell/ $CD25^-$ non-regulatory $CD4^+$ T cell ratio. The discrepancy found on the ratios transferred, compared to what is known to be the physiological ratio, may derive from differences in the homing capacity of the two sub-populations upon cell transfer, as reported on transfers into $Rag_2^{-/-}$ hosts (Annacker et al., 2001) and our own indications upon transfer into $CD3\varepsilon^{-/-}$ hosts (not shown). Thus, the ratios obtained after cell transfer probably do not reflect the injected ratios but are proportionally lower (even if the dose varies according to the injected ratio). The fact that the recovered cell number depends on the injected ratio, suggests that there is a direct

interaction between the two sub-populations, and also suggests that we can recover peripheral $CD4^+$ T cell pools homeostatically controlled at a specific wanted level, if we modulate correctly the injected $25^+/25^-$ $CD4^+$ T cell ratio. This provides data to support the notion that the levels at which peripheral $CD4^+$ T cell homeostasis is attained are related to the fraction of $CD4^+CD25^+$ regulatory T cells present. These cells may act either by preventing peripheral naïve $CD4^+$ T cells to enter the activated/memory pool, as shown by the reconstitution of the normal composition of the peripheral $CD4^+$ T cell pool in the $CD25^{-/-}$ BM chimeras to which the $CD4^+CD25^+$ regulatory T cells were given (Almeida et al., 2002) either by directly controlling the size of the activated/memory compartment, as seen in our cell transfer experiments (Almeida et al., 2002) and suggested by others, using $CD4^+CD45RB^{low}$T cells (Annacker et al., 2000).

12.3- Mechanism of action of $CD4^+CD25^+$ regulatory T cells

The mechanisms responsible for homeostatic regulation may be the same or may differ from the mechanisms responsible for the regulatory role of $CD4^+CD25^+$ T cells in autoimmune or other immune responses. As referred (see additional data and discussion herein), the described mechanisms responsible for *in vitro* suppression by $CD4^+CD25^+$ regulatory T cells of $CD4^+CD25^-$ naïve T cell expansion and the mechanisms responsible for *in vivo* abrogation of autoimmune manifestations after transfer of naïve or $CD25^+$ depleted $CD4^+$ T cell populations differ, as seems to be indicated by the identified role of cytokines in the *in vivo* studies of autoimmune responses regulation by $CD4^+CD25^+$ regulatory T cells and the ruling out of the same cytokines in the *in vitro* studies of suppression. Thus it may be wise to refer to suppressor and regulatory activities of $CD4^+CD25^+$ T cells, or, alternatively, to refer to the $CD4^+CD25^+$ T cell population as suppressor $CD4^+$ T cells instead of regulatory $CD4^+$ T cells, as suggested (Shevach, 2002).

From our failure to identify the mechanisms responsible for suppressive activity by $CD4^+CD25^+$ regulatory T cells *in vivo* (Section B, additional data), and from the difficulties in the identification of a mechanism responsible for all of the described proprieties of the $CD4^+CD25^+$ regulatory T cells (Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2002), I tend to conclude that the regulatory T cells may chose from a number of effector mechanisms, that probably include cell-contact dependent mechanisms of suppression of expansion and cytokine mediated effects on the control of immune responses. This can be due to the action of a multipotent population of $CD4^+CD25^+$ T cells or to the differentiated action of smaller subpopulations that we are still not able to distinguish by cell-surface

markers. In simple terms, considering the $CD4^+CD25^-$ T cells as the equivalent of a prey with a large reproductive ability (Rabbit), the $CD4^+CD25^+$ regulatory T cells may be the equivalent to one specific predator, able to kill by a number of different mechanisms (Man) or to the more vast category of "predators", including many species killing by different specific mechanisms (Wolf, Owl, Snake, etc). Thus, the search for the mechanism(s) responsible for $CD4^+CD25^+$ regulatory T cell action continues, and final answers concerning the mechanism of cell-contact dependent suppression, the role of secreted cytokines and the importance of the APC in the process are still due (Maloy and Powrie, 2001; Shevach, 2002).

12.4- Concluding remarks

We could say that due to the independent homeostatic regulation of the naïve and memory pools (Tanchot and Rocha, 1995; Tanchot and Rocha, 1998), the two cell types do not compete, being allowed to persist and providing the immune system with the ability to deal with new antigens and with the ability to mount efficient secondary responses. Thus, the observed peripheral sub-population structure is the result of the separation of the homeostatic control of different sub-populations. The $CD4^+CD25^+$ regulatory T cell sub-population suggests that the peripheral T cell homeostasis may also be the result of the peripheral sub-population structure, as these cells regulate the size of the naïve and activated/memory $CD4^+$ T cell pools. We have shown that the presence or absence of this particular minor $CD4^+$ T cell sub-population is the difference between uncontrolled T cell homeostasis, with accumulation of large numbers of activated T cells and massive reduction of the peripheral naïve T cell pool and a peripheral T cell pool composed by the normal number of T cells, and the normal fractions of naïve and activated/memory $CD4^+$ T cells (Almeida et al., 2002). We have also shown that these $CD4^+CD25^+$ regulatory T cells are able to control the expansion of cotransferred $CD4^+CD25^-$ T cells, in a dose-dependent manner. In this cell transfer system, all the originally naïve $CD4^+$ T cells become activated, thus the resulting peripheral $CD4^+$ T cell pools do not contain a naïve T cell pool. In this situation, thymic output is absent (host mice are $CD3\varepsilon^{-/-}$), and the mice are lymphopenic, what raises the possibility that homeostatic proliferation may play a role in the expansion of the transferred $CD4^+CD25^-$ T cells. In support of this, it has recently been suggested that homeostatic proliferation plays a role in triggering the expansion of the remaining $CD4^+$ T cells, after $CD4^+CD25^+$ T cell depletion (McHugh and Shevach, 2002). This finding should be taken into account when analysing data obtained after transfer of separated $CD4^+$ T cells into immunodeficient hosts.

Our (and similar data from others) (Annacker et al., 2001; McHugh and Shevach, 2002) results obtained after transfer of CFSE stained $CD4^{+}CD25^{-}CD45RB^{high}$ sorted T cells into $CD3\varepsilon^{-/-}$ hosts, with or without cotransferred $CD4^{+}CD25^{+}$ T cells, do not suggest that suppression of expansion is due to complete block of cell cycle of the $CD4^{+}CD25^{-}$ T cells. Thus, homeostatic proliferation may still be occurring in presence of $CD4^{+}CD25^{+}$ regulatory T cells. However, these cells can still be limiting the extent of homeostatic proliferation or the survival of the newly generated T cells. Altogether, these reflections lead us to consider two sides of the regulatory T cell action. On the one hand, we may consider that the regulatory effects of these cells in the control of autoimmune responses reflect a broader role of these cells in the control of peripheral $CD4^{+}$ T cell numbers. By controlling the expansion of all peripheral naïve T cells, these cells would control the expansion of autoimmune clones within, as seen in the $CD25^{-/-}$ BM chimeras or in the cotransfer system. On the other hand, it must be considered that the control of ongoing autoimmune responses by the $CD4^{+}CD25^{+}$ regulatory T cells will also be reflected in the cell numbers recovered. Thus, the $CD4^{+}CD25^{+}$ regulatory T cells occupy a key positioning in the peripheral sub-population structure of the $CD4^{+}$ T cell pool, and the size of the $CD4^{+}CD25^{+}$ regulatory T cell pool is also probably relevant to the verified equilibrium levels of $CD4^{+}$ T cell homeostasis. Thus, the mechanisms responsible for the differentiation of this specific minor $CD4^{+}$ T cell pool will be responsible for the observed sub-population structure and consequent normal homeostasis, which we observe in the normal peripheral $CD4^{+}$ T cell pools. What can be these mechanisms?

13- THE ROLE OF CYTOKINES IN THE ESTABLISHMENT OF THE PERIPHERAL $CD4^{+}$ T CELL SUB-POPULATION STRUCTURE; IL2 AND $CD4^{+}CD25^{+}$ REGULATORY T CELLS

One of the most relevant T cell interactions for peripheral T cell homeostasis is competition (Freitas and Rocha, 2000). T lymphocytes may compete for a number of putative resources and competition will result in modulation of population sizes (Begon et al., 1990). Thus, resource availability and exploitation take part in the establishment of the observed peripheral sub-population structure.

13.1-Cytokines are resources and receptor expression defines exploitable resources and T cell niche.

In the identified resources category, we should include MHC molecules (Brocker, 1997; Kirberg et al., 1997; Tanchot et al., 1997), antigen (McLean et al., 1997; Smith et al., 2000), and cytokines (Ku et al., 2000; Lantz et al., 2000). Thus, different requirements for MHC ligands were shown for peripheral naïve and memory T cells (Tanchot et al., 1997) and similar findings were reported regarding differential requirements for naïve and memory $CD4^+$ T cells in γc dependent signalling (Lantz et al., 2000). The observed peripheral sub-population structure may be the result of niche differentiation of the activated/memory and naïve T cells. This would allow the persistence of the two sub-populations (Freitas and Rocha, 2000), important for the integrity of the main functions of the immune system (chapter 7). Thus, when particular sub-populations of T cells are distinguished by the expression of particular receptors, this may or not be related with the engagement in niche differentiation, as the signals perceived are or not involved in the survival or proliferation of the cell. As seen in the introduction section (8.6), cytokines using the γc receptor chain have been involved in survival and homeostasis of peripheral T lymphocytes. The fact that the $CD4^+CD25^+$ regulatory T cells have as distinctive phenotype the expression of the α chain of the IL2R, responsible for the assemblage of the high affinity receptor for IL2 (Nelson and Willerford, 1998) suggested to us that IL2 could be important for the development or survival of these cells.

13.2- IL2 and $CD4^+CD25^+$ regulatory T cells

As referred above (chapter 12) we have found that $CD4^+CD25^+$ regulatory T cell presence was essential for the maintenance of peripheral T cell homeostasis, as shown by their ability to regulate the peripheral composition and size of the $CD4^+$ T cell pools in the $CD25^{-/-}$ BM chimeras (Almeida et al., 2002). As the $IL2^{-/-}$ mice develop a lymphoproliferative syndrome with many shared features with the phenotype of the $CD25^{-/-}$ mice (Sadlack et al., 1995; Sadlack et al., 1993; Willerford et al., 1995), we wondered if the cause would be the same, namely, the absence of an essential sub-population of $CD4^+CD25^+$ regulatory T cells. Indeed, the $IL2^{-/-}$ mice were known to lack peripheral $CD4^+CD25^+$ T cells (Papiernik et al., 1998), though the significance of this observation could be questioned by the need of IL2 for upregulation of the IL2Rα chain (Nelson and Willerford, 1998). IL2 could either be necessary for the development of the $CD4^+CD25^+$ regulatory T cells, for their survival in the peripheral

pools or for both. By sorting the few (1-3%) of $CD4^+CD25^+$ T cells present in the peripheral pool of $IL2^{-/-}$ mice, we were able to demonstrate that contrary to what had been suggested (Wolf et al., 2001), $IL2^{-/-}$ mice can generate these cells, thus their reduced peripheral number most probably reflects impaired survival of these cells in the peripheral pools. We have provided more evidence for this in our mixed BM chimera system (Almeida et al., 2002) by demonstrating that mixed BM cells from $CD25^{-/-}$ and $IL2^{-/-}$ donors are able to generate a normally sized $CD4^+CD25^+$ regulatory T cell compartment, that could only have developed from the $IL2^{-/-}$ donor BM precursors. The need for IL2 for the survival of these cells and the inability of the $CD4^+CD25^+$ regulatory T cells to produce IL2 (Thornton and Shevach, 1998), suggest that IL2 produced by expanding $CD4^+CD25^-$ naïve T cells would contribute to their own regulation, providing a feedback mechanism for the control of some immune responses. It could also be the reason for our results on the dependency on the initial ratios in the cell transfer experiments (Almeida et al., 2002). Also, the lack of IL2 in host mice where $CD4^+CD25^+$ regulatory T cells were “parked” could partially explain their impaired suppressor ability, while large amounts of IL2 produced by the expanding $CD4^+CD25^-$ T cells in mice where $CD25^-$ were parked, would explain the excellent suppressive capacity of $CD4^+CD25^+$ T cells transferred at 7 weeks (Almeida et al., 2002). Thus, IL2 seems to be a resource with particular relevance for this particular sub-population of $CD4^+$ T cells, and IL2 could be produced by expanding $CD4^+$ T cells, or by other cells present (Granucci et al., 2001). It is yet to be found if the amount of IL2 present in the peripheral pools correlates with the size of the $CD4^+CD25^+$ regulatory T cell subpopulation or if it is just a matter of presence or absence, as it is yet to be clarified what is the fraction of peripheral $CD4^+CD25^+$ T cells that does not belong to the regulatory lineage. I would here suggest that one way to conserve a constant sub-population of regulatory T cells would be to index their numbers to the size of the peripheral IL2 producing activated T cell pool, providing a self-regulating system that would allow and require the presence of an activated T cell pool, controlled at a specific size.

13.3- Concluding remarks

The fact that one specific sub-population of $CD4^+$ T cells (the $CD4^+CD25^+$ regulatory T cells) depends on a specific cytokine (IL2) for survival and the strong correlation of this specific lineage of $CD4^+$ T cells with the expression of the receptor for this cytokine, seems to suggest that this population has developed to explore a specific niche. It is of interest that in the $CD25^{-/-}$/B6Ly5.1 mixed BM chimeras where the representation of the normal B6Ly5.1 was smaller (5% of normal B6Ly5.1 diluted in 50% $CD25^{-/-}$ and 45% $TCR\alpha^{-/-}$) we observed a

small but consistent competitive advantage only in the peripheral $CD4^+$ T cell compartment of the normal B6Ly5.1 origin $CD4^+$ T cells. This competitive advantage of the normal B6Ly5.1 $CD4^+$ T cells could be explained by the ability to differentiate a specific sub-population of $CD4^+$ T cells, expressing the CD25 receptor, and thus able to explore a niche barren to the $CD25^{-/-}$ $CD4^+$ T cells. This illustrates the role of resources in the establishment of peripheral T cell sub-population structure and homeostasis.

That the size of this niche is dependent on the presence of other cells producing the cytokine and that this cytokine is also involved in their own growth, seems to suggest, more than a predator prey interaction, a parasitic kind of interaction. Unfortunately, this reasoning may be too simplistic, as $CD4^+CD25^+$ T cells have been shown *in vitro* not to suppress $CD4^+CD25^-$ T cell expansion by consumption of IL2, but rather seem to inhibit IL2 production at the transcriptional level (Thornton and Shevach, 1998).

Taking this case as an example it should be investigated if other sub-populations exist that rely on some specific resources (cytokines) for their survival as a possible strategy for the identification of other sub-populations with a relevant role in homeostasis or in other processes involved in the immune system physiology or function.

14-GENERAL CONCLUSIONS AND DISCUSSION

Basic components of peripheral T cell homeostasis are processes that contribute continuously to input, output or equilibrium in the peripheral T cell pools. As it is maintained throughout the life span of the individual (Scollay et al., 1980), thymic output is a basic component of peripheral T cell homeostasis. As it varies with age, it is a component whose relevance also varies. We should assume that this brings consequences for the other components of peripheral T cell homeostasis. Thus, the influence degree of other components of peripheral T cell homeostasis, like peripheral cell division, may be affected by the variation of thymic output, corresponding to a larger fraction of the daily input of T cells into the peripheral T cell pools in the aged situation, where thymic output is reduced. As the peripheral T cell pools are not equal in their contribution for the immune system’s functions, the fact that different components of peripheral T cell homeostasis contribute differently for discrete sub-populations of T cells is relevant to the immunocompetence of the individual. Thus, if naïve and memory T cell compartments are considered separately, we have found evidence to establish that thymic output is of higher relevance for the naïve than to the memory T cell compartment (Almeida et al., 2001). When combined with the known tendency of cells originated by peripheral division to integrate the peripheral T cell pool of cells with an

activated/memory phenotype (Ernst et al., 1999; Kieper and Jameson, 1999; Mackall et al., 1993), we can conclude that the different contribution by different components of peripheral T cell homeostasis for the maintenance of the total peripheral T cell pool will result in the increase of the representation of the activated/memory phenotype pool, as has been described in the aged situation (Barrat et al., 1997; Ernst et al., 1990). It is still not clear how this is reflected in the immunocompetence of the individual, but it has been suggested that the cells found in the aged individuals have reduced functional responses (reviewed in Linton et al., 1997).

As the naïve and activated/memory T cell compartments have independent homeostatic regulation, these effects are attenuated. Thus, the diminishing thymic export in the adult situation will not be immediately translated into a higher representation of the activated/memory pools, unless a lymphopenic situation is generated. The first effects of the reduced thymic export will be probably reflected in the average life-span of the existing naïve T cells, due to a reduction in the number of competitor cells. However, as the time lapse increases, putative intrinsic limits for naïve T cell life span (Freitas and Rocha, 1993; Sprent, 1993; Sprent and Basten, 1973; von Boehmer and Hafen, 1993) and the increase in the probability of activation (Linton et al., 1996) will tend to cause a decrease in peripheral naïve T cell number. As the total peripheral T cell number is maintained constant, this decrease must be compensated by an increase in the size of the peripheral activated/memory T cell compartment (Barrat et al., 1997; Ernst et al., 1990). While the reduction of thymic export supplies a plausible cause for the reduction of the size of the peripheral naïve T cell pool, the reasons for the increase in size of the activated/memory T cell compartment are less clear. It could be that this increase is the reflex of changes in the environment, providing an increase in the niche size available for activated/memory T cells. It could also be that with time the activated/memory T cell sub-population could generate, due to competition processes for a limited niche, diverse sub-populations, allowing the exploitation of a vaster niche.

Thus, another basic component of peripheral T cell homeostasis is lymphocyte competition (Freitas and Rocha, 2000). The role of lymphocyte competition in the maintenance of the observed equilibrium probably bypasses the role of the intrinsic life span of a cell. Thus, the average life span of a given cell will be probably more affected by the presence or absence of other cells, as lymphocytes compete for survival and growth signals, than by their intrinsic life span. Another role of competition is the drive it provides for the diversification of sub-populations of specialized individuals (Schluter, 1994). This will be reflected in the peripheral organization of the mature T cell pools. The diversification of smaller sub-populations with specialized resource usage and immune proprieties opens the door for the existence of complex interactions between the different sub-populations of T

cells. These may include mutualism, parasitism and facilitation (Freitas and Rocha, 2000), and result in succession in specific microenvironments (Freitas and Rocha, 2000).

We have characterized one particular interaction between $CD4^+$ T cell subpopulations, with a major impact in peripheral $CD4^+$ T cell homeostasis and in the organization of the peripheral mature T cell pools. We suggest that the $CD4^+CD25^+$ regulatory T cells and their effects in the suppression of expansion of $CD4^+CD25^-$ naïve T cells are another basic component of the homeostasis of the peripheral $CD4^+$ T cell pool. By the demonstration that their presence is dependent upon the presence of IL2, and that the ability to differentiate minor sub-populations may be translated into competitive advantage, we demonstrate that receptor expression may be an ability for resource usage and allow niche exploitation and that this can be translated into the representation of given populations of T cells.

Thus, I would conclude suggesting that thymic output and peripheral T cell division are basic components of T cell homeostasis providing cell income, that competition for resources is another basic component of peripheral T cell homeostasis, limiting the contribution of each of the former and driving the differentiation of specific T cell sub-populations with different proprieties. These latter will build complex interactions that will provide a vast number of different drives away from the equilibrium point or regulatory feedback mechanisms and further contribute to the dynamic nature of the daily and microenvironment specific variations in peripheral T cell numbers. In the majority of cases, this will be translated into peripheral T cell homeostasis and immunocompetence.

15- IMPLICATIONS FOR THE HUMAN CASE

As referred, one of the purposes of the identification of the mechanisms responsible for T cell homeostasis, is to reconstitute normal peripheral T cell homeostasis in situations where its impairment results in deterioration in the immunocompetence or in the health condition of the individual.

15.1-The Thymus and peripheral T cell reconstitution

Although differences exist, most considerations made above apply to the human case. Thus, our study is relevant for the understanding of the aging situation in the human individual and for the protocols of immune reconstitution after irradiation procedures or subsequent to HIV

infection and treatment. In elderly humans, thymic output is also reduced but present (Douek et al., 1998; George and Ritter, 1996; Jamieson et al., 1999; Steinmann et al., 1985) and the peripheral T cell pools are also biased towards an activated/memory phenotype. The highest dependency of the peripheral naïve T cell pool on thymic output, and the prioritary replenishment of the activated/memory T cell pool may hinder attempts to reconstitute a sufficiently diverse peripheral T cell pool after T cell depletion. Besides, the suggestion from our analysis of thymus cellularity that thymic size may be a function of precursor establishment may imply that the strategy for the reconstitution of a “full-size” thymus may hinge on the identification of the factors involved in thymic seeding by BM derived precursors.

One of the distinctive features of HIV infection of the thymus is the premature atrophy of the organ (Haynes et al., 2000) and the hallmark of HIV infection is the reduction in $CD4^+$ T cell counts. The reduction in the $CD4^+$ T cell counts is accompanied by a rise in $CD8^+$ T cells numbers, a situation that leads to the reversion of the $CD4^+/CD8^+$ T cell ratios (Margolick and Donnenberg, 1997; Roederer et al., 1997). It had been suggested that this is a reflex of a phenomenon termed “blind T cell homeostasis” (Margolick and Donnenberg, 1997; Margolick et al., 1995). In short, it suggests that homeostatic processes acting to counter T cell depletion would do so recurring to both $CD4^+$ and $CD8^+$ T cell proliferation, and as in HIV infection $CD4^+$ T cells are selectively infected, this would result in the observed increased in the proportion of $CD8^+$ T cells. These studies are not always easy to interpret, as they rely on T cell blood counts, that represent a small fraction (around 2%) of the peripheral T cell numbers and selective trapping in the lymph nodes of $CD4^+$ T cells due to HIV infection, could bias blood results. It is not clear to what extent are the $CD4^+$ and $CD8^+$ T cell pools shared, but it may be relevant that in the described HIV situation, the majority of the peripheral T lymphocytes belong to the activated/memory pool. It could be that the highest overlap for $CD4^+$ and $CD8^+$ T cell populations occurs precisely in the activated/memory compartment. Together with the development of peripheral $CD4^-CD8^-$ T cells this would explain difficulties in the observation of an increase in peripheral $CD8^+$ T cell numbers in young $CD4^{-/-}$ mice or of an increase in peripheral $CD4^+$ T cell numbers in $CD8^{-/-}$ mice. In our study (Almeida et al., 2001) we observed that the peripheral $CD4^+/CD8^+$ ratio did not correlated neither with the size of the peripheral SP thymocyte compartment neither with the exported $CD4^+/CD8^+$ ratio, being slightly variable but independent of the degree of replenishment of the peripheral T cell pool, and consequently, of the representation of the activated/memory pool. In this case, the main interest of our observation lies on the implications for the reconstitution of the peripheral T cell pools after well succeeded control of the infection. The same will be verified for peripheral reconstitution by BM transplant,

following irradiation. Efficient reconstitution of the peripheral T cell pools may thus be dependent on the ability to increase to minimal values the numbers of thymic emigrants.

15.2- CD4$^+$CD25$^+$ regulatory T cells in Homeostasis, Autoimmunity and tumour immunotherapy

The identification of CD4$^+$CD25$^+$ regulatory T cells in humans is recent (Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Shevach, 2001), but it bears great interest, due to the large spectre and the nature of the processes that may be affected by their action. We have shown (Almeida et al., 2002) that these cells are major players in peripheral T cell homeostasis. Thus, in situations of pathogenesis linked to disruption of homeostatic mechanisms acting to limit maximal numbers of peripheral T cells, the integrity of the CD4$^+$CD25$^+$ peripheral T cell compartment should be investigated. Though less probably, our results also suggest the possibility that situations of immunodeficiency may be the result of a too high representation of CD4$^+$CD25$^+$ regulatory T cells in the peripheral T cell pools. Our observations also point to the importance of the regeneration of this particular sub-population of CD4$^+$ T cells when attempting to restore the peripheral T cell pools of immunodeficient individuals. Besides these considerations, the transposition from the known regulatory activity of this population in the control of autoimmune diseases assures intense investigation on these cells. The unveiling of the mechanisms responsible for regulatory and suppressor activities of these cells will provide important clues for the development of new tools and strategies in the treatment and prevention of autoimmune disease. For this, it is also important to understand what are the mechanisms responsible for their maintenance in the peripheral pools of the individual. We have provided evidence that strongly suggests a requirement for IL2 in peripheral survival of CD4$^+$CD25$^+$ regulatory T cells (Almeida et al., 2002).

Another aspect that has also been suggested from studies performed in mice, is the involvement of these CD4$^+$CD25$^+$ regulatory T cells in the suppression of antitumour responses. It has been shown that the depletion of the CD4$^+$CD25$^+$ regulatory T cells could enhance antitumour responses (Shimizu et al., 1999). Other regulatory T cells, namely the Tr cells (Groux et al., 1997; Groux and Powrie, 1999), which share some features with the CD4$^+$CD25$^+$ regulatory T cells have been successfully expanded *in vitro*, and similar strategies could be developed to attempt to expand the CD4$^+$CD25$^+$ T cells as well. For all this, the study of CD4$^+$CD25$^+$ will continue to interest a large community of investigators.

SECTION D

PERSPECTIVES

16- THE THYMUS

If some questions were close to being answered, namely about the possibility for homeostasis driven phenomena in thymic development, a number of additional questions are raised from our observations (Almeida et al., 2001). We have observed a tendency for the increase of the fraction of activated/memory phenotype cells in both the peripheral $CD4^+$ and $CD8^+$ T cell pools. What are the mechanisms responsible for these observations? The suggestion that homeostatic proliferation is the major contributor to this situation has not been formerly shown. BrDU or similar studies could provide further proof for increases in the fraction of proliferating cells in mice with reduced thymic exports.

The phenotype of the peripheral T cell pools obtained with the experimental system that we developed mimics the situation found in the aged situation. This system can thus be used to investigate the causes and consequences of aging in the immune system. It would be interesting to access if in our mice reconstituted with a low fraction of competent BM cells the functional characteristics of the peripheral lymphocytes found are impaired as it seems to be the case in the aged environment (Linton et al., 1997).

One important feature of the referred experimental system is the ability to measure the impact of thymic export in different peripheral T cell pools. We have used the system for the study of the impact of reduced thymic export in the naïve and activated/memory compartments. We will be using the same system to evaluate the possible impact of reduced thymic export in the size and representation of the peripheral $CD4^+CD25^+$ regulatory T cell pool. Our results seem to indicate that reconstitution by a single transfer of 10^5 $CD4^+CD25^+$ regulatory T cells 2 weeks after BM reconstitution is an efficient way to prevent development of lymphoid hyperplasia and disease in $CD25^{-/-}$ BM chimeras for large periods, but results were clearly less relevant when the same number of cells was given 4 weeks after BM reconstitution (Almeida et al., 2002). Also, our results with BM chimeras where the fraction of wt competent BM was 5% or 10% (Almeida et al., 2002) suggest a lower limit in the export of cells from the $CD4^+CD25^+$ regulatory T cell lineage needed to control the peripheral T cell pools. The investigation of the minimal numbers needed to seed the peripheral $CD4^+CD25^+$ T cell pool and of the extent of thymic independent pathways for regeneration and expansion of this subset can provide answers on the mechanisms responsible for the establishment of the peripheral $CD25^+/CD25^-$ equilibrium.

17- $CD4^+CD25^+$ REGULATORY T CELLS

By the identification of the $CD4^+CD25^+$ regulatory T cell pool as an essential peripheral $CD4^+$ T cell pool for peripheral T cell homeostasis, we advance in the understanding of the mechanisms responsible for peripheral $CD4^+$ T cell homeostasis. On the other hand, we raise a number of questions concerning the multitude of actions of these cells. If it is true that the described features of these cells *in vivo* and *in vitro*, (regarding their regulatory activities and their suppressive effects, respectively) may be reflected in the observations concerning cell numbers and homeostasis it may also be that those observations are the result of the action of these cells in the control of immune responses. It urges to identify the mechanisms by which the $CD4^+CD25^+$ regulatory T cells exert their functions and also to identify the drive for T cell expansion that is implicated in all different situations. Thus, in all the cell transfer systems reported, the possible involvement of homeostatic proliferation should be investigated, as it has been recently suggested that homeostatic proliferation is essential for the development of autoimmune manifestations after peripheral $CD4^+CD25^+$ T cell depletion (McHugh and Shevach, 2002).

Another issue left open lies on the complete understanding of the kinetics of the $CD25^+$/$CD25^-$ interaction. With our sequential and secondary cell transfer experiments, we were able to establish that the order, the lag time and the presence or absence of the reciprocal phenotype population had dramatic effects on the outcome, seen as cell numbers recovered or as autoimmune manifestations. It remains to be established if the homing capacity of the $CD25^+$cells is impaired the same way upon transfer to empty hosts or to hosts of a previous transfer of $CD25^-$ naïve $CD4^+$ T cells. It can be that due to the ongoing proliferation of the $CD25^-$ naïve cells more cells will home or maintain CD25 expression. The assessment of localization of these cells may prove to be extremely relevant for the understanding of the phenomena involved. The same way, the study of migration related molecules and receptors (chemokines, adhesion molecules, chemokine receptors) in these cells may also provide relevant data.

Upon co-transfer into immunodeficient hosts, we observed that $CD4^+CD25^+$ regulatory T cells were able to suppress the expansion of the co-transferred $CD25^-$ cells in a dose-dependent manner. Thus, we obtained mice with peripheral T cell pools of given sizes, dependent on the initial ratio transferred. These resulting $CD4^+$ T cell pools are constituted by $CD25^+$ and $CD25^-$ cells, and these cells are all non-naïve, as assessed by the CD45RB marker. The numbers obtained seem to be stable, but long-term kinetics need to be assessed to understand if these equilibrium values represent equilibrium values for an

ongoing interaction between the two populations present, or if they represent final stages of an initial interaction, that it is not ongoing at the recovering time points.

As in our sequential transfer protocol the recovered cells of an initial $CD25^+$ transfer are not all suppressive it should be addressed what are the differences occurring in this population, in particular, what are the differences occurring in this $CD25^+$ T cell population upon transfer alone into immunodeficient hosts or when transferred with naïve $CD4^+CD25^-$ T cells. It has been shown that the $CD25^+$ regulatory T cells need to be activated via their TCR in order to exert suppressor functions. It may also turn out that the $CD25^+$ regulatory T cell sub-population only differentiates into an effector regulatory stage in the presence of $CD25^-$ cells. Possible differences could be seen in the effector functions (seen at the cytokine level or cell-surface molecule expression) or in the tissue localization.

The survival requirements of the $CD4^+CD25^+$ T cells and the influence of IL2 in the survival and in the size of the peripheral $CD4^+CD25^+$ T cell pool need to be further described. If we have provided evidence to suggest that the presence/absence of IL2 is the difference between presence/absence of $CD4^+CD25^+$ regulatory T cells, the ultimate experiments to prove this statement will require the transfer of $IL2^{-/-}$ $CD4^+CD25^+$ regulatory T cells into $IL2^{-/-}$ T cell deficient hosts. The same way, in order to establish that the source of IL2 used by the $CD4^+CD25^+$ regulatory T cells is the $CD4^+CD25^-$ T cell population, we will use $CD25^{-/-}IL2^{-/-}$ BM as donor cells in $Rag^{-/-}$ or $Rag^{-/-}IL2^{-/-}$ hosts, in order to identify the origin of the IL2 required for $CD4^+CD25^+$ T cell survival. Once this is established, we should be able to vary the amount of IL2 present in order to investigate whether to an increase in the IL2 present corresponds an increase in the size of the peripheral $CD4^+CD25^+$ T cell pool.

Finally, the possible lineage specific effects on $CD4^+$ but not in the $CD8^+$ of the $CD4^+CD25^+$ that we have found have not been found in other recent *in vivo* (Murakami et al., 2002) or *in vitro* (Piccirillo and Shevach, 2001) studies. The reasons for this will be investigated.

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