UNIL | Université de Lausanne
Faculté de biologie
et de médecine

# CHARACTERIZATION OF $CD4^+$ $CD25^+$ T CELLS IN SOLID ORGAN TRANSPLANT RECIPIENTS

**Thèse**

**présentée à la Faculté de Biologie et de Médecine de**

**l'Université de Lausanne pour l'obtention**

**du grade de**

**docteur en médecine et ès sciences (MD-PhD)**

**par**

**Laure VALLOTTON**

Médecin diplômée de la Confédération Helvétique
Originaire de Aeschi/SO

**Jury**

Prof. Amalio Telenti, Président et Répondant de la commission MD-PhD
Prof. Giuseppe Pantaleo, Directeur de thèse
Prof. Pedro Romero, Expert
Dr. Jean Villard, Expert

**LAUSANNE**
**2009**

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

**Département de Médecine**
**Service d'Immunologie et d'Allergie**
**Directeur : Professeur Giuseppe Pantaleo**

# CHARACTERIZATION OF $CD4^+$ $CD25^+$ T CELLS IN SOLID ORGAN TRANSPLANT RECIPIENTS

**Thèse**

**présentée à la Faculté de Biologie et de Médecine de**

**l'Université de Lausanne pour l'obtention**

**du grade de**

**docteur en médecine et ès sciences (MD-PhD)**

**par**

**Laure VALLOTTON**

Médecin diplômée de la Confédération Helvétique
Originaire de Aeschi/SO

**Jury**

Prof. Amalio Telenti, Président et Répondant de la commission MD-PhD
Prof. Giuseppe Pantaleo, Directeur de thèse
Prof. Pedro Romero, Expert
Dr. Jean Villard, Expert

**LAUSANNE**
**2009**

UNIL | Université de Lausanne
Faculté de biologie et de médecine

**Ecole Doctorale**
**Doctorat MD-PhD**

# Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

| | | |
|---|---|---|
| ***Président*** | Monsieur Prof. | Amalio **Telenti** |
| ***Directeur de thèse*** | Monsieur Prof. | Giuseppe **Pantaleo** |
| ***Répondant MD-PhD*** | Monsieur Prof. | Amalio **Telenti** |
| ***Experts*** | Monsieur Prof. | Pedro **Romero** |
| | Monsieur Dr | Jean **Villard** |

le Conseil de Faculté autorise l'impression de la thèse de

**Madame Laure Vallotton**

médecin diplômé de la Confédération Helvétique

intitulée

**Characterization of CD4$^+$ CD25$^+$T cells in solid organ transplant recipients**

Lausanne, le 3 avril 2009

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Amalio Telenti

# TABLE OF CONTENTS

# REMERCIEMENTS

Une thèse est avant tout un travail collectif; j'aimerais donc remercier un certain nombre de personnes sans lesquelles tout ceci n'aurait pas été possible.

En premier lieu, je tiens à remercier mon directeur de thèse, le Prof. Giuseppe Pantaleo, qui m'a donné l'opportunité et les moyens de réaliser ce travail de thèse au sein du Laboratoire d'Immunopathologie du SIDA.

J'aimerais également remercier mon "co-"directeur de thèse, le Prof. Manuel Pascual, qui s'est toujours montré très enthousiaste et motivant, et a toujours répondu présent pour m'encourager à présenter les résultats de mon travail lors de divers colloques et congrès. Grâce à lui, une collaboration fructueuse a pu s'établir entre notre laboratoire de recherche et le Centre de Transplantation d'Organes du CHUV.

Je tiens à remercier Laura Codarri et Donatella Ciuffreda qui étaient à l'origine de ce projet de recherche ; elles m'ont accordé leur confiance et enseigné les techniques de cytométrie de flux, de tri et de culture cellulaire qui m'ont permis de mener à bien ce projet.

Cette thèse s'est appuyée sur diverses études cliniques qui se sont déroulées depuis 2004 au CHUV de Lausanne ainsi qu'aux HUG de Genève. J'éprouve donc une immense reconnaissance envers tous les patients (ils sont plus d'une centaine) qui ont accepté d'y participer. Sans eux, je n'aurais pas eu de résultats à présenter… Merci également aux équipes médicales, infirmières et administratives de ces deux sites ; à Lausanne, les docteurs Jean-Pierre Venetz., Massimiliano Fontana, Ghaleb Nseir, Sam Rotman, Sébastien Kissling, Anca Antonino, Carine Berutto, Roberto Bullani, John David Aubert, et les infirmières Christiane Rotzetter, Anne-Laure Berger, Silvia Ciganek, ainsi que Brigitte Léger, Madeleine Arslan et Catherine Toffel ; à Genève, les docteurs Karine Hadaya et Leo Buhler, ainsi que les infirmières de la consultation de néphrologie.

Merci au Fonds National Suisse de la Recherche Scientifique qui m'a octroyé une bourse dans le cadre du programme MD-PhD, m'offrant la possibilité de découvrir le monde de la recherche scientifique fondamentale.

Si une thèse est avant tout un challenge professionnel, il ne faut pas oublier tous les "à-côtés", qui permettent de maintenir un équilibre de vie.

J'aimerais donc remercier mes collègues de labo à BT-02, BT-06 et au BH-19, pour m'avoir appris les subtilités des techniques de recherche scientifique. Mais surtout ils ont su créer une ambiance de travail chaleureuse et motivante, rythmée d'apéros plus ou moins spontanés, de fêtes plus moins prétextes (Epiphanie, Chandeleur, Saint-Martin, Saint-Nicolas, Noël) et de joutes estivales, même au mois de décembre…

Merci particulièrement à Lucienne, Isabelle, Gonzalo et Miguel : grâce à vous, tout "roulait" à BT-02 mais aussi en-dehors, car vous aviez une solution à tout, chacun dans votre domaine ! En vrac (vous vous reconnaîtrez certainement), merci d'avoir partagé avec moi un bureau, des conseils culinaires et cinématographiques, la gestion du CODAFLIPS, les sorties 28 Forever, au Jagger's et à la Cravache, le téléphone (coups de fil du 022 827 30 60), sans oublier le sport (gym, course à pied, plongée, ski, raquettes, peau de phoque) et les cappuccinos de l'ortho.

Un grand merci à mes camarades du programme MD-PhD, Camilla, Mario, Vincent et Yannis ; ensemble nous avons eu le courage de reprendre des études après notre final de médecine, puis d'affronter le monde de la Biologie…

Merci à mes amis de longue date de la 3AX1, en particulier Anne et Denis, Alex et Friederike, Samuel et Mélanie, Sophie, pour leur amitié, leur soutien et tous les moments que nous avons passés ensemble depuis bientôt 15 ans. Merci aussi à mes camarades d'études de médecine, Juliane, Delphine, Nelson et Anik.

Merci au Dr. Philippe Michel, qui m'a toujours soutenue et écoutée quand les choses étaient un peu plus difficiles.

J'aimerais bien sûr remercier mes parents et ma grand-maman pour m'avoir donné les moyens de réaliser mes désirs et encouragé dans mes choix. Merci à mes beaux-parents, Mélo et Stéphane de m'avoir si gentiment accueillie dans la famille.

Enfin, *last but not least*, toute ma reconnaissance va à Raphaël, qui partage ma vie depuis déjà pas mal de temps ; merci à toi pour tout ce que tu me donnes et tout ce que tu es, merci pour ton amour, ta patience et ton compréhension… je sais que ce n'est pas toujours facile de me supporter… Et je me réjouis de découvrir avec toi une nouvelle étape dans notre vie, avec notre p'tit/e loulou/te qui devrait pointer le bout de son nez d'ici quelques jours…

# 1. SUMMARY

Although important progresses have been achieved in the therapeutic management of transplant recipients, acute and chronic rejections remain the leading causes of premature graft loss after solid organ transplantation. This, together with the undesirable side effects of immunosuppressive drugs, has significant implications for the long-term outcome of transplant recipients. Thus, a better understanding of the immunological events occurring after transplantation is essential.

The immune system plays an ambivalent role in the outcome of a graft. On one hand, some T lymphocytes with effector functions (called alloreactive T cells) can mediate a cascade of events eventually resulting in the rejection, either acute or chronic, of the grafted organ; on the other hand, a small subset of T lymphocytes, called regulatory T cells, has been shown to be implicated in the control of these harmful rejection responses, among other things. Thus, we focused our interest on the study of the balance between circulating effectors (alloreactive) and regulatory T lymphocytes, which seems to play an important role in the outcome of allografts, in the context of kidney transplantation. The results were correlated with various variables such as the clinical status of the patients, the immunosuppressive drugs used as induction or maintenance agents, and past or current episodes of rejection. We observed that the percentage of the alloreactive T lymphocyte population was correlated with the clinical status of the kidney transplant recipients. Indeed, the highest percentage was found in patients suffering from chronic humoral rejection, whilst patients on no or only minimal immunosuppressive treatment or on sirolimus-based immunosuppression displayed a percentage comparable to healthy non-transplanted individuals. During the first year after renal transplantation, the balance between effectors and regulatory T lymphocytes was tipped towards the detrimental effector immune response, with the two induction agents studied (thymoglobulin and basiliximab).

Overall, these results indicate that monitoring these immunological parameters may be very useful for the clinical follow-up of transplant recipients; these tests may contribute to identify patients who are more likely to develop rejection or, on the contrary, who tolerate well their graft, in order to adapt the immunosuppressive treatment on an individual basis.

# 2. INTRODUCTION

## *A short and non-exhaustive history…*

The first description of transplantation in humans is found in Chinese texts, as soon as in the 4th century BC. Tsin Yue-Jen, a surgeon, switched the hearts of two soldiers and said that both patients survived, but gave no reason for the transplant… In the next centuries, great advances were made in the understanding of human anatomy, physiology and surgery. In 1900-1902, the German scientist Karl Landsteiner discovered the principle of the four blood groups.

**Figure 1.**

**Sir Peter Medawar.**

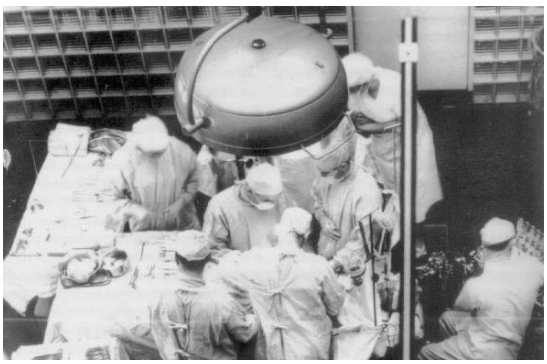


**Figure 2.**

**The first successful kidney transplantation performed by Joseph E. Murray in 1954 in Boston.**

In the 1940's, Sir Peter Medawar (1915-1987) at the University of London experimented with the immunological basis of organ rejection [**Figure 1**]. He was awarded his Nobel Prize of Medicine in 1960 for his work on tissue grafting and his discovery of acquired immunological tolerance, a model that paved the way to successful organ and tissue transplantation (his first publication: Gibson T and Medawar PB, 1943).

The first truly successful – defined as lasting more than 6 months – transplantation was performed in 1954 in Boston [**Figure 2**]. Joseph E. Murray transplanted a kidney from one identical twin to another without any immunosuppressive medication. The recipient died 8 years later and the donor was still alive in 2005. Joseph E. Murray won the Nobel Prize of Medicine in 1990.

In 1958, the French immunologist Jean Dausset published his results on the major histocompatibility complex (MHC) and its role in graft rejection. He received the Nobel Prize of Medicine in 1980.

The 1960's were marked by a lot of successes in the field of human organ transplantation: first successful cadaveric kidney transplant (1962), lung transplant (1963), pancreas transplant (1965), liver transplant (1967), and heart transplant (1967). At the same period, the first long-term dialysis treatments were developed and a dialysis centre opened in Seattle.

In the 1970's, the Swiss biologist Jean-François Borel discovered cyclosporine A, a compound produced by fungi; its unique immunosuppressive properties revolutionized the long-term management of transplant recipients (Rüegger A *et al*., 1976). Years of clinical studies revealed that given as part of a cocktail, primarily with steroids, it selectively suppresses T lymphocytes and prevents the rejection of transplanted organs; cyclosporine A was finally approved by the FDA in 1983.

Until now, a lot of advances have been made in the field of transplantation. An end-stage disease is not anymore a death sentence. It is possible to replace many organs by a cadaveric or living one and to improve significantly the life expectancy and the quality of life of transplant recipients. This success is essentially due to the development of new immunosuppressive compounds, the improvement of surgical techniques and the better understanding of the immunological mechanisms involved in transplantation. However, the achievement of a state of tolerance to the transplant is the only approach that is likely to provide long-term graft survival without the problems associated with life-long global immunosuppression.

There is accumulating evidence that T cell-mediated dominant control of self-reactive T cells contributes to the maintenance of immunological self-tolerance and that its alteration can cause autoimmune disease. Efforts to delineate such a regulatory T cell population have revealed that $CD25^{high}$ cells in the $CD4^{+}$ population in normal naïve animals bear the ability to prevent autoimmune disease *in vivo* and, upon antigenic stimulation, that they suppress the activation and proliferation of other T cells *in vitro*. The $CD4^{+}$ $CD25^{high}$ regulatory T cells, which are naturally anergic and suppressive, appear to be produced by the normal thymus as a functionally distinct subpopulation of T cells. They play critical roles not only in preventing autoimmunity but also in controlling tumor immunity and transplantation tolerance.

## *2.1. The human immune system*

### 2.1.1. Role of the immune system

Historically, Edward Jenner (1749-1823) is considered as the "father of immunology", the science of our body's defense against foreign invaders. The immune system is a collection of immune processes within an organism that protect against disease by identifying and killing foreign invaders such as microorganisms, tumor cells and imported foreign tissues and organs. Components of the immune system include various cell types originating in the bone marrow [**Figure 3**], specialized organs and tissues, and immunomodulatory proteins, all interacting in an organized and dynamic network.

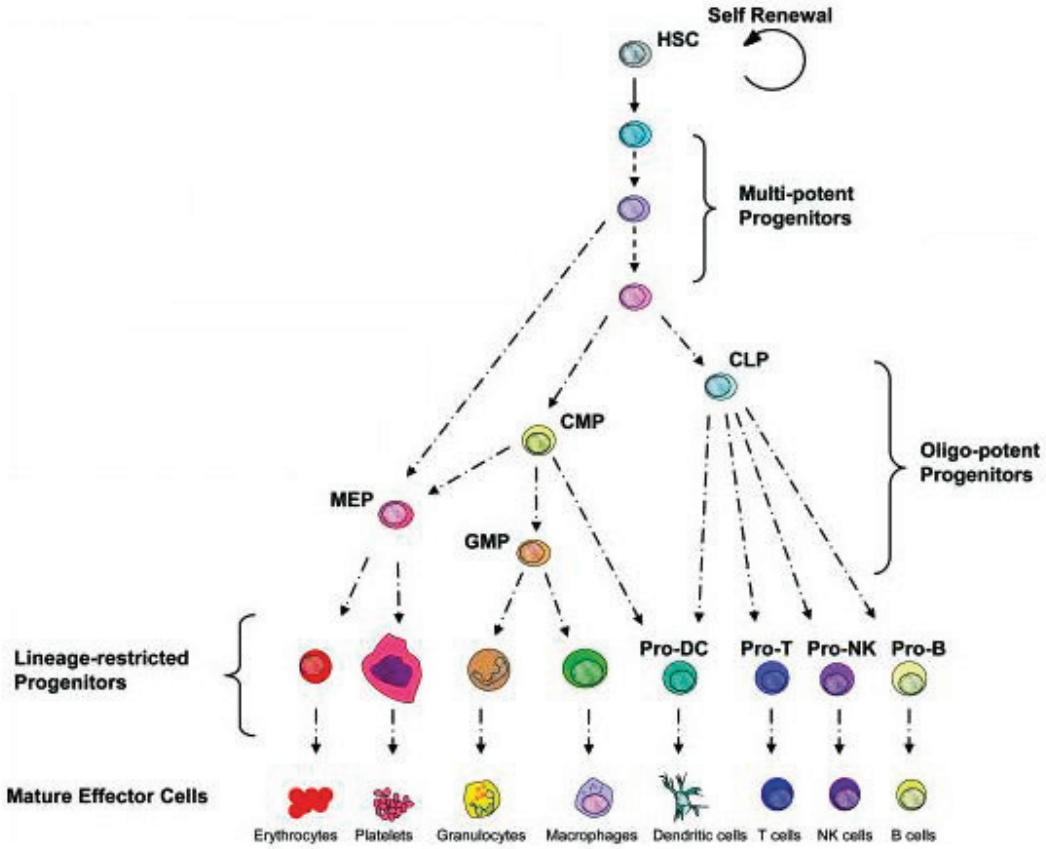



**Figure 3. Model of the hematopoietic developmental hierarchy (Bryder D *et al.*, 2006).**

**HSC: hematopoietic stem cell; CMP: common myeloid progenitor; MEP: megakaryocyte/erythrocyte progenitor; GMP: granulocyte/macrophage progenitor; CLP: common lymphoid progenitor; Pro-DC: dendritic cell progenitor; Pro-T: T cell progenitor; Pro-NK: NK cell progenitor; Pro-B: B cell progenitor.**

The immune system can be divided roughly into two distinct components, namely the innate and the adaptive immune system.

## 2.1.2. Innate immunity

The innate immune system is an evolutionarily ancient form of host defense against infections, and can be found in nearly all forms of life, including plants and invertebrates. It is the first line of defense of the organism, after breakdown of the physical barriers offered by the skin and the mucosal surfaces of the airways, the intestines and the genital sphere. It is composed of various types of cells, derived from common myeloid progenitors in the bone marrow: natural killer (NK) cells, mast cells, eosinophils, basophils, dendritic cells (DCs), and phagocytic cells including macrophages (in tissues) and neutrophils (in the blood).

Activation of the innate immune system results in the upregulation of co-stimulatory molecules and in the production of cytokines (proteins released by cells that affect the behavior of other cells that bear receptors for them) and chemokines (proteins released by cells that attract other cells). In turn, these inflammatory mediators initiate a local process of inflammation, and may also trigger the activation of the complement cascade, a system of plasma proteins that activates proteolytic reactions on microbial surfaces (but not on host cells), coating these surfaces with fragments that are recognized and bound by phagocytic receptors on macrophages. Responses elicited by the innate immune system are non-specific and do not lead to long-term immunological memory. Many infections are handled successfully by the innate immune system and cause no disease.

Finally, the innate immune system plays a crucial part in the initiation and subsequent direction of adaptive immune responses by a process known as antigen presentation, and in the control of infections during the first 4-7 days, the delay required by the adaptive immune system to be set up efficiently.

## 2.1.3. Adaptive immunity

The adaptive immune system is evolutionarily more recent and is present only in jawed vertebrates. It develops as a more specific line of defense and is triggered by the innate immune system with a substantial lag time between exposure and maximal response. The adaptive immune response displays an improved recognition of the pathogen, which is retained after the elimination of the pathogen; this phenomenon is called "immunological memory" and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered.

Adaptive immune responses depend upon specialized antigen-presenting cells (APCs), called dendritic cells, and upon lymphocytes, which derive from common lymphoid progenitors in the bone marrow and are divided into B lymphocytes (B cells, mature in the bone marrow) and T lymphocytes (T cells, mature in the thymus) based on their sites of differentiation and on their antigen receptors. They also depend on other cellular mediators shared with the innate immune system, such as phagocytic cells and NK cells.

Lymphocytes are remarkable in being able to mount a specific immune response against virtually any foreign antigen. This is possible because each individual lymphocyte matures bearing a unique variant of a prototype antigen receptor, so that the population of T and B lymphocytes collectively expresses a huge repertoire of receptors that are highly diverse in their antigen-binding sites. In theory, there are lymphocytes of at least $10^8$ different specificities in an individual at any one time.

B cells are the mediators of the so-called "humoral immunity". They are generated and mature in the bone marrow and bear on their cell surface the B cell antigen receptor (BCR), which is a membrane-bound form of the antibody that the B cell will secrete after activation and differentiation to plasma cells. B cells protect the organism against extracellular pathogens and their toxic products, by producing antibodies, which are Y-molecules whose arms form two identical antigen-binding sites. Antibodies can protect from pathogens or their toxic products by binding to them, thereby preventing their access to cells that they might infect or destroy ("neutralization"); another way of antibody-mediated protection is that antibodies enable a phagocytic cell to ingest and destroy the bound pathogen ("opsonization"); finally, antibodies might activate the complement cascade, which leads to the formation of pores in the targeted pathogen and to its direct destruction. Eventually, neutralization, opsonization and complement activation all result in the scavenging and degradation of the pathogen or the toxic product by macrophages.

On the other hand, T cells mediate the so-called "cellular immunity". They are also generated in the bone marrow, but mature and differentiate in the thymus. They exhibit on their cell surface a highly specific receptor, the T cell antigen receptor (TCR). T cells are needed to control intracellular pathogens (which are inaccessible to B cells and antibodies), such as some bacteria, parasites and all viruses. Through their TCR, they recognize pathogen-derived antigens presented by the major histocompatibility complex (MHC) of APCs. Since we focused our study on T cells, they will be discussed extensively in the next part.

Although the immune system is mainly specialized to recognize foreign peptides derived from pathogens (infections), it might also be activated in the presence of non pathogen-derived antigens, such as antigens provided by an allograft (transplantation), tumor-derived peptides (cancer), environmental antigens (allergy) and even auto-antigens (autoimmunity).

## *2.2. T cells*

### 2.2.1. Generation and maturation of T cells

Before birth, T lymphoid progenitor cells are generated in the bone marrow from hematopoietic stem cells, and subsequently migrate to the thymus where they will complete their maturation and differentiation to become T cells. After birth, the thymus is a continuous source of new lymphocytes, which migrate to populate the peripheral lymphoid tissues; however, after puberty, the thymus progressively shrinks and T cell numbers are maintained through division of mature T cells, outside of the central lymphoid organs.

An extensive review of thymic development, anatomy and physiology can be found in Rezzani R *et al*. (2008). Briefly, the thymus provides a network of specialized nonlymphoid stromal cells that interact intimately with the developing lymphocytes, providing signals through secreted growth factors and cell surface molecules. It consists of two distinct anatomical regions – an outer cortical region, the cortex, and an inner medulla. The cortex contains only immature thymocytes and scattered macrophages, whereas more mature thymocytes, along with macrophages and dendritic cells, are found in the medulla. Developing thymocytes pass through a series of distinct phases that are marked by changes in the status of TCR genes and expression of the TCR molecules, and by changes in expression of cell surface proteins such as the CD3 complex and the co-receptor proteins CD4 and CD8 [**Figure 4**]. Two distinct lineages of T cells – α:β and γ:δ, which have different types of TCR – are produced early in T cell development; later, α:β T cells develop into two distinct functional subsets, CD4 and CD8 T cells. When hematopoietic precursors first enter the thymus from the bone marrow, they lack most of the surface molecules characteristic of mature T cells (CD3, CD4, CD8), and their receptor genes are unrearranged; they are called "double negative" (DN) thymocytes. Afterwards, immature thymocytes undergo several transitions (DN1-DN4), leading to expression of both CD4 and CD8, and of a non-functional TCR complex called "pre-TCR" (Godfrey DI *et al*., 1993); they are called "double positive" (DP) thymocytes. After this stage, which takes place in the cortex of the thymus, thymocytes acquire a fully assembled TCR expressed at high levels, and subsequently undergo a crucial dual screening step, namely positive and negative selection (Robey E *et al*., 1994). During positive selection, thymocytes which bind with adequate affinity to self-antigen/self-MHC complexes on thymic cortical stromal cells are rescued from death by receiving survival signals, whilst those thymocytes that bind with too low affinity die by apoptosis; the fate of thymocytes is also determined during positive selection: they are committed to become either $CD4^+$ cells (if they were positively selected by MHC class II molecules) or $CD8^+$ cells (if they were positively selected by MHC class I molecules). Negative selection is mediated by thymic medullary epithelial cells and DCs and occurs during and after the double-positive stage: thymocytes that react with a too high avidity with self-antigens are deleted by

apoptosis, thus eliminating potentially harmful autoreactive T cells ("central tolerance"). About 98% of thymocytes die during the development process by failing either positive selection or negative selection, whereas the only 2% that survive exit the thymus as single positive, $CD4^+$ or $CD8^+$, mature naïve T cells.

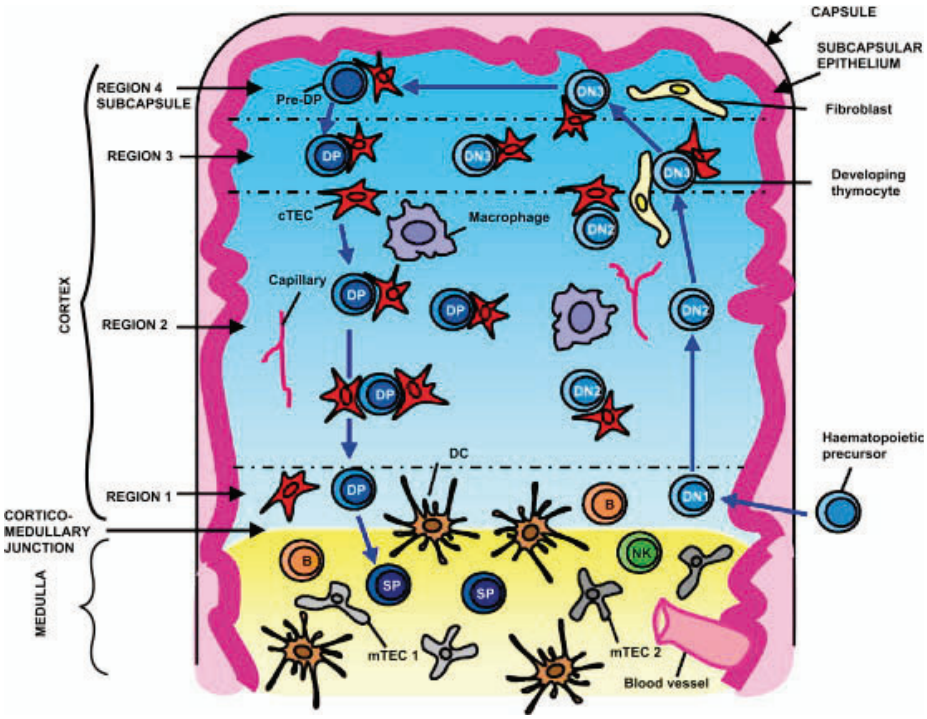



**Figure 4. Thymus structure and function (Rezzani R *et al.*, 2008).**

**DN: double negative; DP: double positive; SP: single positive; cTEC: cortical thymic epithelial cell; mTEC: medullary thymic epithelial cell; DC: dendritic cell; NK: natural killer.**

Once T cells have left the central lymphoid tissues, they are carried in the blood to the peripheral lymphoid tissues. If they do not encounter their specific antigen and become activated within a peripheral lymphoid tissue, they leave the tissue and recirculate via lymph and blood, continually reentering lymphoid tissues until either antigen is encountered or the T cell dies.

## 2.2.2. Organization of the mature T cell pool

Mature recirculating T cells that have not yet encountered their antigens are known as naïve T cells. In order to be activated, naïve T cells must recognize a foreign peptide bound to a self MHC molecule in addition to the concomitant delivery of a co-stimulatory signal by specialized APCs, driving the clonal expansion of naïve T cells and their differentiation into effector cells.

### *2.2.2.1. Antigen processing and presentation*

The development of an adaptive immune response is not initiated directly at the site of infection, but instead in secondary lymphoid tissues such as lymph nodes, Peyer's patches of the gut, tonsils and the spleen. Pathogen-derived antigens (or other "foreign" antigens and even auto-antigens in the case of autoimmunity) are transported to these secondary lymphoid tissues by the lymph or, more rarely, by the blood. These antigens are ingested by professional APCs (DCs, macrophages and B cells), processed into peptides and delivered at their cell surface by their MHC molecules, along with co-stimulatory molecules, for presentation to T cells.

MHC molecules are divided into two classes, class I and class II, based upon their structure, tissue distribution and source of peptide antigen, as well as upon their interactions with T cell subsets. In humans, they are called "HLA", for "Human Leucocyte Antigen" and are encoded on chromosome 6. There are six major HLA loci encoded within the MHC.

The class I HLA molecules are encoded by three distinct genetic loci known as HLA-A, -B, and -C. Class I molecules comprise a single heavy chain, non covalently complexed to a smaller molecule known as β2-microglobulin (Parham P *et al*., 1987; Natarajan K *et al*., 1999). The heavy chain has three domains, α1, α2, and α3. The α1 and α2 domains create the antigen-binding cleft, the floor of which is formed by a β-pleated sheet that is overlaid by two α-helical walls. The α3 domain and β2-microglobulin form a membrane-proximal scaffold that interacts with CD8 on T cells [**Figure 5A**]. Class I molecules are expressed on most nucleated cell types as well as on platelets, and all three locus products are expressed in a co-dominant fashion.

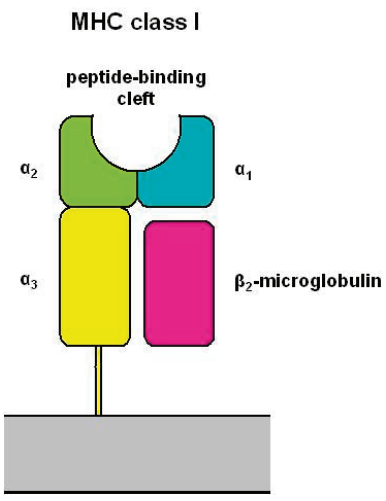



**Figure 5A. Structure of the MHC class I molecule.**

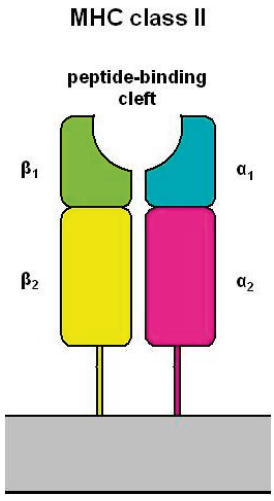



**Figure 5B. Structure of the MHC class II molecule.**

The class II molecules include HLA-DR, -DQ, and -DP, encoded by distinct genetic loci clustered in the class II region of the MHC. The overall protein structure adopted by class II molecules is similar to that of class I, but it is achieved by the association of two membrane-bound chains known as α and β (Madden DR, 1995). The α and β chains assemble non-covalently to create an antigen-binding cleft located above a conserved membrane-proximal structure that can interact with CD4 on T cells [**Figure 5B**]. Class II molecules are constitutively expressed only on DCs, macrophages and B cells, and all three locus products are expressed in a co-dominant fashion.

It is generally admitted that MHC class I molecules present peptides of 8-11 amino acids of length, obtained after degradation of cytoplasmic proteins, to CD8 T cells, whereas MHC class II molecules present longer peptides (11-25 amino acids), originating from extracellular proteins taken up by the APCs, to CD4 T cells.

#### *2.2.2.2. Naïve T cell activation and clonal expansion*

There are mainly three mandatory requirements for the optimal antigen-specific activation and subsequent clonal expansion of naïve T cells, preventing unspecific activation as well as expansion of inefficient T cells; it is known as the "three-signal model".

First, as already explained, activation of naïve T cells is triggered when TCR and either CD4 or CD8 co-receptors bind to peptide/MHC complexes presented by professional APCs ("signal 1"). The strength of the TCR signal has a quantitative influence on T cell activation and differentiation (Sloan-Lancaster J *et al*., 1993; Viola A *et al*., 1996). Second, a co-stimulatory signal is required, provided by both the cell surface molecules displayed on APCs and the soluble factors ("signal 2"). The absence of a second signal promotes T cell anergy, which is a state of antigen-specific nonresponsiveness, or apoptosis (DeSilva DR *et al*., 1991; Jenkins MK *et al*., 1991; Norton SD *et al*., 1991; Harding FA *et al*., 1992). Several co-stimulatory signaling molecules have been described, and they generally belong to two families: the B7 family (reviewed in Greenwald RJ *et al*., 2005) [**Figure 6**] and the tumor-necrosis factor (TNF) family of receptors (reviewed in Watts TH, 2005).

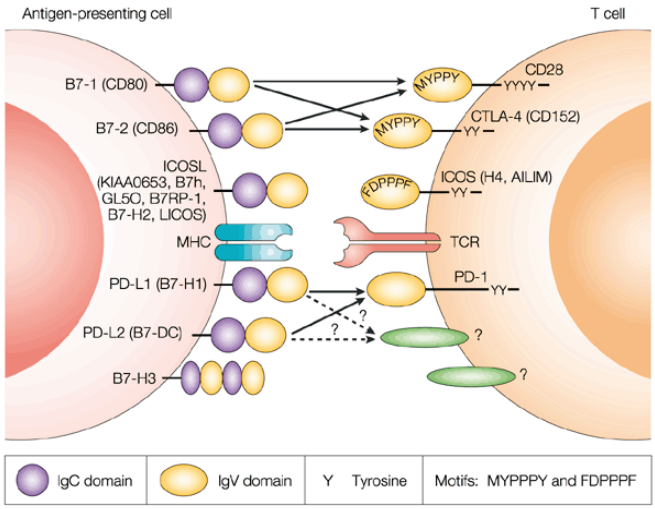



**Figure 6. Structures of the B7-1/B7-2 – CD28/CTLA-4 superfamily members (Sharpe AH *et al.*, 2002).**

**CTLA-4: cytotoxic T lymphocyte antigen 4; ICOS: inducible T cell co-stimulator; ICOSL: ICOS ligand; MHC: major histocompatibility complex; PD-1: programmed death 1; PD-L1, PD-L2: PD-1 ligands 1 and 2; TCR: T cell receptor.**

The combination of both TCR–peptide/MHC complex and co-stimulatory molecules interactions provides optimal signal to naïve T cells, initiating a complex intracellular signaling cascade leading to the activation of transcription factors such as NF-κB, NFAT and AP-1 (Mustelin T *et al.*, 2003) [**Figure 7**]. Ultimately, T cell activation leads to the induction of new gene synthesis that results in the differentiation, proliferation and effector actions of T cells.

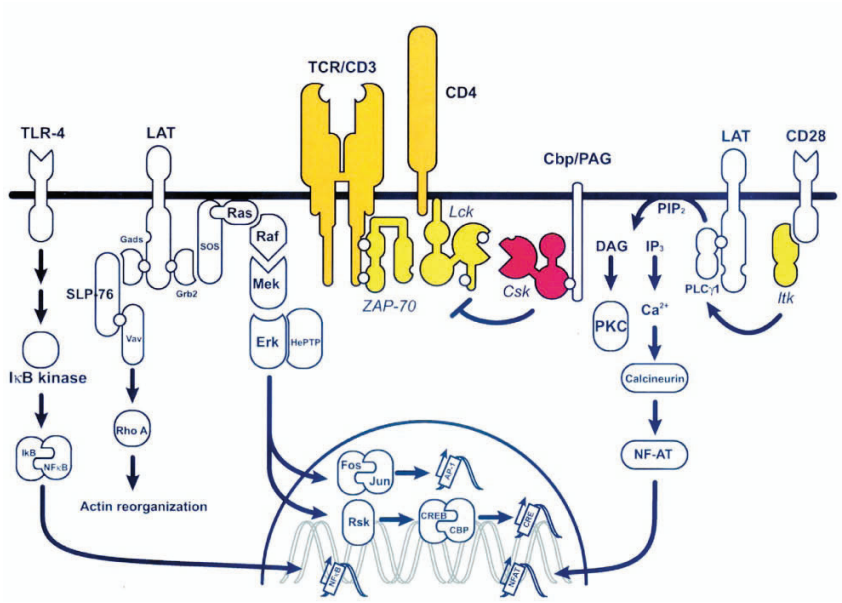



**Figure 7. Intracellular signaling pathways and kinases involved in T cell activation (Mustelin T *et al.*, 2003).**

Among others, T cell activation induces the synthesis of IL-2 along with the α chain of the IL-2 receptor (CD25). The IL-2 receptor has three chains: α, β, and γ (Minami Y *et al*., 1993). Resting T cells express a form of this receptor composed of β and γ chains that binds IL-2 with moderate affinity, allowing resting T cells to respond to very high concentrations of IL-2. Association of the α chain with the β and γ chains creates a receptor with a much higher affinity for IL-2, allowing the cell to respond to very low concentrations of IL-2. Binding of IL-2 to the high-affinity receptor then triggers progression through the rest of the cell cycle (Hatakeyama M *et al*., 1989), by activating the "target of rapamycin" pathway to provide "signal 3". IL-2 also promotes the differentiation of these cells into armed effector T cells.

### *2.2.2.3. Memory T cell subsets and effector functions*

Once activated, T cells acquire new functions and properties that render them able to fight efficiently the triggering stimulus. We have already seen that T cells generated in the thymus can be divided into two main classes, depending on the co-receptor expressed on their cell surface and the class of MHC molecules recognized, namely CD4 and CD8 T cells, which display distinct functions.

CD4 T cells, display a broad range of effector functions [**Figure 8**]. Historically, two different subsets were first identified in 1986, T helper 1 cells ($T_H1$) and T helper 2 cells ($T_H2$) (Mosmann TR *et al*., 1986). However, in 1995, a new subset with particular functional properties was identified, called regulatory T cells (Treg) (Sakaguchi S *et al*., 1995). Finally, in 2005, a fourth lineage of effector CD4 T cells was identified, which were able to produce IL-17; they are known as $T_H17$ cells (Park H *et al*., 2005). CD4 T cells subsets fates and functions will be discussed in more detail in the next parts.

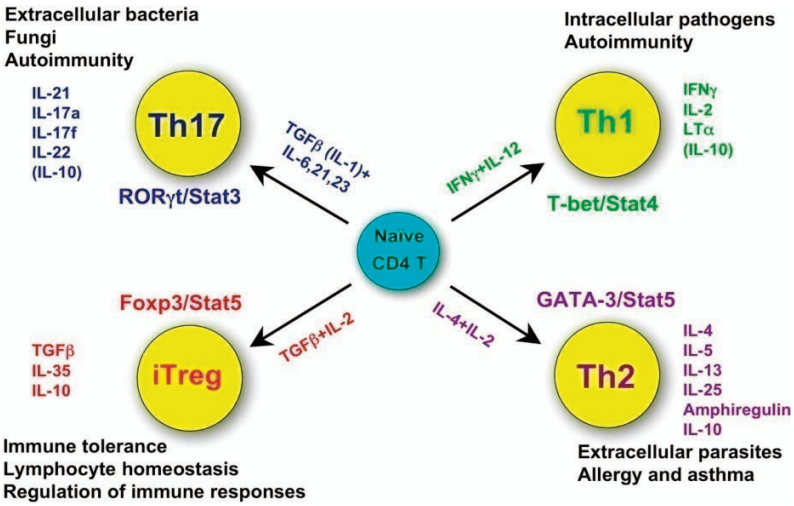



**Figure 8. Summary of the four CD4 T helper cell fates: their functions, their unique products, their characteristic transcription factors, and cytokines critical for their fate determination (Zhu J and Paul WE, 2008).**

On the other hand, CD8 T cells, also called cytotoxic T cells (CTLs), have the ability to kill target cells that display peptide fragments of cytoplasmic pathogens, most notably viruses, but also of tumor cells and allogeneic cells, bound to MHC class I molecules at the cell surface. $CD8^+$ T lymphocytes cause lysis of target cells by two mechanisms: membranolysis, in which secreted molecules such as perforin and granzymes form pores in the membrane of target cells (Berke G, 1994), and apoptosis, mediated through the triggering of apoptosis-inducing (Fas-like) surface molecules on the target cell (Hashimoto S *et al*., 2000).

## *2.3. CD4 T cells*

### 2.3.1. $T_H1$ cells

$T_H1$ cells have been shown to be responsible for cell-mediated immunity. They mediate immune responses against intracellular pathogens (Mosmann TR *et al*., 1989; Paul WE and Seder RA, 1994). In humans, $T_H1$ cells play a particularly important role in resistance to mycobacterial infections, and are also responsible for the induction of some autoimmune pathologies and of delayed-type hypersensitivity. They mainly produce interferon γ (IFNγ), tumor necrosis factor α (TNFα), lymphotoxin α (LTα), interleukin-12 (IL-12) and IL-2. IFNγ is important in activating macrophages to increase their microbicidal activity.

The presence of IL-12, produced by maturing DCs, as well as of type I and II IFN, produced by NK cells and DCs, drives the polarization of naïve T cells towards a $T_H1$ phenotype, by activating specific transcription factors. The transcription factor T-bet is the master regulator of $T_H1$ differentiation (Szabo SJ *et al*., 2000). In addition, Stat1 plays a major role in the IFNγ-mediated induction of T-bet (Lighvani AA *et al*., 2001) and Stat4, an IL-12 signal transducer, is important for amplifying $T_H1$ responses (Kaplan MH *et al*., *Nature*, 1996) and for inducing direct IFNγ production in activated CD4 T cells. $T_H1$ cells express IL-12Rβ2 upon TCR activation and then maintain its expression thanks to IL-12 and IFNγ stimulation (Szabo SJ *et al*., 1997). Among chemokine receptors, CxCR3 (Sallusto F *et al*., 1998) and CCR5 (Loetscher P *et al*., 1998) show a striking preferential expression on $T_H1$ cells.

### 2.3.2. $T_H2$ cells

$T_H2$ cells are responsible for humoral immunity, through secretion of specific cytokines that induce B cell proliferation and differentiation into antibody-secreting plasma cells. They mediate host defense against extracellular pathogens including bacteria and parasites such as helminthes (Mosmann TR *et al*., 1989; Paul WE and Seder RA, 1994). They are also important in the induction and persistence of asthma and other allergic diseases. They mainly produce IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25.

The presence of IL-4 in the environment drives the polarization of naïve T cells towards a $T_H2$ phenotype and represses the expression of $T_H1$ cytokines, by inducing the expression of

the master regulator GATA-3 (Pai SY *et al*., 2004) through activation of Stat6 (Kaplan MH *et al*., *Immunity*, 1996). IL-4Rα expression is up-regulated during $T_H2$ differentiation. Among chemokine receptors, CCR3 (Sallusto F *et al*., 1997), CCR4 (Bonecchi R *et al*., 1998) and CCR8 (D'Ambrosio D *et al*., 1998) are preferentially expressed in $T_H2$ cells.

### 2.3.3. $T_H17$ cells

The recently identified $T_H17$ lineage is responsible for, or participates in, the induction of many organ-specific autoimmune diseases such as experimental autoimmune encephalitis (Komiyama Y *et al*., 2006), collagen-induced arthritis (Murphy CA *et al*., 2003) and inflammatory bowel disease (Yen D *et al*., 2006), as well as for the protection against extracellular bacteria in mice (Rudner XL *et al*., 2007) and against fungi in humans (Palm NW and Medzhitov R, 2007). They have been termed $T_H17$ because of their ability to secrete high amounts of the pro-inflammatory cytokine IL-17 (Park H *et al*., 2005), which mediates inflammation through the ubiquitously expressed IL-17 receptor (Spriggs MK, 1997).

RORγt is the transcription factor responsible for the differentiation of $T_H17$ cells (Ivanov II *et al*., 2006), as demonstrated by the fact that overexpression of RORγt induces IL-17 production and that RORγt-deficient cells produce very little IL-17. Recently, it was also shown that RORα is up-regulated in $T_H17$ cells (Yang XO *et al*., 2008) and that deficiency in both RORγt and RORα completely abolishes IL-17 production. Finally, Stat3, which is the main signal transducer for IL-6, IL-21 and IL-23, is indispensable for IL-17 production (Harris TJ *et al*., 2007). In mice, IL-6 and TGFβ act synergistically to drive the differentiation of $T_H17$ cells, whilst the absence of IL-6 triggers the differentiation of regulatory T cells (Chen W *et al*., 2003). The apparent link between these two populations suggests that differentiation of one or the other lineage critically depends on the encountered cytokine milieu.

In addition to the three above-described effector lineages, CD4 T cells in the thymus can differentiate into a fourth subset, namely regulatory T cells (Treg), which has the unique property to repress immune responses (so-called suppressive capacity). We will discuss the phenotypic and functional properties of regulatory T cells in the next part.

## *2.4. Treg cells*

T cells able to suppress immune responses have been described in the early 1970s under the term of "suppressor T cells" by Gershon RK *et al*., who concluded that: "[in mice, some] thymocytes are capable of suppressing the antigen-induced response of other thymocytes without the mediation of B cells or their product (i.e., antibody)" (Gershon RK and Kondo K, 1970). In 1982, it was demonstrated that the development of autoimmune oophoritis induced by neonatal thymectomy could be prevented by a single injection of spleen cells or

thymocytes from normal adult female mice (Sakaguchi S *et al*., 1982). The subject regained interest in the mid-1990s when Sakaguchi S *et al*. showed that a minor population (5-10% in mice) of $CD4^+$ T cells, which co-expresses the IL-2 receptor (IL-2R) α chain (CD25), is crucial for the control of autoreactive T cells *in vivo*. They demonstrated that when $CD4^+$ cell suspensions prepared from mice lymph nodes and spleens were depleted of $CD25^+$ cells by specific monoclonal antibodies, and then inoculated into athymic mice, all recipients spontaneously developed evident autoimmune diseases (such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis); reconstitution of $CD4^+$ $CD25^+$ cells within a limited period after transfer of $CD4^+$ $CD25^-$ cells prevented these autoimmune developments in a dose-dependent fashion (Sakaguchi S *et al*., 1995). They were also the first to demonstrate that CD25 could be used as a marker for the so-called suppressor cells. Subsequent *in vitro* studies by several groups showed that $CD4^+$ $CD25^+$ T cells are both hyporesponsive and suppressive (Thornton AM and Shevach EM, 1998; Takahashi T *et al*., 1998). $CD4^+$ $CD25^+$ regulatory T cells were discovered originally in mice, but a population with identical phenotypic and functional properties has also been defined in humans (Levings MK *et al*., 2001; Dieckmann D *et al*., 2001; Jonuleit H *et al*., 2001; Taams LS *et al*., 2001).

Regulatory T cells can be divided into "natural" Treg, which develop in the thymus as a specific T cell lineage, and "adaptive" Treg, which differentiate in the periphery from naïve T cells under specific conditions. Initially, regulatory T cells were thought to mediate tolerance to self antigens, thus preventing the development of autoimmune diseases; however, experimental evidences strongly suggest that they also play a central role in immune responses in the context of microbial infections (Suvas S and Rouse BT, 2006), allergic disorders (Xystrakis E *et al*., 2007), cancers (Wang HY and Wang RF, 2007), materno-fœtal tolerance (Aluvihare VR *et al*., 2004), and transplantation (Yong Z *et al*., 2007).

## 2.4.1. Phenotypic characteristics of Treg

Regulatory T cells display several cell surface as well as intracellular markers that permit to identify them among $CD4^+$ T cells. For a purpose of clarity, we will focus our description on the so-called "natural" Treg (nTreg).

### *2.4.1.1. CD25*

CD25, as mentioned previously, was the historical marker of Treg cells. It corresponds to the IL-2R α chain. However, it is not exclusively expressed by Treg cells, but also by CD4 and CD8 T cells upon activation. Furthermore, the expression profile of CD25 on peripheral CD4 T cells is not easy to interpret, particularly in humans, since there is not a clear distinction between cells which do not express CD25 ($CD25^{neg}$) and those expressing CD25 ($CD25^{pos}$), but rather a continuous spread from CD25 negative cells to cells expressing high levels of

CD25 ($CD25^{high}$); three populations can be distinguished based on the CD25 expression level: $CD4^+$ $CD25^{neg}$ T cells (no or very low expression of CD25), $CD4^+$ $CD25^{int}$ T cells (intermediate expression of CD25), and $CD4^+$ $CD25^{high}$ T cells (high expression of CD25) [**Figures 9A** and **9B**].

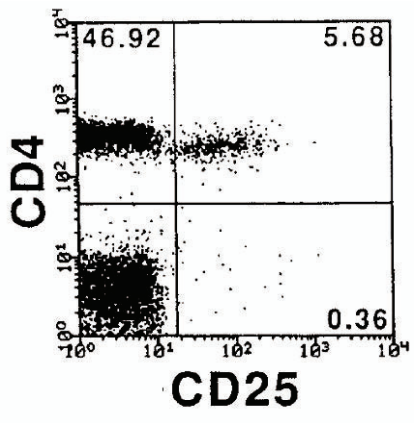



**Figure 9A. Representative cytometric expression profile of $CD4^+$ $CD25^+$ T cells, in mice (Sakaguchi S *et al.*, 1995).**

**In mice, there is a clear distinction between $CD4^+$ $CD25^{neg}$ and $CD4^+$ $CD25^{pos}$ T cells.**

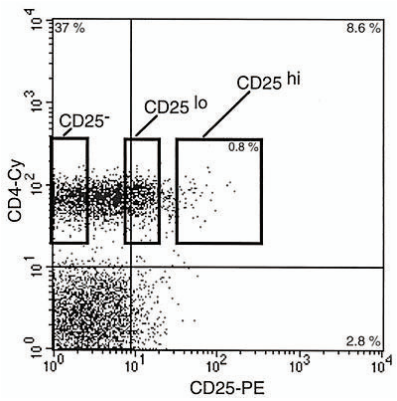



**Figure 9B. Representative cytometric expression profile of $CD4^+$ $CD25^+$ T cells, in humans (Baecher-Allan C *et al.*, 2001).**

**In humans, three populations can be distinguished: $CD4^+$ $CD25^{neg}$, $CD4^+$ $CD25^{int}$ and $CD4^+$ $CD25^{high}$ T cells.**

This distinction is important, since only $CD4^+$ $CD25^{high}$ T cells display the characteristic suppressive capacity of regulatory T cells (this point will be discussed in more details later) [**Figure 10**].

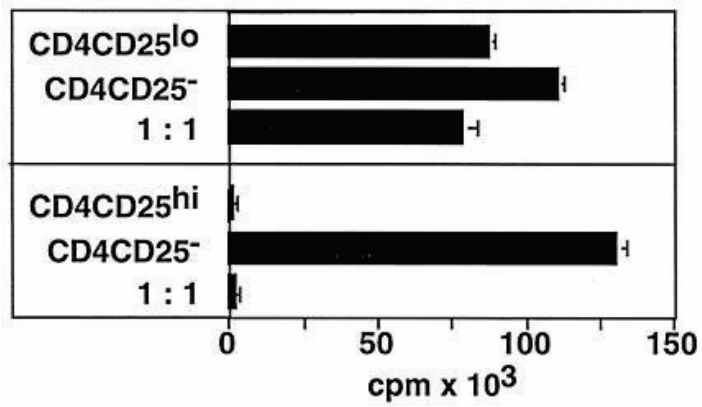



**Figure 10. Proliferative and suppressive capacity of $CD4^+$ $CD25^{neg}$, of $CD4^+$ $CD25^{int}$, and of $CD4^+$ $CD25^{high}$ T cells (Baecher-Allan C *et al.*, 2001).**

***Upper panel*: upon polyclonal stimulation (plate-bound anti-CD3), both $CD4^+$ $CD25^{neg}$ and $CD4^+$ $CD25^{int}$ T cells proliferated vigorously; co-culture of these two populations did not display inhibition of proliferation.**

***Lower panel*: upon polyclonal stimulation (plate-bound anti-CD3), only $CD4^+$ $CD25^{neg}$ T cells proliferated whilst $CD4^+$ $CD25^{high}$ T cells were hyporesponsive; in co-culture, $CD4^+$ $CD25^{high}$ T cells strongly inhibited the proliferation of $CD4^+$ $CD25^{neg}$ responder T cells.**

Traditionally, mouse and human Treg cells have been characterized as $CD4^+$ $CD25^+$ cells. Indeed, mouse Treg cells can be effectively isolated based on staining for $CD4^+$ $CD25^+$ $CD45RB^{low}$ expression. By contrast, the purity of isolated human Treg cells has always been an issue because T cells upregulate CD25 expression upon activation (Fontenot JD *et al*., *Immunity*, 2005). Consequently, the search for Treg-cell-specific cell surface markers, particularly in humans, has continued with a growing number of candidates proposed.

### *2.4.1.2. FoxP3*

The transcription factor FoxP3 ("forkhead box P3") is considered to date as the most reliable marker for identification of regulatory T cells and is critically implicated in their development, maintenance and function (Fontenot JD *et al*., 2003; Hori S *et al*., 2003). It belongs to the forkhead/winged-helix family of transcription factors [**Figure 11**].


**Figure 11. Functional structure of the FoxP3 protein (Campbell DJ and Ziegler SF, 2007).**

The FoxP3 transcription factor protein is composed of at least four functionally distinct domains (Gajiwala KS and Burley SK, 2000): beginning at the N-terminus, a transcriptional repression domain containing a proline-rich region (amino acids (aa) 1-193), a zinc-finger motif of uncertain function (aa 200-223), a dimerization domain containing a leucine-zipper-like motif (aa 240-261), and a forkhead DNA binding domain (aa 338-421) at the C-terminus.

The critical role of FoxP3 on the development and function of regulatory T cells was first suggested in 2001 when the autoimmune Scurfy mice (Brunkow ME *et al*., 2001) and a human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) patient (Bennett CL *et al*., 2001; Wildin RS *et al*., 2001) were found to have mutations in the FoxP3 gene. Both Scurfy mice and IPEX patients present mutations in the FoxP3 gene leading to a dysfunctional or truncated protein. Scurfy mice completely lack natural regulatory T cells (Fontenot JD *et al*., 2003; Khattri R *et al*., 2003) and suffer from an aggressive lymphoproliferative disorder, with prominently activated CD4 T cells infiltrating many organs such as the lymph nodes, spleen, liver, skin and lungs (Godfrey VL *et al*., 1991). The development of this lymphoproliferative disorder could be prevented by the neonatal transfer of $CD4^+$ $CD25^+$ T cells (Smyk-Pearson SK *et al*., 2003). In IPEX patients, the FoxP3 protein is usually present but dysfunctional, permitting the generation of regulatory T cells, which are

normal in number and phenotype but display an altered suppressive capacity (Bacchetta R *et al*., 2006). In mice, FoxP3 expression is strictly restricted to regulatory T cells. By contrast, in humans, it is possible that FoxP3 expression can be induced following activation of naïve CD4 T cells, although it is still controversial if *de novo* FoxP3 expression is correlated with stable acquisition of Treg features or not (Walker MR *et al*., 2003; Roncador G *et al*., 2005; Gavin MA *et al*., 2006; Wang J *et al*., 2007). Recent studies have demonstrated the presence of epigenetic modifications of several regions in the FoxP3 locus exclusively occurring in natural regulatory T cells, both in mice (Floess S *et al*., 2007) and humans (Baron U *et al*., 2007). An evolutionarily conserved region within the 5' non-coding part of the gene, named TSDR (Treg-specific demethylated region), was shown to contain CpG motifs, which are completely demethylated in regulatory T cells, but methylated in naïve and effector T cells; regulatory T cells induced *in vitro* display only incomplete DNA demethylation despite high FoxP3 expression, suggesting that expression of FoxP3 must be stabilized by epigenetic modification to result in a permanent suppressor cell lineage commitment. These modifications may be useful in discriminating natural regulatory T cells from transiently FoxP3-expressing activated T cells (Janson PC *et al.*, 2008).

FoxP3 acts mainly as a transcriptional repressor (through its N-terminal proline-rich repressor domain), by binding to NFAT and NF-κB and therefore inhibiting the expression of their target genes (among others, genes coding for the cytokines IL-2, IL-4 and IFNγ) (Schubert LA *et al*., 2001; Bettelli E *et al*., 2005; Wu Y *et al*., 2006). Recently, it was also shown that FoxP3 could directly interact with AML1/Runx1, suppressing IL-2 and IFNγ production (Ono M *et al*., 2007). On the other hand, FoxP3 is responsible for the transcription of several genes specifically associated with the Treg phenotype, among which CD25, CTLA-4 and GITR (Williams LM and Rudensky AY, 2007) [**Figure 12**].

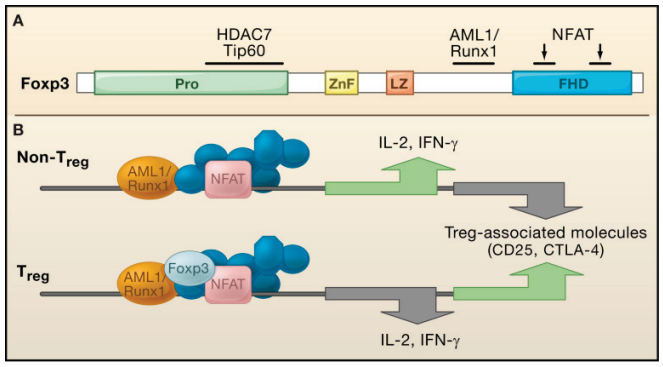



**Figure 12. Control of Treg function by FoxP3 (Sakaguchi S *et al*., 2008).**

**(A) Structure of the FoxP3 protein. Bars indicate the binding sites for other transcription factors (AML1/Runx1, NFAT) or chromatin-remodeling enzymes (HDAC7, Tip60).**

**Pro: proline-rich region; ZnF: zinc-finger domain; LZ: leucine-zipper domain; FHD: forkhead box domain.**

**(B) The transcriptional complexes involving NFAT and AML1/Runx1 activate or repress the genes encoding cytokines and several cell surface molecules in Treg and non-Treg, depending on the presence of FoxP3.**

In conclusion, FoxP3 is the master regulator of regulatory T cells, by inhibiting the expression $T_H1$-, $T_H2$- and $T_H17$-associated genes, by stimulating the expression of Treg-associated genes, and by stabilizing Treg characteristic features.

Although FoxP3 seems to be the "ideal" marker for identifying regulatory T cells, it has the drawback to be localized in the nucleus, which implies permeabilization of the cell membrane for its detection and is incompatible with the further use of the isolated cells in culture experiments. This has prompted efforts to identify other specific markers of Treg that are expressed on the cell surface, in addition to CD25.

#### *2.4.1.3. CTLA-4*

The CD28/CTLA-4 interacts with their ligands, the B7 family, to provide a second signal for T cell activation/anergy. In contrast to CD28, which provides an activatory co-stimulation signal, CTLA-4 (CD152) mediates down-regulation of immune responses by interacting with B7.1 (CD80)/B7.2 (CD86) on target cells (Thompson CB and Allison JP, 1997). CTLA-4 is constitutively expressed only by Treg cells (Takahashi T *et al*., 2000), and upregulated upon activation in conventional T cells (Walunas TL *et al*., 1994). The interaction between CTLA-4 and B7 molecules offers co-stimulation signal and results in Treg activation (Read S *et al*., 2000). This suggests that CTLA-4 is involved in the regulatory activity of Treg cells, although CTLA-4-deficient mice display Treg that are able to mediate suppression (Read S *et al*., 2006).

#### *2.4.1.4. GITR*

GITR belongs to the tumor necrosis factor receptor (TNFR) superfamily, which also includes 4-1BB, OX40 (CD134) and CD95 (Nocentini G *et al*., 1997). It is expressed on various lymphocytes at various levels, but high surface expression of GITR is restricted to resting Treg cells in the periphery and the thymus (Shimizu J *et al*., 2002). However, like CD25, GITR expression is upregulated on $CD25^-$ T cells following activation, rendering it not as a specific marker of Treg.

#### *2.4.1.5. CD39*

CD39, also known as ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1 [NTPDase 1]), is the most prominent ectoenzyme of the immune system. The function of CD39 is to effectively remove toxic extracellular ATP by converting it to ADP or AMP; CD39 is thought to work together with CD73, which catalyzes the conversion of AMP to adenosine. In the immune system, extracellular ATP functions as a "natural adjuvant" that exhibits multiple proinflammatory effects: it is released by damaged cells as an indicator of trauma and cell death. It was recently shown that CD39 is expressed primarily by $FoxP3^+$ regulatory T cells and that its expression is driven by the Treg-specific transcription factor FoxP3 (Borsellino G *et al*., 2007). Subsequently, Deaglio S *et al*. (2007) demonstrated that

the concomitant expression of CD39 and CD73 on regulatory T cells contributes to the generation of the immunosuppressive factor adenosine, which is known to exert an inhibitory effect on T cell responses.

#### *2.4.1.6. CD127*

CD127, the α chain of the IL-7 receptor, has been recently reported to be very useful in discriminating regulatory and activated cells among $CD4^+$ $CD25^{high}$ T cells in healthy individuals (Seddiki N *et al*., 2006; Liu W *et al*., 2006). In addition, the study conducted by Liu et al. suggests a link between CD127 and FoxP3: indeed they could show that FoxP3 physically interacts with the promoter region of CD127, which could explain the down-regulation of CD127 observed in Treg cells. The absence of CD127 on the surface of Treg cells is also in accordance with the observation that Treg survival critically depends on the presence of IL-2 (secreted by other T cells), but not on the presence of IL-7, which is essential for the survival of conventional T cells (Von Freeden-Jeffry U *et al*., 1995). To date, the combination of CD4, CD25 and CD127 surface markers should allow for quantitative studies of regulatory T cells as well as for enrichment of live regulatory T cells for functional analyses and/or expansion *in vitro* (Hartigan-O'Connor DJ *et al*., 2007).

#### *2.4.1.7. Other markers*

Besides the aforementioned markers, additional molecules have been shown to be more or less associated with the Treg lineage, but none of them has been described as really specific of regulatory T cells. Among others, we can mention: programmed cell death-1 (PD-1, down-regulated on Treg in mice and humans; Raimondi G *et al*., 2006), lymphocyte activation gene-3 (LAG-3, the mRNA of which is upregulated in Treg cells; Huang CT *et al*., 2004), neuropilin-1 (Nrp1, constitutively expressed on Treg cells; Bruder D *et al*., 2004), membrane-bound TGFβ (expressed on Treg cells; Nakamura K *et al*., 2001), and folate receptor 4 (FR4, upregulated on Treg cells; Yamaguchi T *et al*., 2007).

### 2.4.2. Functional characteristics of Treg

#### *2.4.2.1. Suppressive function*

*In vivo* and *in vitro*, regulatory T cells are characterized by an anergic state (that can be partially reversed by high-doses of IL-2) and by a suppressive function. They inhibit multiple stages of target cell activity [**Figure 13**]. Indeed, natural Treg cells are able to suppress the proliferation of naïve T cells and their differentiation into effector T cells. They can also suppress effector activities of differentiated $CD4^+$ and $CD8^+$ T cells, and the function of NK cells, NK T cells, B cells, macrophages, osteoclasts, and DCs (reviewed in Von Boehmer H, 2005; Shevach EM *et al*., 2006; Miyara M and Sakaguchi S, 2007; Tang Q and Bluestone JA, 2008).

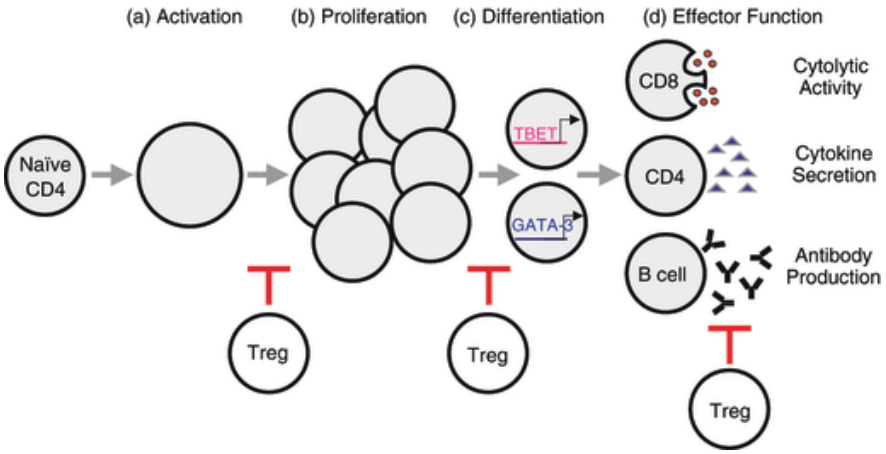



**Figure 13. Regulatory T cells may exert their suppressive activity at different stages during the development of an immune response, from activation of a T cell to its effector function (Sojka DK *et al.*, 2008).**

The mechanisms of Treg action remain poorly understood and contentious. Differences between *in vitro* and *in vivo* requirements, particularly with regard to the inhibitory cytokines IL-10 and TGFβ, have fuelled the controversy. *In vitro*, Treg cells suppress the proliferation and cytokine production (in particular of IL-2) of responder T cells when the two populations are co-cultured and stimulated by antigen in the presence of APCs (Takahashi T *et al.*, 1998; Thornton AM and Shevach EM, 1998). Once activated by a particular antigen, Treg cells can suppress responder T cells irrespective of whether they share antigen specificity (Takahashi T *et al.*, 1998).

Three mechanisms of Treg-mediated suppression have been proposed; they include cell-contact-dependent suppression, secretion of immunosuppressive cytokines, and local competition for growth factors [**Figure 14**]. More than one mechanism of Treg-mediated suppression may operate for the control of a particular immune response, in a synergistic and sequential manner. All these mechanisms require close spatial proximity between the suppressor and suppressed cells, either in the target tissue or the regional lymph nodes.

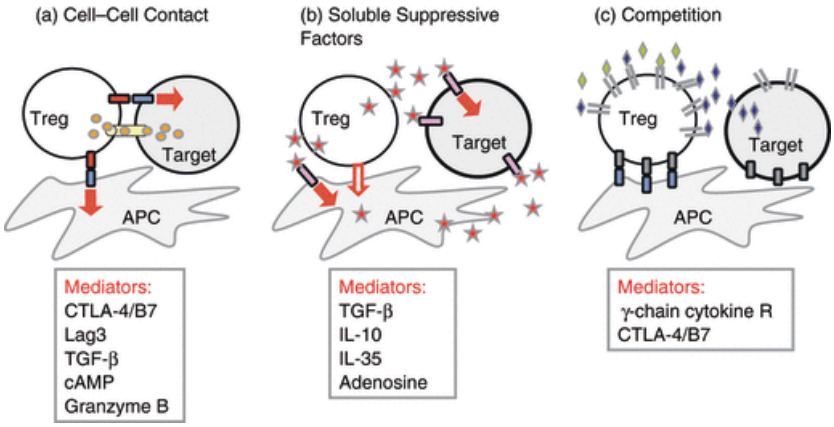



**Figure 14. Proposed mechanisms of regulatory T cell suppression (Sojka DK et al., 2008).**

(a) The cell-contact-dependent mechanism of suppression is still controversial. Transwell experiments have shown that direct cell-cell contact is indispensable for effective suppression. Several cell surface molecules on Treg have been proposed for this direct interaction, among which GITR (Shimizu J *et al*., 2002), CTLA-4 (Paust S *et al*., 2004), membrane-bound TGFβ (Nakamura K *et al*., 2001), LAG-3 (Huang CT *et al*., 2004) and the cytolytic molecules Fas and granzyme B (Janssens W *et al*., 2003; Cao X *et al*., 2007; Zhao DM *et al*., 2006). Another pathway is the modulation of cyclic adenosine monophosphate (cAMP) levels in the target cells; indeed, elevation of cAMP levels is associated with inhibition of cellular proliferation, differentiation and cytokine gene expression, including IL-2 and IFNγ (Minguet S *et al*., 2005).

(b) Treg cells have been shown to produce immunoregulatory molecules such as TGFβ and IL-10, which directly affect the activity of cytotoxic T cells and APCs. TGFβ1 reduces cytokine secretion by activated $CD4^+$ T cells (Zheng SG *et al*., 2004), without limiting their capacity to expand and without inducing their apoptosis, whilst IL-10 enhances the response of activated T cells to TGFβ1 through regulation of TGF receptor expression (Cottrez F and Groux H, 2001). Thus, combined effects of TGFβ and IL-10 inhibit the activity of effector T cells with minor effect on their expansion. Recently, a study in mice has shown that natural Treg cells predominantly produce immunosuppressive IL-35, a new member of the IL-12 family (Collison LW *et al*., 2007).

(c) The constitutive expression of CD25 by Treg cells gives them an initial competitive advantage for the consumption of IL-2 over naïve T cells, which express CD25 only after TCR stimulation. It was recently shown that Treg-mediated competition for growth factors leads to cytokine deprivation-induced apoptosis in the target effector cells, both *in vitro* and *in vivo* (Pandiyan P *et al*., 2007). However, Treg-mediated suppression cannot be entirely explained by the competitive consumption of IL-2; indeed, Treg cells can suppress the autoimmune response of IL-2R-deficient T cells (Fontenot JD *et al*., *Nat Immunol*, 2005), and provision of exogenous IL-2 in *in vitro* co-cultures enables target T cells to proliferate in the presence of Treg whilst endogenous target T cell production of IL-2 remains suppressed (Thornton AM *et al*., 2004; Sojka DK *et al*., 2005).

Many of the suppressive mechanisms that have been discussed here are not essential for Treg activity and may play a supportive role to an unidentified master regulatory function. To accommodate the variety of immune functions regulated by Treg cells [**Figure 13**], they may use a suppressive mechanism that targets an essential and common step in lymphocyte activation.

The suppressive activity of Treg cells has to be counterbalanced in cases where mounting an effective immune response is required, such as in the case of microbial infections. This inhibition of Treg activity can be either achieved by negatively regulating the Treg cells

themselves or by positively arming the target cells against Treg suppressive activity. A variety of proinflammatory signals, such as Toll-like receptor triggering (Liu G and Zhao Y, 2007) and direct inhibition by TNFα (Valencia X *et al*., 2006), can modulate Treg function. On the other hand, elevating the strength of target T cells activation level, through increasing antigen dose or provision of costimulatory signals (by upregulation of CD80 and CD86), has been shown to abrogate Treg-mediated suppression (George TC *et al*., 2003).

## 2.4.3. Adaptive regulatory T cells

Besides naturally-occurring regulatory T cells which develop in the thymus and display a diverse TCR repertoire that is specific for self antigens (Fontenot JD, *J Exp Med*, 2005; Hsieh CS *et al*., 2004), other subsets of regulatory T cells can also be "induced", "adapted" or "converted" from effector T cells during inflammatory processes in peripheral tissues, or experimentally generated for therapeutic purposes (Hawrylowicz CM and O'Garra A, 2005; Izcue A *et al*., 2006; Roncarolo MG *et al*., 2006). These regulatory cells do not always express FoxP3, are induced in the periphery much like $T_H1$ or $T_H2$ cells, and are generated to self and foreign antigens. Adaptive Treg cells appear to rely primarily on immunosuppressive cytokines to mediate suppression both *in vivo* and *in vitro*, although they have also been reported to suppress in a cell-contact dependent manner similar to natural Treg cells. It is likely that these different populations of Treg cells work together in a coordinated manner to regulate immune responses. To date, two subsets of adaptive Treg cells have been well characterized, T regulatory 1 ($T_r1$) and T helper 3 ($T_H3$) cells.

### *2.4.3.1. $T_r1$ cells*

$T_r1$ cells are a predominantly IL-10-expressing population that have been shown to cure models of inflammatory bowel disease in mice (Groux H *et al*., 1997; Vieira PL *et al*., 2004). Unlike natural Treg, $T_r1$ cells are able to produce IL-10 and TGFβ and do not express FoxP3, yet their properties *in vitro* are very similar to those of $FoxP3^+$ Treg cells. For example, they exhibit diminished proliferation in response to antigenic stimulation, exert cell contact-dependent suppression, scarcely produce IL-2, and display an activated cell surface phenotype (Vieira PL *et al*., 2004). These cells can be generated in several manners, such as by activating CD4 cells in the presence of IL-10, by culturing CD4 cells in the presence of dexamethasone and vitamin D3, by activating naïve CD4 cells through the TCR and the complement regulatory protein CD46, or by culturing them with IFNα (Groux H *et al*., 1997; Allan SE *et al*., 2005; Barrat FJ *et al*., 2002). Interestingly, $T_r1$ cells generated *in vivo* by combined IL-10 and rapamycin treatment in the presence of antigen were able to transfer antigen-specific tolerance to naïve untreated mice (Battaglia M *et al*., *Diabetes*, 2006).

#### *2.4.3.2. $T_H3$ cells*

$T_H3$ cells are also a suppressive CD4 population that secrete TGFβ and are able to inhibit immune responses *in vivo* (Chen Y *et al.*, 1994). They were initially identified in Peyer's patches and in the mesenteric lymph nodes. These cells have been implicated in models of oral tolerance and can be generated *in vitro* by culture in the presence of TGFβ (Verbsky JW, 2007).

### 2.4.4. Regulatory T cells in clinical situations

Naturally arising regulatory T cells have been initially shown to actively maintain immunological self-tolerance. Deficiency in or dysfunction of these cells may be a cause of autoimmune disease. However, it was subsequently appreciated that a reduction in their number or function can also elicit tumor immunity, whereas their antigen-specific population expansion can establish transplantation tolerance. The other clinical situations in which tolerance mediated by regulatory T cells has been implicated are allergy, microbial infections, and fœto-maternal tolerance [**Figure 15**].

Increasing Treg numbers and/or enhancing their suppressive function may be beneficial for treating autoimmune diseases and for preventing allograft rejection. On the other hand, depletion of Treg cells and/or inhibition of their function could enhance immunity against tumors and chronic infectious agents. As said previously, we focused our interest on $CD4^+$ $CD25^+$ T cells in the context of human solid organ transplantation.

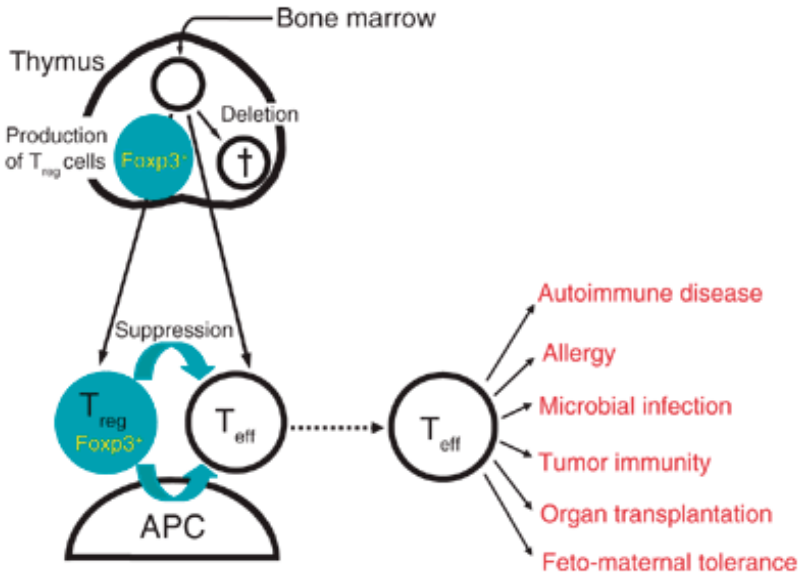



**Figure 15. Clinical situations in which Treg-mediated tolerance is implicated (Sakaguchi S, 2005).**

## *2.5. Transplantation immunology*

Transplantation immunology refers to an extensive sequence of events that occurs after an allograft or a xenograft is removed from a donor and then transplanted into a recipient. Tissue is damaged at both the graft and the transplantation sites. An inflammatory reaction follows immediately, and innate immune responses are initiated. Cellular components of the immune system are believed to produce mediators that perpetuate the inflammation and promote maturation of adaptive immune responses. Innate immunity is triggered when highly conserved molecules on the surface of injured cells engage receptors expressed by cells of the innate immune system. Eventually, damage is controlled through tissue repair and reinforcement; if damage is nonpathologic, the graft survives.

Antigen-independent causes of tissue damage (i.e., ischemia, hypothermia, reperfusion injury) are the result of mechanical trauma as well as of disruption of the blood supply when the graft is harvested. In contrast, antigen-dependent causes of tissue damage involve immune-mediated damage, which can eventually lead to graft rejection and organ loss.

### 2.5.1. Basic rules of graft rejection

Graft rejection is an immunological response mediated primarily by T cells. It results from a complex process involving both innate and acquired immune systems. The basic rules of tissue grafting were first elucidated by skin transplantation between inbred strains of mice (Billingham RE *et al*., 1954), leading to the following definitions:

- autograft: graft between different sites on the same animal or person;
- syngeneic graft: graft between genetically identical animals or people (e.g., identical twins);
- allograft: graft between genetically unrelated individuals of a same species;
- xenograf: graft between individuals of different species.

Only autograft and syngeneic graft are accepted without immunosuppressive treatments, whilst allograft and xenograft are rapidly rejected. Another observation that was made at that time is that when a mouse is grafted for a second time with skin from the same donor, it rejects the second graft faster (within 4-6 days instead of 10-13 days). This is called a second-set rejection and this accelerated response is MHC-specific; skin from a second-donor of the same MHC type is rejected equally fast, whereas skin from a MHC-different donor is rejected in a first-set pattern. Furthermore, naïve mice that are given T cells from a sensitized donor behave as if they had already been grafted [**Figure 16**].

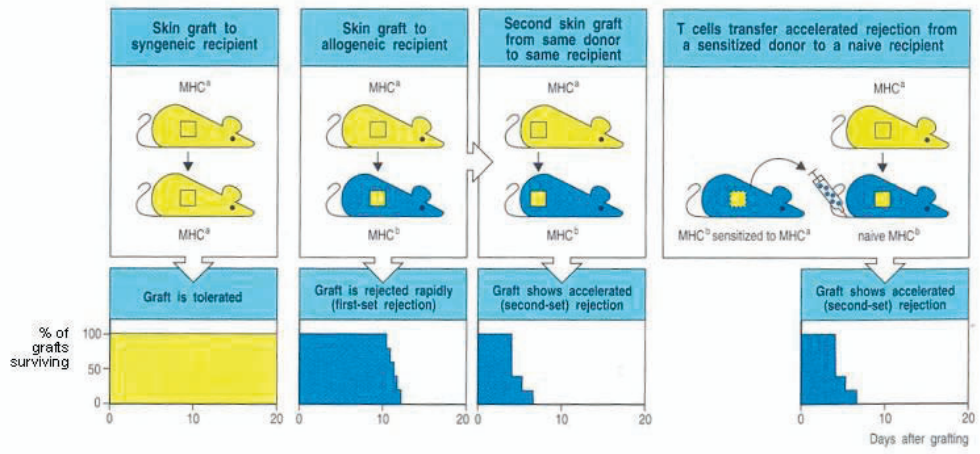



**Figure 16. Skin graft rejection is the result of a T cell-mediated anti-graft response (Janeway CA *et al*., 2001).**

**First panel: skin graft to syngeneic recipient, leading to indefinite acceptance.**

**Second and third panels: skin graft to allogeneic recipient; first-set rejection (second panel) and second-set rejection (third panel).**

**Fourth panel: T cell transfer from sensitized donor to naïve recipient, leading to second-set rejection.**

Graft rejection is the result of an alloreactive response, which can be defined as an immune response directed against nonself allogeneic antigens (alloantigens), developing in a transplant recipient. The alloreactive response can be divided into three successive stages, similarly to the "three-signal model": first, the recognition of alloantigens by naïve host T cells; second, the activation and the expansion of alloreactive T cells; and third, the effector phase, which consists in the destruction process itself.

## 2.5.2. Nature of alloantigens

Transplants can express two types of alloantigens, defined as major and minor based on the speed with which skin grafts were rejected between species before the molecular nature of alloantigens was discovered. Major alloantigens consist in allogeneic MHC class I and class II molecules borne by donor APCs or present on the graft and processed by recipient APCs. They represent the major barrier to successful transplantation. Minor alloantigens are constituted by allopeptides that are presented by either class I or class II MHC molecules shared by both donor and recipient (Simpson E, 1998); they derive from proteins that are either only present in the donor, such as the male antigen H-Y, or present in both but displaying differences in amino-acid sequence between donor and recipient (Zelenika D *et al*., 1998). In the context of transplantation, host T cells can recognize MHC antigens through two non-mutually exclusive different pathways: the direct and indirect pathways of alloantigens recognition.

## 2.5.3. Pathways of MHC alloantigens recognition

Before they can cause rejection, naïve alloreactive T cells must be activated by APCs (of donor or recipient origin) with adequate co-stimulation [**Figure 17**].

In the direct pathway of allorecognition, APCs of donor origin carried by the graft migrate via the lymph to regional lymph nodes, where their intact allo-MHC can activate those host T cells that bear the corresponding TCR (Sherman LA and Chattopadhyay S, 1993). The activated alloreactive effector T cells are then carried back to the graft, which they attack directly. The allo-MHC class I antigens are recognized by the TCR of $CD8^+$ T cells, whereas the allo-MHC class II antigens are recognized by the TCR of $CD4^+$ lymphocytes.

In the indirect pathway of allorecognition, allogeneic proteins borne by the graft are taken up and processed by the recipient's own APCs, and presented to T cells by self-MHC molecules. Among the graft-derived peptides are the minor H antigens and also peptides from the foreign MHC molecules themselves, either class I or class II, which are a major source of the polymorphic peptides recognized by the recipient's T cells (Simpson E *et al*., 2002). $CD4^+$ T cells specific for allogeneic peptides presented by self class II MHC molecules dominate the indirect pathway of T cell allorecognition (Fangmann J *et al*., 1992; Liu Z *et al*., 1992); actually, this corresponds to the physiological processing of soluble extracellular proteins that APCs are performing continuously.

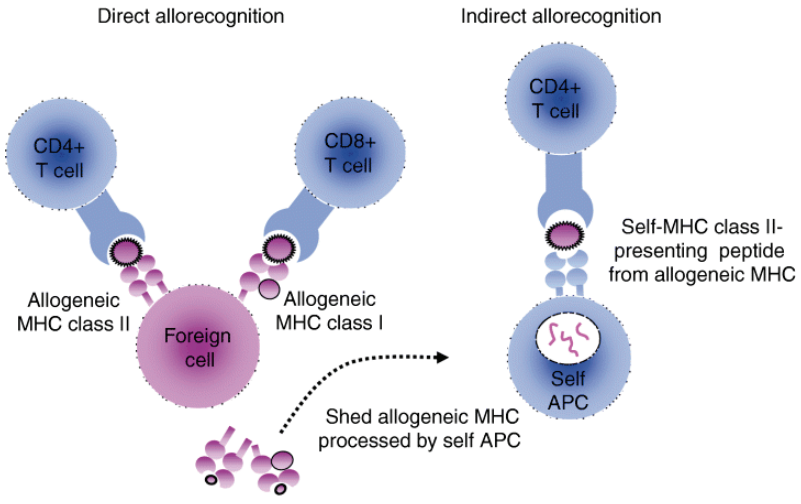



**Figure 17. Direct and indirect pathways of T cell allorecognition (Whitelegg A and Barber LD, 2004).**

The vast majority of T cells that respond to allogeneic MHC molecules do so via the direct recognition pathway. T cells with direct allospecificity account for more than 90% of the total alloimmune repertoire (Benichou G *et al*., 1999). Direct T cell allorecognition is the principle player in the initial vigorous immune response to allogeneic cells that causes acute rejection (Pietra BA *et al*., 2000) and is unique to transplantation. With time, the direct alloresponse

declines (Baker RJ *et al*., 2001). This may reflect gradual loss by natural cell death of the allogeneic DCs that activate the direct alloresponse (Lechler RI and Batchelor JR, 1982). Self-APCs, however, continue to acquire allogeneic MHC from foreign cells for processing and presentation by self class II MHC molecules. Indirect T cell allorecognition therefore comes to the fore and plays an important role in late onset chronic rejection (Vella JP *et al*., 1997; SivaSai KS *et al*., 1999).

## 2.5.4. Role of professional APCs

In the context of transplantation, both direct and indirect pathways of T cell allorecognition can provide the first step in the activation of naïve T cells ("signal 1"). During priming by professional APCs, naïve alloreactive T cells also receive costimulatory signals ("signal 2"), allowing transduction of several intracellular signals, which lead to transcription of numerous genes involved in immune activation, such as chemokines and cytokines genes, and also of costimulatory molecules themselves ("signal 3").

In the setting of the direct pathway of alloantigens recognition, naïve alloreactive T cells first encounter donor APCs in the T cell area of lymph nodes or spleen. As a consequence of ischemia/reperfusion injuries due to the transplantation, donor APCs residing in the graft become rapidly activated and migrate out of the graft to recipient lymphoid tissues. At the same time, they undergo a maturation process consisting in the increased expression of MHC and costimulatory molecules. Both activation and maturation of donor APCs make them fully suitable for subsequent T cell activation.

In the setting of the indirect pathway, immature circulating recipient APCs migrate from the blood into the graft through the combined effects of the chemokines released by the graft itself and the increased expression of adhesion molecules on graft endothelial cells. There, they ingest and process donor MHC molecules before they return to the lymph nodes or spleen where they initiate the priming of T cells (Lechler R *et al*., 2001). Of note, mice lacking lymph nodes and spleen are able to accept cardiac allografts indefinitely (Lakkis FG *et al*., 2000).

As a consequence of T cell activation, effector elements are generated that mediate graft injury and are responsible for the clinical manifestations of allograft rejection. They include cellular (T cell cytotoxicity) and/or humoral (alloantibodies) components, as described hereafter.

## 2.5.5. Alloreactive T cell cytotoxicity

Once activated, T cells acquire cytotoxic properties that enable them to kill their targets. The two main cytotoxic mechanisms are the perforin/granzyme and the Fas/Fas-ligand (FasL) systems [**Figure 18**].

The perforin/granzyme pathway is mainly used by CD8 T cells and NK cells. Perforin and granzymes are rapidly synthesized after CD8 T cell activation, and stocked as intracellular granules (Shresta S *et al*., 1998). Upon CD8/TCR-allo-MHC molecule recognition, a tight junction is formed, allowing intracellular cytotoxic granules to fuse with the target cell membrane. Perforin molecules are able to form pores in the target cell membrane, through which granzyme molecules penetrate in the cytoplasm and the nucleus, leading to DNA fragmentation and apoptosis of the target cell (Russell JH and Ley TJ, 2002). This chain of events is known as the granzyme exocytosis pathway. Both perforin and granzymes molecules are needed for maximal cytotoxicity of CD8 T cells; indeed, perforin-deficient mice display impaired *in vitro* target cell lysis, whilst granzymes A and B knockout mice are able to make cell membrane pores but do not induce target cell apoptosis (Simon MM *et al*., 1997).

Along with the granzyme exocytosis pathway, CD8 CTLs can also utilize the Fas-dependent pathway to induce cytolysis and apoptosis (Ju ST *et al*., 1994). In the Fas-dependent pathway, FasL is either packaged into cytotoxic granules with perforins and granzymes or is trafficked directly to the activated effector cell surface for binding to the target cell. Whereas Fas is constitutively expressed on most cell surfaces (Peter ME and Krammer PH, 1998), FasL is essentially inducible. Binding of FasL on the effector cell to Fas on the target cell membrane triggers apoptosis ultimately through the same effector mechanisms as in the granzyme exocytosis pathway.

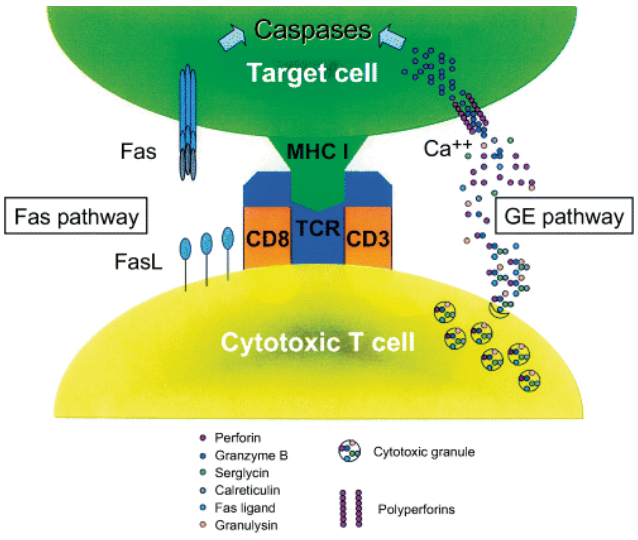



**Figure 18. Mechanisms of cytotoxic T lymphocyte-induced graft damage (Rocha PN et al., 2003).**

**FasL: Fas ligand; GE: granzyme exocytosis pathway; MHC: major histocompatibility complex; TCR: T cell receptor.**

CD4 T cells may also eliminate cells expressing MHC class II antigen through a Fas-dependent or Fas-independent mechanism (Williams NS and Engelhard VH, 1996). In addition, CD4 $T_H1$ cells can mediate delayed-type hypersensitivity (DTH) responses via the production of soluble mediators, such as IFNγ and TNF, that function to activate and guide immune cells, including monocytes and macrophages, to the site of injury. This activation

causes a further amplification of cytokine and chemokine production, along with generation of proteolytic enzymes, nitric oxide, and other soluble factors that perpetuate the local inflammatory response. These mediators may also directly alter graft function by modulating vascular tone, permeability and integrity, or by promoting platelet aggregation.

## 2.5.6. Alloantibodies

Alloreactive CD4 T cells also promote B cell maturation and differentiation into antibody-secreting plasma cells via CD40-CD40 ligand interactions. Alloantibodies that are produced by these B cells exert most of their detrimental effects on the graft by activating the complement and coagulation cascades (Brauer RB *et al*., 1995). Alternatively, antibodies can bind Fc receptors on NK cells or macrophages and cause target cell lysis via antibody-dependent cell-mediated cytotoxicity (ADCC) [**Figure 19**]. Alloantibodies may be present already before transplantation, through transfusions, pregnancy, or previous transplantation, or may develop *de novo* (Brändle D *et al*., 1998). Preformed alloantibodies (anti-HLA or anti-blood group A or B) are able to immediately bind to graft endothelial cells after revascularization, activating the complement and coagulation cascades and leading to hyperacute rejection and transplant loss from graft infarction (Baldwin WM 3rd *et al*., 2001).

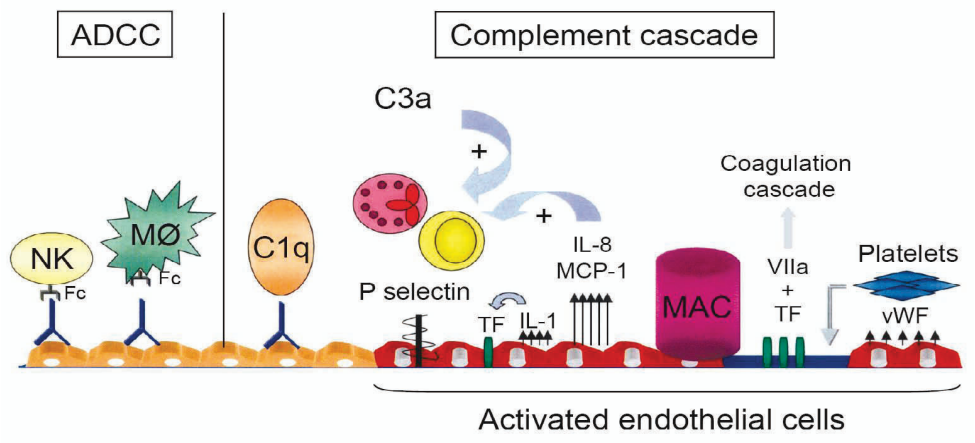



**Figure 19. Mechanisms of antibody-mediated graft damage (Rocha PN *et al*., 2003).**

**ADCC: antibody-dependent cell-mediated cytotoxicity; MCP-1: monocyte chemotactic protein-1; vWF: von Willebrand factor.**

In conclusion, there are many distinct effector pathways that can by themselves lead to the same outcome, namely allograft rejection. One consequence of this redundancy of the alloimmune effector response may be the difficulty to set up a reliable *in vitro* assay that may help to monitor the tolerance state of allograft recipients.

## 2.5.7. Types of allograft rejection

The following discussion will be focused on what happens in the context of renal transplantation since our study considered almost exclusively renal transplant recipients. On a temporal point of view, one can distinguish three different types of allograft rejection: hyperacute, acute, and chronic rejection. The past two decades have seen 1-year renal allograft survival increase from 50% to nearly 90% in cadaveric donors and 95% in living related donors (Cecka JM, 2002), this increase being largely due to the use of calcineurin inhibitor (CNI)-based immunosuppression (Starzl TE *et al*., 1981), the better treatment of acute rejection episodes, and the improvement in tissue typing and organ preservation techniques. However, 10-year survival rates fall dramatically to 51% and 68% respectively, of which 50-80% are attributable to chronic rejection.

### *2.5.7.1. Hyperacute rejection*

As mentioned earlier, hyperacute rejection (HAR) is the consequence of the presence of large quantities of preformed circulating alloantibodies against ABO or MHC proteins that bind these antigens on graft endothelial cells and activate the classic complement pathway. The actions of complement and inflammatory mediators transform the endothelium from a protective barrier between the blood and extravascular tissues into a procoagulant, chemoattractive and adhesive interface that promotes inflammation. Once endothelial cells are damaged, the underlying matrix is exposed, activating the extrinsic coagulation pathway. The end result of these processes is widespread intravascular thrombosis, haemorrhage and tissue injury. Pathological examination reveals obstruction of small vessels by platelet thrombi, interstitial haemorrhage and severe injury to endothelial cells, with immunoglobulin and complement deposits. HAR remains a major barrier to xenotransplantation. Given the lack of effective treatment, it almost invariably leads to allograft loss within minutes to few hours after transplantation, at least in the case of renal transplantation, since heart and liver transplants seem relatively resistant to HAR. However, thanks to the advent in blood typing and T cell cross-match testing, hyperacute rejection has become a very rare event in the clinical practice.

### *2.5.7.2. Acute rejection*

The precise pathogenesis of acute rejection (AR) is still controversial. It was historically considered as a pure cell-mediated process, since experiments had shown that mice lacking T cells were unable to reject fully mismatched allografts and that reconstitution of these mice with T cells restored the rejection process (Mombaerts P *et al*., 1992). However, it is now clear that acute rejection may also be antibody-mediated and represents a distinct clinicopathologic entity (Colvin RB, 2007). The gold standard for diagnosis of acute rejection (cellular or humoral) remains the histopathologic examination of a renal allograft biopsy (90% sensitivity). Standardization of interpretation of rejection-related histopathology has

evolved over the past decade, primarily due to the introduction of the Banff criteria in 1993 (Solez K *et al*., 1993), which were regularly revised since then [see **Appendix I** for the latest update (Solez K *et al*., 2007)]. Acute rejection may occur days, weeks, or even years after transplantation.

#### 2.5.7.2.1. Acute T cell-mediated rejection

Clinically, patients with acute T cell-mediated rejection (TCMR) present with a sudden rise in serum creatinine, fluid retention, and sometimes fever and graft tenderness. With current induction regimens and immunosuppressive therapies (both largely directed at T cells), TCMR incidence has dropped to 5-10% in the first year after renal transplantation in unsensitized patients. On pathological examination, it is manifested by a massive infiltration of mononuclear cells, essentially T cells, in the interstitium, tubules ("tubulitis") and sometimes arteries ("endarteritis") [**Figure 20**].

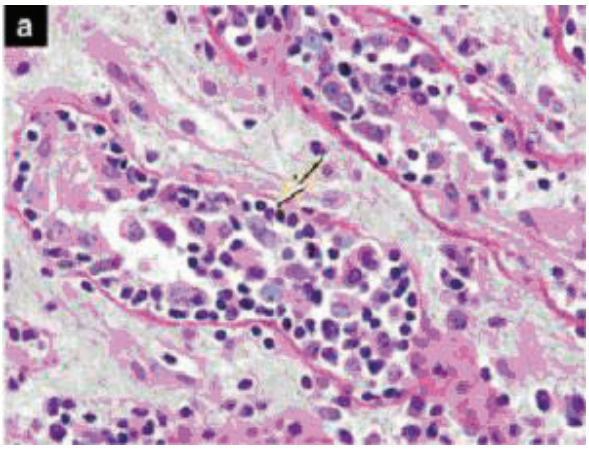



**Figure 20. Pathological examination of a renal biopsy with acute T cell-mediated rejection (Cornell LD *et al*., 2008).**

**Multiple mononuclear cells infiltrate the tubules ("tubulitis", *arrow*).**

Both tubulitis and endarteritis lesions are T cell-dependent and may occur in the absence of B cells and alloantibodies (Jabs WJ *et al*., 2003; Russell PS *et al*., 1997). During acute rejection, a variety of chemokines are produced in the graft, including CxCL10, CCL2, CCL3, CCL4, CCL5 and lymphotactin (Segerer S *et al*., 2001). Tubules are also a source of chemokines CCL2, CCL3, CCL4, CCL5, CxCL8 (IL-8) and $Cx_3CL1$, and cytokines TNFα, TGFβ and IL-6 (Robertson H *et al*., 2000; Cockwell P *et al*., 2002; Ali S *et al*., 2005; Al-Lamki RS *et al*., 2001).

#### 2.5.7.2.2. Acute antibody-mediated rejection

Acute humoral rejection (AHR) accounts for approximately 25% of all acute rejection episodes, but may be mixed with a component of T cell-mediated rejection. Risk factors for developing AHR include presensitization (i.e., presence of anti-donor HLA antibodies before transplantation) and decreased immunosuppression (e.g., due to non-compliance). Besides HLA molecules, other proteins can serve as targets if expressed on graft endothelial cells,

including ABO blood group antigens, putative endothelial alloantigens (Collins AB *et al*., 2006) and even auto-antibodies (e.g., those to angiotensin II type 1 receptors (Dragun D *et al*., 2005)). Like patients with TCMR, those with AHR present with an abrupt rise in serum creatinine. A renal biopsy is required to distinguish both entities. Pathologically, the kidney usually shows an accumulation of neutrophils and monocytes in peritubular and glomerular capillaries (Tinckam KJ *et al*., 2005; Fahim T *et al*., 2007). Recognition of AHR has become easier with the advent of C4d staining (Feucht HE *et al*., 1993), an inactive fragment of C4b of the classic complement pathway [**Figure 21**].

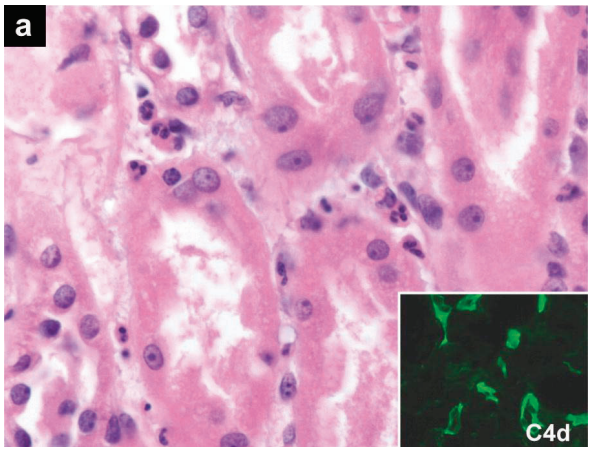



**Figure 21. Pathological examination of a renal biopsy with acute antibody-mediated rejection (Cornell LD *et al*., 2008).**

**Peritubular capillaries show margination of neutrophils. C4d is detected in these capillaries by immuno-fluorescence (*inset*).**

C4d *per se* has no known functional role, but remains bound in the tissue for several days after immunoglobulin and C1 have been released. C4d deposits in the majority of the peritubular capillaries as an intense ring pattern, detectable using immunofluorescence microscopy (Regele H *et al*., 2002). Circulating donor-specific alloantibodies (DSAs; to donor HLA class I or II antigens) are present in 88-95% of the patients who have C4d deposition (Colvin RB, 2007). However, AHR can occur in the absence of demonstrable circulating antibodies, which is probably due to the absorption of alloantibodies by the graft (Martin L *et al*., 2005).

### *2.5.7.3. Chronic rejection*

Clinically, chronic rejection (CR) manifests as a progressive deterioration in renal function, usually associated with hypertension and proteinuria, in the absence of another specific pathology. It is the most important cause of renal graft failure after the first year following transplantation. At five years, CR affects 30% to 40% of renal transplants, whilst it generally admitted that at 10 years more than 50% of kidney transplant recipients suffer from CR. The diagnosis of CR is made purely upon histological findings. It may occur by cellular or humoral mechanisms, or both. Chronic changes can be seen in the glomeruli, vessels, tubules, and interstitium. Histological features of chronic rejection include transplant glomerulopathy, peritubular capillaropathy, transplant arteriopathy, and, less specifically, interstitial fibrosis

and tubular atrophy. The pathogenesis of CR depends on multiple immunological as well as non-immunological factors.

Immunological factors that contribute to the development of CR include acute rejection episodes, especially those arising more than three months after transplantation (Nankivell BJ *et al*., 2001). In addition, there is increasing evidence that subclinical rejection, defined as histologically proven acute rejection in the absence of functional graft deterioration, is an important factor in predicting the subsequent development of CR (Veronese FV *et al*., 2004). HLA mismatching (Kasiske BL, 1997) as well as presensitization and production of anti-HLA antibodies after transplantation (Davenport A *et al*., 1994) correlate both with increased risk of CR. In this regard, detection of C4d deposits in peritubular capillaries may be a useful diagnostic marker (Mauiyyedi S *et al*., 2001).

Non-immunological risk factors for CR development include delayed-graft function (DGF; Cosio FG *et al*., 1997), ischemic-reperfusion injury (Szabo A and Heemann U, 1998), increasing donor age (Halloran PF *et al*., 1999), cytomegalovirus (CMV) infection (Humar A *et al*., 1999), cadaveric donor with brain death (Koo DD *et al*., 1999), toxicity of CNIs (cyclosporine or tacrolimus; Nankivell BJ *et al*., 2004), and donor/recipient comorbidities such as hypertension and hypercholesterolemia (Paul LC and Benediktsson H, 1995; Fernandez-Miranda C *et al*., 1997).

Although the risk factors are well recognized, the precise pathogenesis of CR remains incompletely understood. Both immunological and non-immunological factors seem to result in activation of the transplant vascular endothelium, which sets in motion a cascade of events leading to vascular dysfunction, and, ultimately, organ dysfunction. These events include induction of a procoagulant state, release of proinflammatory cytokines, expression of adhesion molecules, leucocyte activation, vasoconstriction, and capillary leakage (Karimova A and Pinsky DJ, 2001). The efflux of proinflammatory cytokines enhances the migration and localization of neutrophils, monocytes, macrophages and T lymphocytes to the site of endothelial injury. Additionally, there is increased expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1) and of MHC class I and II molecules (Fuggle SV *et al*., 1993). Experimental models have shown the importance of macrophages and T cells in the generation of fibrosis in CR (Pilmore HL *et al*., 2000). There is some evidence that the nature of infiltrating T cells ($T_H1$ or $T_H2$) may contribute to the progression of CR; unfortunately, at present, there is contradictory evidence as which subset is associated with the generation of CR or which is protective (Uboldi de Capei M *et al*., 2004; Nocera A *et al*., 2004). Macrophages secrete a wide range of profibrotic cytokines (TNFα, IL-1β, PDGF, bFGF, TGFβ) and matrix proteins, resulting in the characteristic accumulation of extracellular matrix seen in CR. One putative mechanism of fibrosis is by epithelial-mesenchymal transition (EMT) of tubular cells to an activated myofibroblast that migrates into the interstitium (Robertson H *et al*., 2004).

Despite a better understanding of the physiopathology of CR, there are to date no known drug regimens that inhibit or reverse its progression. Efforts should be made to prevent CR, with measures directed to the controllable risk factors such as presensitization and HLA histocompatibility. But efforts should also be made to detect subclinical rejection and insufficient immunosuppression beyond the early phase after transplantation, since sub-detectable immune activation is for sure the major immunological cause of CR. Detection strategies should be able to identify and predict immunological events prior to the manifestation of clinical parameters indicating graft failure. The available methods of immune monitoring as well as the future directions will be briefly discussed in the next section.

## 2.5.8. Tools for immune monitoring

A major challenge in the follow-up of transplant recipients is in the adequate "tuning" of the patients' immune function. The anti-allograft immune response must be suppressed in order to prevent the occurrence of rejection; on the other hand, over-immunosuppression is associated with undesirable side effects, such as opportunistic infections and development of malignancies, and must be avoided.

In order to be useful, the candidate tests need to be accurate, specific and widely adoptable with a rapidity of data interpretation. To fulfill these criteria, they need to be tested in large patient cohorts, over adequate observation periods and over a range of disease severities and types. Ideally, they should be detectable in a noninvasive manner, for example in the blood or urine in the case of renal transplantation.

To date, the most obvious but most basic tests are measurement of serum creatinine, creatinine clearance and proteinuria, that are used to monitor graft function. However, both are biomarkers of clinical function and cannot provide specific information concerning the state of the recipient's immune system. Indeed, histological biomarkers of graft biopsies according to the Banff classification system are currently the gold standard for diagnosing the status of organ transplants, indirectly reflecting immune reactivity towards the organ transplant (Rush D, 2006). Protocol biopsies (and treatment of subclinical acute rejection episodes with corticosteroids when indicated) have been shown to lead to better outcome (Shishido S *et al*., 2003). Nevertheless, the invasive (and therefore risky) and expensive nature of biopsies are major constraints to their repetitive use as would be required for immune monitoring. Currently, the measurement of immunosuppressive drugs levels in blood is the sole method to monitor transplant immunosuppression. However, it has become clear that even strict compliance to prescribed dosage and drug therapeutic window levels prevent neither over-immunosuppression nor under-immunosuppression and their associated side effects. The analysis of circulating anti-HLA antibodies, including DSAs, by enzyme-linked immunosorbent assay (ELISA) and more recently by the Luminex technology, has also become a routine method of monitoring renal transplant recipients. Indeed, it has been shown

that presence of circulating anti-HLA antibodies (donor-specific or not) has a negative impact on graft outcome (Terasaki PI and Ozawa M, 2004; Langan LL *et al*., 2007). However, they are not highly accurate predictors of graft failure given that they can persist for years before any apparent deterioration of graft function (Lee PC *et al*., 2002), they appear late following transplantation (up to several years) (Hourmant M *et al*., 2005) and they can also be present in patients displaying operational tolerance (Roussey-Kesler G *et al*., 2006). Various cellular assays have been used in the past, such as the mixed leucocyte reaction (MLR) assay, cytotoxic T lymphocyte (CTL) assay and the limiting dilution assay (LDA). Although they can be informative in certain settings (Weimar W *et al*., 2004), they have generally proved too time-consuming for routine use. Subsequently, novel techniques have been developed, including the *transvivo* delayed-type hypersensitivity (DTH) test (VanBuskirk AM *et al*., 2000; Xu Q *et al*., 2007), the enzyme-linked immunospot (ELISPOT) assay (Gebauer BS *et al*., 2002; Hricik DE *et al*., 2003; Poggio ED *et al*., 2004), and blood phenotyping by multicolor flow cytometry and intracellular staining of cytokines (Martínez-Llordella M *et al*., 2007; Louis S *et al*., 2006), among others. Their respective principles, advantages and disadvantages are listed in **Table 1** (adapted from Hernandez-Fuentes MP *et al*., 2003, and Ashton-Chess J *et al*., 2009).

| Assay | Technique / detection | Advantages | Disadvantages |
|---|---|---|---|
| **MLR assay** | Bulk proliferation of recipient cells stimulated by donor cells, measured by $^{3}$H-thymidine incorporation | Direct alloreactivity | Low sensitivity, little reproducibility, little predictive value (Segall M *et al*., 1996) |
| **CTL assay** | Cytolytic activity of *in vivo* primed recipient T cells, measured by $^{51}$Cr release | Reproducibility, large experience in the clinical setting | Assessment of CD8 T cell activity only, long incubation period, use of radioactivity |
| **LDA** | Number of responding cells in a given population | Measures function and frequency | Requires *in vitro* expansion, labor intensive, mathematically complex |
| **DTH assay** | Index of reactivity of T cells to antigens, measured by quantification of resultant swelling in mouse footpad | Direct and indirect alloreactivity, uncover regulation, only assay used to study tolerant patients | Requires mice, cumbersome, large numbers of cells required, measurement of swelling subjective |
| **ELISPOT assay** | Frequency of effector/memory T cells, measured by detection of cytokine production | High sensitivity, relatively easy to use, measures frequency and function, short *in vitro* culture | Counting of cells/spots somewhat subjective |
| **Flow cytometry** | Frequency of specific cells, detected by flow cytometry | Phenotype and function of cells, easy to perform, requires very few cells, multiparametric staining possible | Limited sensitivity, limited experience in clinical transplantation |

**Table 1. Comparison of cellular assays for immunological monitoring (adapted from Hernandez-Fuentes MP *et al*., 2003, and Ashton-Chess J *et al*., 2008).**

Furthermore, there is a real expectation that these tests may be used to identify tolerance as much as to predict rejection. In some cases, the identification of immunological tolerance would allow the partial or even complete cessation of immunosuppression, a highly desirable goal, given the morbidity and mortality associated with long-term administration of these drugs. However, given the complexity and the redundancy of the immune system, multiple mechanisms may contribute to tolerance, and these mechanisms may change over time and differ depending on the tissues involved. Therefore, it is likely that a battery of tests is needed to define the phenotype of tolerance and/or its absence. In the next section, we will review the current state of knowledge in the field of tolerance in solid organ transplantation.

## 2.5.9. Tolerance in solid organ transplantation

In the context of solid organ transplantation, "tolerance" can be defined as a state of antigen-specific unresponsiveness that is sustained in the absence of chronic immunosuppression. Indeed, it is the ultimate goal in transplantation to be able to get rid of lifelong immunosuppression and its associated complications such as increased susceptibility to infections, development of tumors, and cardiovascular and metabolic complications. As soon as in 1953, Billingham, Brent and Medawar conceptualized transplantation tolerance and proved it is possible in laboratory animals (Billingham RE *et al*., 1953). Since then, numbers of publications have demonstrated that tolerance to allografts can be induced in experimental animal models and in human adult recipients, mainly by exploiting the natural mechanisms that maintain immune homeostasis and tolerance to self-antigens. It is now well recognized that immunological tolerance involves central as well as peripheral mechanisms.

Central tolerance is achieved in the thymus during the process of negative selection: thymocytes that react with a too high avidity with self-antigens are deleted by apoptosis (clonal deletion). This mechanism has been exploited by the delivery of donor antigens to the thymus of adult recipients leading to the intrathymic elimination of harmful alloreactive T cell clones. The delivery of donor antigens to the thymus can be achieved either experimentally by the intrathymic injection of donor-derived peptides (Markmann JF *et al*., 1993) or by the induction in the recipient of hematopoietic mixed chimerism with co-existence of cells of both recipient and donor origin (Ildstad ST and Sachs DH, 1984; Sayegh MH *et al*., 1991).

Peripheral tolerance is indispensable to control the few autoreactive cells that escape the negative selection process, as well as the autoreactive cells specific for self-antigens that are not expressed in the thymus. Peripheral tolerance includes schematically four distinct mechanisms: immunological ignorance, anergy induction, apoptosis/deletion, and active regulation.

Immunological ignorance is an uncommon mechanism of tolerance, but can be achieved either because the target antigens are present in too low concentration to trigger an immune response, or because they are sequestrated in locations which are not freely exposed to the

surveillance of the immune system (so-called "immunologically privileged sites", such as the eye, the central nervous system and the testis). In the context of transplantation, it is difficult to introduce donor cells or tissue without alerting the recipient immune system to their presence; however, attempts have been made in this direction by encapsulating cells such as islets of Langerhans before implantation (O'Shea GM and Sun AM, 1986).

T cells can be made non-responsive (anergic) to presented antigens if the T cell engages an MHC molecule without co-stimulatory molecules. Anergy will occur if there is no acute inflammation, leading to no co-stimulatory molecules upregulation due to the low concentration of cytokines. It has been described both *in vitro* and *in vivo*. Some forms of T cell anergy may also result in the development of regulatory activity (Lombardi G *et al*., 1994). In the context of transplantation, T cell anergy can be achieved by blockade of costimulatory signals (Sayegh MH and Turka LA, 1998). Various costimulatory molecules have been targeted in rodent models; the most successful results were obtained with the CD154:CD40 pathway blockade using an anti-CD40L monoclonal antibody (Graca L *et al*., 2000). Unfortunately, the humanized anti-CD40L antibody did not meet the expectations in transplant recipients (Kawai T *et al*., 2000). Another promising target is the CD28:CD80/86 pathway. Excellent results have been obtained with CTLA-4Ig, a fusion protein with specificity to CD80/86 expressed on APCs (Judge TA *et al*., 1999). A modified compound, LEA29Y (balatacept), was tested in phase II clinical trials in kidney transplant recipients, and was shown to be as effective as cyclosporine A (Vincenti F *et al*., 2005), although it did not induce transplantation tolerance.

Apoptosis or deletion of T cells occurs either passively by deprivation of growth factors, such as IL-2, IL-4, IL-9, IL-15 and IL-21, which do no longer stimulate the expression of anti-apoptotic genes (Bcl-2, Bcl-xl), or actively by activation-induced cell death (AICD) when IL-2 binds its receptor and stimulate the expression of the death ligand FasL. The importance of apoptosis in tolerance induction has been demonstrated in transgenic mice expressing constitutively the anti-apoptotic molecule Bcl-xl in T lymphocytes, as well as in IL-2-deficient mice; in both mice strains, T cells were shown to be resistant to apoptosis, and tolerance could not be achieved (Wells AD *et al*., 1999).

Finally, immunoregulation is an active process, whereby one population of cells controls or regulates the activity of another population. Various populations of leucocytes have been described as having the ability to control immune responsiveness to alloantigen stimulation in both the innate and adaptive immunity, among others $CD4^+$ $CD25^+$ regulatory T cells (Hall BM *et al*., 1990; Hara M *et al*., 2001), *in vitro* induced $T_r1$ cells (Groux H *et al*., 1997), TGFβ-producing $T_H3$ cells (Faria AM and Weiner HL, 2005), $CD8^+$ $CD28^-$ cells (Chang CC *et al*., 2002), $CD3^+$ $CD4^-$ $CD8^-$ T cells (Zhang ZX *et al*., 2000), and innate NKT cells (Kronenberg M, 2005). The involvement of regulatory T cells in transplantation tolerance was formally established in 1990 by Hall BM *et al*. in adoptive transfer studies in rats where

transplantation tolerance could be achieved with cyclosporine A. Subsequently, it was established that the suppression was mediated by $CD4^+$ T cells and that regulation could spread to, and recruit, further naïve cohorts of T cells over time as long as donor antigen remained in the system (so-called "infectious tolerance"), allowing the suppression to be sustained life-long (Qin S *et al*., 1993). Another important phenomenon in transplantation tolerance is the so-called phenomenon of "linked suppression»: strain A recipients tolerating grafts from strain B show a capacity to accept grafts from (BxC)F1 donors; in time, some animals accepting (BxC)F1 grafts become tolerant of C. This demonstrates that once regulators have been induced to a limited set of alloantigens, they can spread tolerance to other antigens in the same tissue (Davies JD *et al*., 1996). Treg cells can be found inside the tolerized graft (Graca L *et al*., 2002), and these cells can have indirect allospecificity for donor antigens (Hara M *et al*., 2001). In patients transplanted with lung (Meloni F *et al*., 2004), liver (Demirkiran A *et al*., 2006), or kidney grafts (Salama AD *et al*., 2003), a positive correlation between graft survival and the number of circulating Treg cells has been shown. Based on all these observations, it is now widely accepted that Treg cells play a pivotal role for the induction of transplantation tolerance in rodents as well as in human beings. Therefore, this has led to an increasing interest in the possibility of using Treg cells as a biological therapy to induce tolerance to alloantigens. Several immunotherapeutic strategies implicating the use of Treg cells have been developed, some of which being already tested in human clinical trials.

### 2.5.10. Potential therapeutic use of regulatory T cells in transplantation

Experimental studies in the past and in recent years have demonstrated the proof-of-principle that the adoptive transfer of Treg cells isolated from animals subject to different tolerizing protocols (costimulation blockade, donor-specific transfusion, CD4 T cell depleting or nondepleting antibodies) can prolong allograft survival in different transplantation models (reviewed by Kang SM *et al*., 2007). The advantages of Treg-based immunotherapy over conventional treatments are numerous, including the potential for antigen specificity with the lack of general immunosuppression, the possibility of inducing physiological long-lasting regulation *in vivo*, and the fact that the Treg-based immunotherapy can be individualized for each patient, with very limited side effects.

In mice, adoptive transfer of freshly isolated Treg cells together with a bone marrow allograft has been shown to ameliorate graft-versus-host disease (GVHD) and facilitate engraftment of the bone marrow (Joffre O *et al*., 2004). The same results were obtained with adoptive transfer of *ex vivo* polyclonally expanded donor Treg cells (Hoffmann P *et al*., 2002). Thanks to these promising results generated in animal models and to the lack of antigen-specific requirement for the transferred Treg cells, bone marrow transplantation is the setting for the

first human clinical trial, either with *ex vivo* generated Treg cells (group of B. Blazar and C. June, University of Minnesota, USA; unpublished data), or with freshly isolated unmanipulated Treg cells (group of M. Edinger, Regensburg, Germany; unpublished data). Another approach using $T_r1$ cells generated *ex vivo* upon stimulation with alloantigens in the presence of IL-10 and TGFβ has been shown to be effective in reducing the incidence of GVHD after bone marrow transplantation (Zeller JC *et al*., 1999). Again, this has led to the initiation of a clinical trial in humans (group of M.-G. Roncarolo, San Raffaele Scientific Institute, Milan, Italy; unpublished data).

In contrast to what has been reported in GVHD, there are to date no reports showing that the transfer of freshly isolated Treg cells can prevent the rejection of allogeneic solid organ transplantation. Several studies have demonstrated that Treg cells generated *in vivo* in transplanted animals by various approaches (such as by treatment with vitamin D3 and mycophenolate mofetil (Gregori S *et al*., 2001)) do transfer tolerance in secondary transplant recipients. It is likely that the lack of data proving efficacy after transfer of freshly isolated Treg cells may be due to unreported unsuccessful experiments and to the requirement of antigen-specific Treg cells for protection (Lee MK 4$^{th}$ *et al*., 2004). On the other hand, it has been shown that $T_r1$ cells generated *in vivo*, in mice transplanted with allogeneic pancreatic islets and treated with rapamycin and IL-10, transfer antigen-specific tolerance to secondary transplant recipients (Battaglia M *et al*., *Diabetes*, 2006). However, at present, the lack of data clearly showing that transferred Treg cells protect from allograft rejection in several preclinical animal models represents a major hurdle to the use of Treg-based immunotherapy in solid organ transplantation in humans.

Despite these encouraging results, it is necessary to continue research in the field of detection of immunological "tolerance signature", in order to detect operational tolerance and to adapt immunosuppressive therapies to individual characteristics, as well as in the field of induction regimens and maintenance immunosuppressive drugs, in order to understand their effects on survival and proliferation of immune cells, including Treg cells.

## 2.5.11. Immunosuppressive agents

Immunosuppressive agents are used for induction (intense immunosuppression in the initial days after transplantation), maintenance, and reversal of established rejection. Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic, or blocking lymphocyte response pathways [**Figure 22**].

Immunosuppression has three effects: the therapeutic effect of suppressing rejection, the undesired consequences of immunodeficiency, with development of malignancies and infections, and the nonimmune toxicity to other tissues. Immunodeficiency leads to characteristic infections, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and BK polyomavirus infections, and cancers, such as post-transplantation lymphoproliferative

disease (PTLD), which are directly correlated with the intensity of immunosuppression. Nonimmune toxicity is agent-specific and is often related to the mechanism that is used, because each agent or class of drugs targets molecules with physiologic roles in nonimmune tissues.

Immunosuppressive drugs can be classified into small-molecule drugs, depleting and nondepleting protein drugs (polyclonal and monoclonal antibodies), fusion proteins, intravenous immune globulins (IVIg), and corticosteroids [**Table 2**].

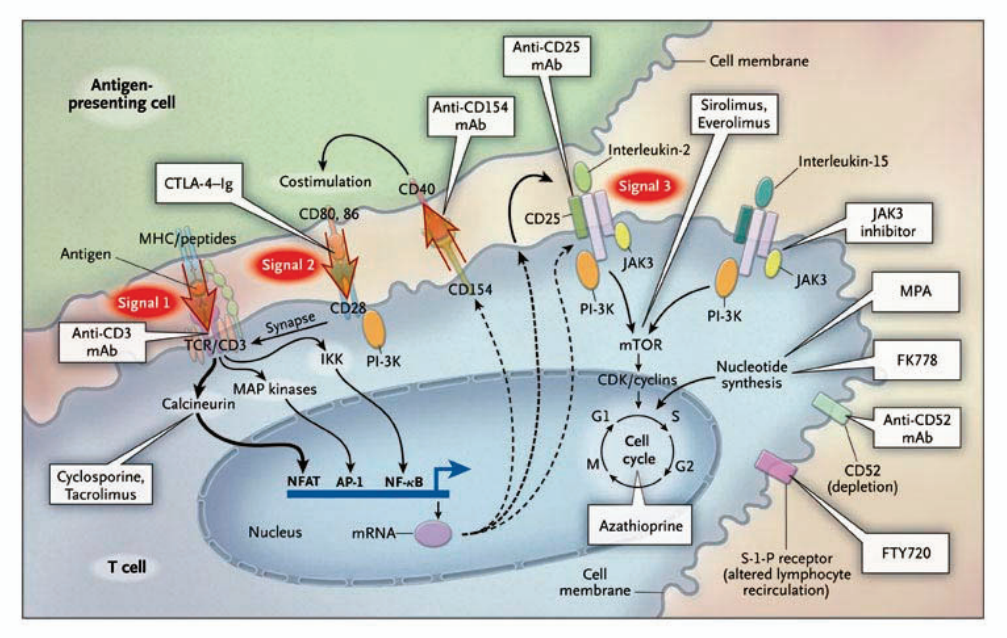



**Figure 22. Individual immunosuppressive drugs and sites of action in the three-signal model of alloimmune response (Halloran PF, 2004).**

**MPA: mycophenolic acid (active principle of MMF); FTY720: fingolimod.**

**Small-molecule drugs**

*Immunophilin-binding drugs*

Calcineurin inhibitors

Cyclophilin binding drugs: cyclosporine A (Sandimmun®)

FKBP12-binding drugs: tacrolimus (Prograf®)

mTOR inhibitors: sirolimus (Rapamune®), everolimus (Certican®)

*Inhibitors of nucleotide synthesis*

Purine synthesis inhibitors: mycophenolate mofetil (CellCept®, Myfortic®)

*Antimetabolites:* azathioprine (Imurek®)

*Sphingosine-1-phosphate-receptor antagonists:* FTY720 (fingolimod)

**Protein drugs**

*Depleting antibodies (against T cells, B cells, or both)*

Polyclonal antibody: horse or rabbit antithymocyte globulin (Atgam® resp. Thymoglobulin®, ATG-Fresenius®)

Mouse monoclonal anti-CD3 antibody (muromonab-CD3; Orthoclone OKT® 3)

Humanized monoclonal anti-CD52 antibody (alemtuzumab; MabCampath®)

B-cell-depleting monoclonal anti-CD20 antibody (rituximab; MabThera®)

*Nondepleting antibodies and fusion proteins*

Humanized or chimeric monoclonal anti-CD25 antibody (daclizumab resp. basiliximab; Zenapax® resp. Simulect®)

Fusion protein with natural binding properties: CTLA-4Ig (LEA29Y, i.e. belatacept)

**Intravenous immune globulins**

**Corticosteroids** (prednisone, prednisolone, methylprednisolone)

**Table 2. Classification of immunosuppressive agents used in solid organ transplantation (adapted from Halloran PF, 2004).**

### *2.5.11.1. Small-molecule drugs*

Most small-molecule immunosuppressive agents are derived from microbial products and target proteins that have been highly conserved in evolution.

Azathioprine (aza) was the first immunosuppressive agent to achieve widespread use in organ transplantation. It is thought to act by releasing 6-mercaptopurine, which interferes with DNA synthesis. Since the introduction of cyclosporine A and mycophenolate mofetil, azathioprine has become a second-line drug.

Cyclosporine A (CsA) is in effect a prodrug that engages cyclophilin, an intracellular protein, forming a complex that then engages calcineurin (Clipstone NA and Crabtree GR, 1992). Calcineurin is normally implicated in the transduction of the signaling pathways in T cells upon MHC/TCR interaction. Its engagement by CsA inhibits the transcription of specific transcription factors implicated in T cell activation, such as NFAT. CsA was the first immunosuppressant to target T cells without inducing myelotoxicity, inflammation, or immune cell depletion, and the use of CsA led to a significant increase in graft and patient survival rates (Kahan BD, 1989; Calne R, 2004).

Tacrolimus (TAC) uses a similar mechanism of action. It engages another immunophilin, FK506-binding protein 12 (FKBP12), to create a complex that inhibits calcineurin with greater potency than does CsA. Recent analyses have not shown a better efficacy of TAC over CsA in terms of rejection prevention (Woodward RS *et al*., 2005). However, TAC is less likely to cause hyperlipidemia, hypertension and cosmetic problems, but more likely to induce post-transplantation diabetes (Vincenti F *et al*., 2007). The use of TAC has increased steadily, and it is now the dominant CNI.

Few *in vitro* studies have described the effect of CNIs on Treg cells. However, it was shown that the induction of FoxP3 mRNA was inhibited in MLR assay (Baan CC *et al*., 2005); this observation was subsequently confirmed at the protein level (Zeiser R *et al*., 2006). *In vivo*, treatment of mice with CsA was shown to compromise the thymic output of Treg cells, resulting in a sharp decrease of Treg cells in peripheral immune compartments (Coenen JJ *et al*., 2007). Together, these data suggest that CNIs are rather detrimental to the generation, survival and function of Treg cells.

Mycophenolate mofetil (MMF) is a prodrug that releases mycophenolic acid (MPA), which inhibits inosine monophosphate dehydrogenase, a key enzyme in purine synthesis. It was shown to be superior to azathioprine in preventing rejection of kidney transplants (Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group, 1996). This drug has largely replaced azathioprine and is widely used in combination with other immunosuppressive agents, because it is simple to use (no monitoring required) and free from organ toxicity and cardiovascular risk. Only one study described the *in vitro* effect of MMF

on Treg cells, showing that this drug did not affect the expression of FoxP3 or the suppressive capacity of these cells (Zeiser R *et al*., 2006).

Sirolimus and everolimus are both target-of-rapamycin (mTOR) inhibitors engaging FKBP12 to create complexes that inhibit mTOR but cannot inhibit calcineurin. These drugs block the signal 3 of the alloimmune response model by preventing cytokine receptors from activating the cell cycle. In addition, mTOR inhibitors are associated with antiproliferative actions (Chapman JR *et al*., 2007). Reported side effects include hyperlipidemia, thrombocytopenia, impaired wound healing, aggravation of proteinuria, mouth ulcers and skin lesions. Furthermore, sirolimus cannot be used in combination with CsA because it increases its nephrotoxicity (Cheng CH *et al*., 2008). Experimental *in vitro* evidences show that mTOR inhibitors do not interfere with the suppressive activity of Treg cells (Game DS *et al*., 2005; Coenen JJ *et al*., 2006), by contrast to CNIs. *In vivo*, mTOR inhibitors were shown to induce de novo expression of FoxP3 in murine alloantigen-specific T cells, in a TGF$\beta_1$-dependent manner (Gao W *et al*., 2007). Also Treg cells expanded *ex vivo* in the presence of sirolimus were able to prevent rejection of β-islet transplants in mice (Battaglia M *et al*., 2005). These results clearly show that mTOR inhibitors favor Treg cells survival and function, maybe tipping the balance from an aggressive towards a more protective type of alloimmune response.

FTY720 (fingolimod) engages lymphocyte sphingosine-1-phosphate receptors after phosphorylation and acts primarily by confining lymphocytes within peripheral lymphoid organs, rendering them incapable of migrating to the sites of inflammation, such as the graft. FTY720 is still under clinical trial in solid organ transplantation (Mansoor M and Melendez AJ, 2008) as well as in multiple sclerosis, although it seems to be associated with more adverse events compared with current standard immunosuppressive regimens (Tedesco-Silva H *et al*., 2005).

#### *2.5.11.2. Depleting antibodies*

Polyclonal antithymocyte globulins (ATGs) are produced by immunizing horses or rabbits with human lymphoid cells, harvesting the IgG, and absorbing out toxic antibodies, such as those against platelets and erythrocytes. The obtained antibodies block several T cell surface-associated proteins (such as CD2, CD3, CD4, CD8, CD11a, CD18, CD25, CD44, CD45, HLA-DR, HLA class I and $\alpha_2$-microglobulin), causing altered function, lysis, and prolonged T cell depletion that lasts beyond one year (Brennan DC *et al*., 1999). Rabbit preparations are favored over horse polyclonal ATGs because of greater potency. Initially approved for the treatment of corticosteroid-resistant acute cellular rejection, polyclonal ATGs are also used as induction agents, usually for 3 to 10 days after transplantation, in high immunological risk recipients (pre-sensitized, high donor/recipient MHC mismatches, Africans Americans). It has been suggested that these agents may favor the development of

transplantation tolerance. Indeed, *in vitro* treatment of human CD4 T cells by thymoglobulin induces the upregulation of Treg-associated genes and proteins such as CD25, CTLA-4, OX40 and FoxP3 (Liu Z *et al*., 2008).

Muromonab-CD3 (OKT3) is an anti-CD3 monoclonal antibody that has been successfully used for more than 20 years as induction agent for patients at high immunological risk, as well as for the treatment of corticosteroid-resistant acute rejection episodes (Opelz G, 1995). However, due to its important side effects (among others the cytokine-release syndrome), ATGs are now usually preferred.

Alemtuzumab is a humanized anti-CD52 monoclonal antibody that was first developed for use in lymphoproliferative diseases, such as refractory B cell chronic lymphocytic leukemia; CD52 is expressed on T cells, B cells, monocytes, macrophages, NK cells and granulocytes (Waldmann H and Hale G, 2005). Alemtuzumab also induces profound and durable lymphopenia. Used as off-label induction agent, there are only three prospective, randomized, but small, studies with one year or less follow-up, and most published series utilize historical control groups with relatively short follow-up. None demonstrated a real advantage of alemtuzumab over other induction agents.

Rituximab is an anti-CD20 chimeric antibody that eliminates most B cells from the circulation. It has been first developed for the treatment of PTLD after solid organ transplantation; subsequently, rituximab has been used off-label for treatment of antibody-mediated rejection and for suppression of preformed alloantibodies in sensitized patients before transplantation (Becker YT *et al*., 2006).

### *2.5.11.3. Nondepleting antibodies and fusion proteins*

Daclizumab (humanized) and basiliximab (chimeric) are both anti-CD25 monoclonal antibodies inhibiting signal 3 of the alloimmune response model. They preferentially target alloreactive T cells activated in the early post-transplantation period, and inhibit the IL-2-mediated proliferation and effector function. They are now commonly used as induction agents in patients at low immunological risk (Vincenti F *et al*., 2006). They do not cause significant lymphocyte depletion and have minimal toxic effects. One *in vitro* study showed inhibition of FoxP3 mRNA induction by daclizumab in allostimulated peripheral blood mononuclear cells (PBMC) (Baan CC *et al*., 2005), whilst another study demonstrated down-regulation of FoxP3 protein expression (Kreijveld E *et al*., 2007). However, in direct co-cultures of Treg cells and effector T cells, basiliximab did not interfere in the suppressive capacity of Treg cells (Game DS *et al*., 2005).

Belatacept (LEA29Y) is a second-generation CTLA-4 immune globulin that is a fusion protein combining CTLA-4 (which engages CD80 and CD86) with the Fc portion of $IgG_1$. It is currently undergoing phase III clinical trials in renal transplantation, and has shown promise as a safe and effective alternative immunosuppression regimen to CNIs and

steroid-based therapies. Phase II trials demonstrated similar efficacy to CsA, with greatly improved renal function. Although its side effects are not yet well characterized, belatacept has shown a decrease of the renal, cardiovascular, and metabolic side effects associated with CNIs (Vincenti F *et al*., 2005). Longer follow-up data have to establish whether belatacept can favor the induction of transplantation tolerance, as was suggested in animal models (Kirk AD *et al*., 1997).

#### *2.5.11.4. Intravenous immune globulins*

Intravenous immune globulins (IVIg) are commercial preparations derived from pooled plasma from more than 100'000 screened donors. It is likely that they contain the entire compilation of human antibodies. IVIg products were initially developed for treatment of immune deficiency disorders. More recently, they have been used in kidney transplantation for decreasing panel reactive antibodies in highly sensitized patients (Gloor JM *et al*., 2003) and for the treatment of antibody-mediated rejection (Moger V *et al*., 2004). Their multiple mechanisms of action are the subject of various studies, but they are not entirely clear. They reduce alloantibodies, inhibit inflammatory cytokine production, block antibody production, and may inhibit complement activation. In desensitization protocols, IVIg are used either alone at high dose or in combination with plasmapheresis at lower dose.

#### *2.5.11.5. Corticosteroids*

Corticosteroids, which include prednisone, prednisolone and methylprednisolone, are pleiotropic hormones acting as agonists of glucocorticoids receptors. They are used fort their potent anti-inflammatory and immunomodulatory action. Their effects are mainly transcriptional through DNA-binding and protein-protein interactions of the steroid-receptors complex, targeting transcription factors such as AP-1 and NF-κB, which are involved in transcription of pro-inflammatory cytokine and chemokine genes such as IL-2, TNFα and IFNγ.

As discussed for the respective drugs, there are evidences that that some of the most widely used immunosuppressive drugs have clear and distinct effects on Treg cells, among other immune cell populations. However, it is not clear yet how immunosuppression affects the balance between regulatory (protolerogenic) and effector (pathogenic) T cells *in vivo*. Overall, the results from *in vitro*, animal, and clinical studies show that immunosuppressive drugs can have detrimental but also beneficial effects on Treg cells. Together, these data indicate that the use of specific immunosuppression as well as their timing and dosing have to be taken into account when elaborating strategies to induce and maintain transplantation tolerance. However, by contrast to the clear results obtained *in vitro*, the impact of immunosuppressive drugs in patients is much less clear and requires more clinical research before firm conclusions can be drawn.

# 3. AIMS OF THE PROJECT

## *3.1. Characterization of $CD4^+$ $CD25^+$ T cells in healthy individuals and solid organ transplant recipients*

As emphasized in the Introduction part, there is now no doubt that $CD4^+$ $CD25^+$ regulatory T cells (Treg) can play a positive role in the acceptance of a transplanted organ, since it has been demonstrated that they are able to effectively suppress allograft rejection *in vivo* (reviewed by Jiang S *et al*., 2006). However, when this project was designed five years ago, a lot of controversial data were published on the phenotype and functionality of Treg cells in human transplant patients. One limitation in the study of Treg cells, especially in humans, was the lack of a really specific phenotypic marker of this lineage of lymphocytes. The combination of CD4 and CD25, with a gating strategy on the cells positive for CD4 and expressing the highest level of CD25, was widely accepted as identifying the Treg population, by analogy with experiments made in mice. Recently, two additional markers have been identified that help in the isolation of the Treg population: FoxP3 and IL-7Rα (CD127). Indeed, using one or both of these markers, it was found that the majority of $CD4^+$ $CD25^{high}$ T cells, but not all , have the typical suppressive properties of the Treg population, in healthy human volunteers (Seddiki N *et al*., 2006; Liu W *et al*., 2006).

The aim of the project was to study by flow cytometry the phenotypic and functional characteristics of subsets of $CD4^+$ $CD25^{high}$ T cells, namely regulatory T cells and activated T cells, in healthy humans as well as in solid organ transplant recipients. Parts of the following results were published in the *Journal of Experimental Medicine* in July 2007 (Codarri L *et al*., 2007).

# 4. RESULTS

## *4.1. Characterization of CD4$^+$ CD25$^+$ T cells in healthy individuals*

Before studying CD4$^+$ CD25$^+$ T cells subsets in solid organ transplant recipients, it was of interest to analyze the control situation found in healthy donors. For this purpose, we analyzed the phenotype and frequency of these cells using flow cytometry, and their function by a mixed lymphocyte reaction (MLR) assay.

### 4.1.1. Phenotype of CD4$^+$ CD25$^+$ T cells in healthy individuals

The analysis of CD4$^+$ CD25$^+$ T cells by flow cytometry requires the use of monoclonal fluorescent antibodies and a thorough gating strategy. Monoclonal antibodies that we use to identify CD4$^+$ CD25$^+$ T cells and their subsets are directed against CD4, CD25, CD45RO, CD127 and FoxP3, among others. The first step is to gate on the lymphocyte population on a FSC / SSC dot plot [**Figure 23**]. Then, on a dot plot displaying CD4 and CD25, the CD4 positive T cell population can be divided roughly into three distinct populations, based on the CD25 expression level: a CD25 negative population (CD25$^{neg}$), a population expressing intermediate levels of CD25 (CD25$^{int}$), and a population expressing the highest level of CD25 (CD25$^{high}$) [**Figure 24**].

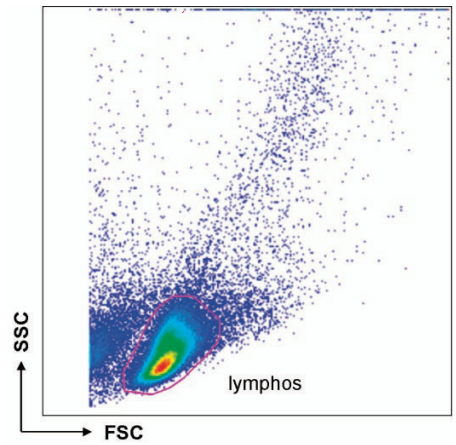



**Figure 23. FSC/SSC dot plot.**

**The lymphocyte population is defined as a compact population of cells of relatively small size (FSC, forward scatter) and of weak granularity (SSC, side scatter).**

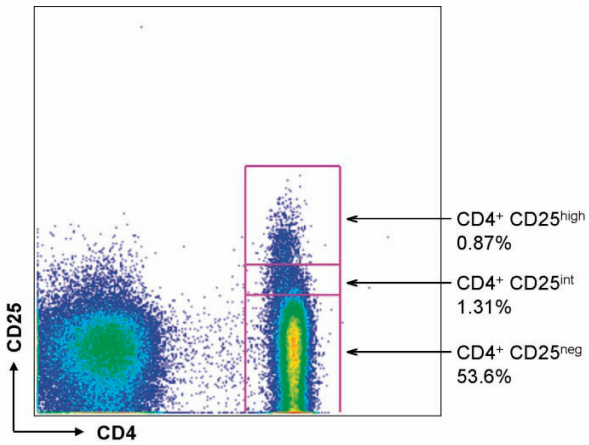



**Figure 24. CD4/CD25 dot plot, gated on lymphocytes.**

**CD4 positive T cells can be divided into three distinct populations based on the CD25 expression level: CD25$^{neg}$, CD25$^{int}$, and CD25$^{high}$.**

As discussed in the Introduction part, only the CD25$^{high}$ population of CD4$^+$ T cells displays regulatory features, and for this reason, the rest of our observations were focused on this subset of CD4$^+$ T cells, that we will name CD4$^+$ CD25$^{high}$ T cells.

In a first step to characterize the $CD4^+$ $CD25^{high}$ T cells, we tested several healthy volunteers to see whether a correlation with the gender or the age exists. Overall, we found a mean of 1.62% of $CD25^{high}$ cells within $CD4^+$ T cells (range: 0.20-4.09%; median: 1.46%); there was no statistical difference between female and male healthy volunteers (females, mean: 1.45%, range: 0.20-2.50%; males, mean: 1.86%, range: 0.21-4.09%) [**Figure 25**].

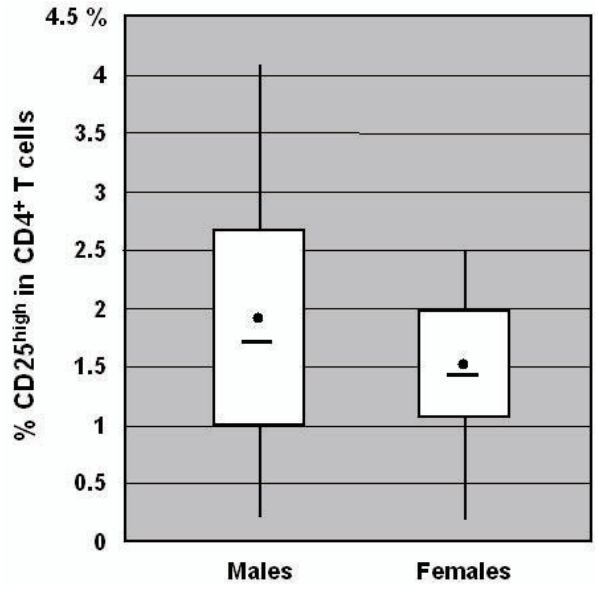



**Figure 25. Percentage of $CD4^+$ $CD25^{high}$ T cells in 14 male and 19 female healthy volunteers.**

**Minimal value, 25th percentile, median (—), mean (•), 75th percentile and maximal value are depicted.**

It was also of interest to investigate the stability of the percentage of $CD4^+$ $CD25^{high}$ T cells over time in healthy donors. For this purpose, we assessed the frequency of these cells in 3 healthy subjects over a 1-year period, at 0, 1, 3, 6, and 12 months. We found that in fact the percentage of $CD4^+$ $CD25^{high}$ T cells showed significant variations in a single individual; the coefficient of variation (CV) was found to be 32.2% in average (range: 19.0-50.4%) [**Figure 26A**]. By contrast, the percentage of $CD4^+$ T cells remained quite stable over the 1-year period, with a mean CV of 6.9% (range: 5.4-8.7%) [**Figure 26B**].

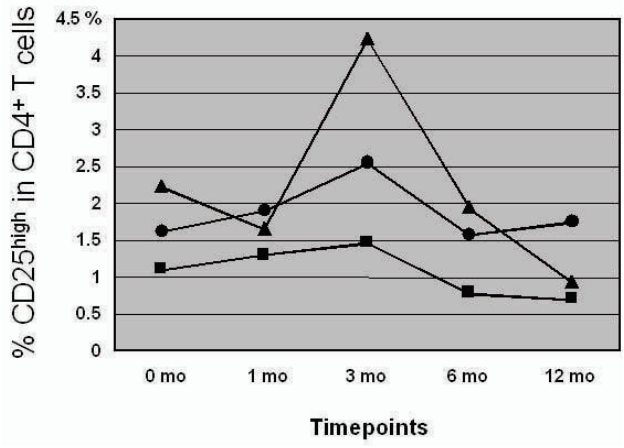



**Figure 26A. Kinetic of $CD4^+$ $CD25^{high}$ T cells over a 1-year period in 3 healthy subjects.**

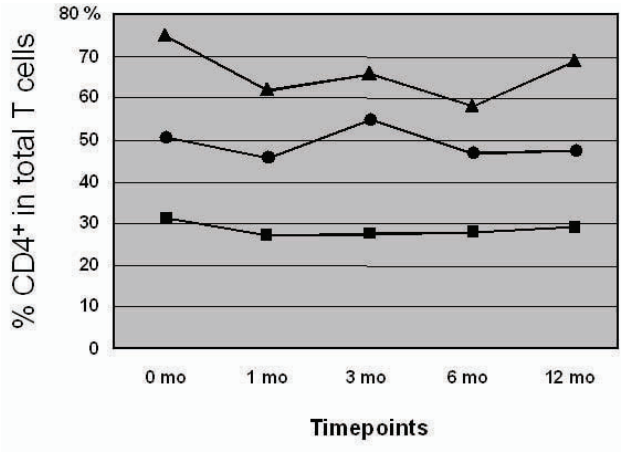



**Figure 26B. Kinetic of $CD4^+$ T cells over a 1-year period in 3 healthy subjects.**

## 4.1.2. Function of $CD4^+$ $CD25^+$ T cells in healthy individuals

We also assessed the in vitro suppressive capacity of $CD4^+$ $CD25^{high}$ T cells in four healthy volunteers. To do so, we sorted the $CD4^+$ $CD25^{high}$ (regulatory cells) and $CD4^+$ $CD25^{neg}$ (responder cells) populations by flow cytometry and irradiated allogeneic PBMC (stimulator cells); stimulator and responder cells were put in culture either alone (positive control) or in the presence of regulatory cells, at a ratio of 2:1:1 respectively. The proliferation of the responder cells was assessed at day 7 by $^3$H-thymidine incorporation. The mean percentage of inhibition exerted by regulatory cells was calculated on the basis of triplicates, as follows:

$$\%\,\text{inhibition} = 100 - \left( \left( \frac{\text{proliferation of responder cells in presence of regulatory cells}}{\text{proliferation of responder cells alone}} \right) \times 100 \right)$$

We found a mean percentage of suppressive capacity of $CD4^+$ $CD25^{high}$ T cells of 86.5% (range: 70.2-99.1%) [**Figure 27**], which is consistent with values reported in the literature.

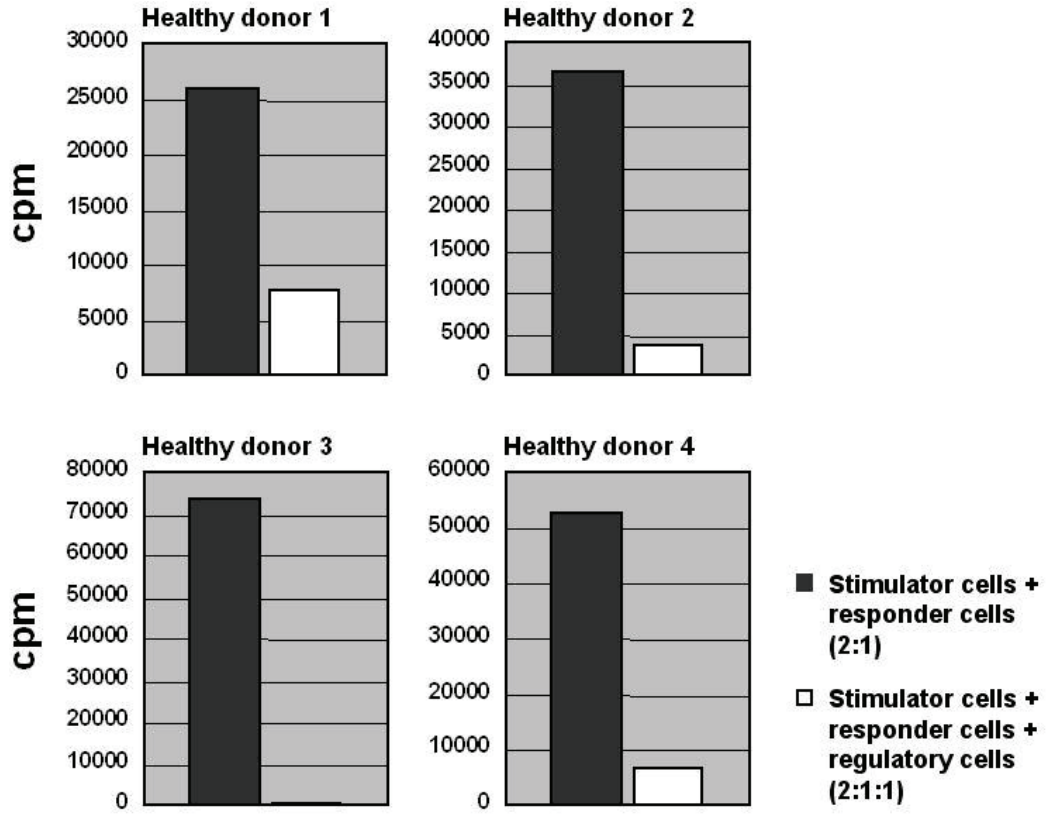



**Figure 27. Suppressive capacity of $CD4^+$ $CD25^{high}$ T cells in four healthy volunteers.**

**The mean percentage of inhibition was 70.2% in healthy donor 1, 89.5% in healthy donor 2, 99.1% in healthy donor 3, and 87.2% in healthy donor 4.**

## 4.1.3. Definition of functionally different subsets among $CD4^+$ $CD25^{high}$ T cells

During the past years, it has become increasingly clear that the population of $CD4^+$ $CD25^{high}$ T cells is not a homogeneous population in terms of phenotype and function. Indeed, CD25 is not a specific marker expressed only by regulatory T cells, but is also a marker of recent activation of both CD4 and CD8 T lymphocytes (Waldmann TA, 1989). For this reason, the identification of other markers to distinguish regulatory and recently activated T cells is mandatory, especially in settings of immune activation such as autoimmune inflammatory disorders and organ transplantation.

As emphasized in the Introduction part, several markers have been shown to be more or less specific to regulatory T cells, and among them, the transcription factor FoxP3 and the interleukin-7 receptor α chain (IL-7Rα, CD127) seem to have the highest specificity. We will

not come back to FoxP3, which was already extensively described in the Introduction part as well as in the literature since its discovery in 2003 (Hori S *et al*., 2003; Fontenot JD *et al*., 2003; Ramsdell F, 2003). On the other hand, the use of CD127 as a discriminatory marker is more recent, the first descriptions having been published in 2006 (Seddiki N *et al*., 2006; Liu W *et al*., 2006). Interestingly, at the same period (2005-2006), we were also working on the possible usefulness of CD127, based on a publication showing infiltration of mononuclear cells expressing both CD25 and CD127 in idiopathic dilated cardiomyopathy, an autoimmune disease (Holzinger C *et al*., 1995). Our observations in healthy individuals were consistent with the above-mentioned two publications, and we will summarize them below.

### *4.1.3.1. Phenotype*

Using CD127 staining, in addition to CD4 and CD25, permits to reveal two well-defined populations, a $CD127^{low}$ one and a $CD127^{high}$ one, among $CD4^+$ $CD25^{high}$ T cells. In addition, we found it helpful to add a fourth marker, CD45RO, which is expressed on cells with a memory phenotype: it permits to discriminate between naïve and memory regulatory T cells, and is useful for defining populations in sorting experiments. The first step in the gating strategy is to gate on $CD4^+$ $CD25^{high}$ T cells and then to display a dot plot CD127 / CD45RO: three distinct populations can then be clearly identified [**Figure 28**].

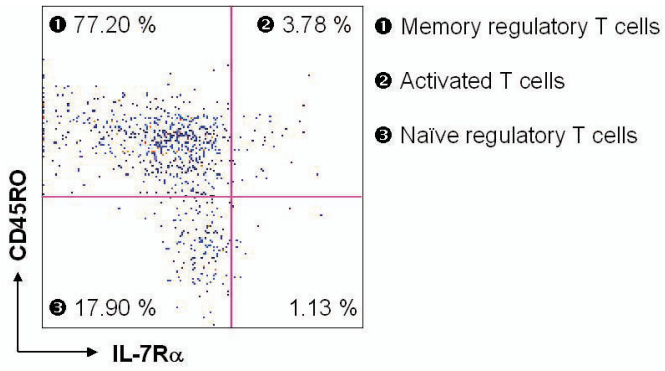



**Figure 28. CD127 / CD45RO dot plot, gated on $CD4^+$ $CD25^{high}$ T cells, of one representative healthy volunteer.**

We performed these analyses in 73 healthy donors. Overall, the vast majority of $CD4^+$ $CD25^{high}$ T cells were found to be $CD127^{low}$ (mean ± std deviation: 91.55 ± 4.37%), whilst the $CD127^{high}$ population represented only 8.45% of $CD4^+$ $CD25^{high}$ T cells. When using CD45RO as an additional marker, the $CD45RO^+$ $CD127^{low}$ sub-population represented 74.93% of $CD4^+$ $CD25^{high}$ T cells, the $CD45RO^-$ $CD127^{low}$ sub-population 16.62%, and the $CD45RO^+$ $CD127^{high}$ sub-population 5.97% [**Figure 29**].

| $CD4^+$ $CD25^{high}$ | | mean ± std deviation |
|---|---|---|
| $CD45RO^+$ $CD127^{low}$ | | 74.93 ± 12.10% |
| $CD45RO^+$ $CD127^{high}$ | | 5.97 ± 3.11% |
| $CD45RO^-$ $CD127^{low}$ | | 16.62 ± 10.96% |
| $CD45RO^-$ $CD127^{high}$ | | 2.49 ± 2.32% |

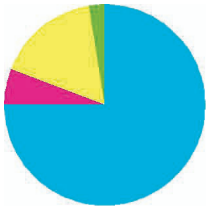


**Figure 29. Repartition of the four $CD4^+$ $CD25^{high}$ T cell sub-populations based on the expression of CD45RO and CD127.**

### *4.1.3.2. Function*

It was important to confirm the respective function of these subsets, and for this purpose we assessed the *in vitro* suppressive capacity of the $CD45RO^+$ $CD127^{low}$ and $CD45RO^+$ $CD127^{high}$ sorted $CD4^+$ $CD25^{high}$ sub populations, as described elsewhere. This assay using thymidine incorporation as read-out showed that only the $CD127^{low}$ sub-population was able to mediate *in vitro* suppression (mean percentage of inhibition: 86.2%), whilst the presence of the $CD127^{high}$ sub-population did not affect the proliferation of the responder cells [**Figure 30**]. This result confirms that the regulatory T cells are contained within the $CD127^{low}$ sub-population of $CD4^+$ $CD25^{high}$ $CD45RO^+$ T cells and that CD127 can be safely used as a discrimination marker to distinguish regulatory T cells from activated T cells, at least in healthy donors, in accordance with previously published data (Seddiki N *et al*., 2006; Liu W *et al*., 2006).

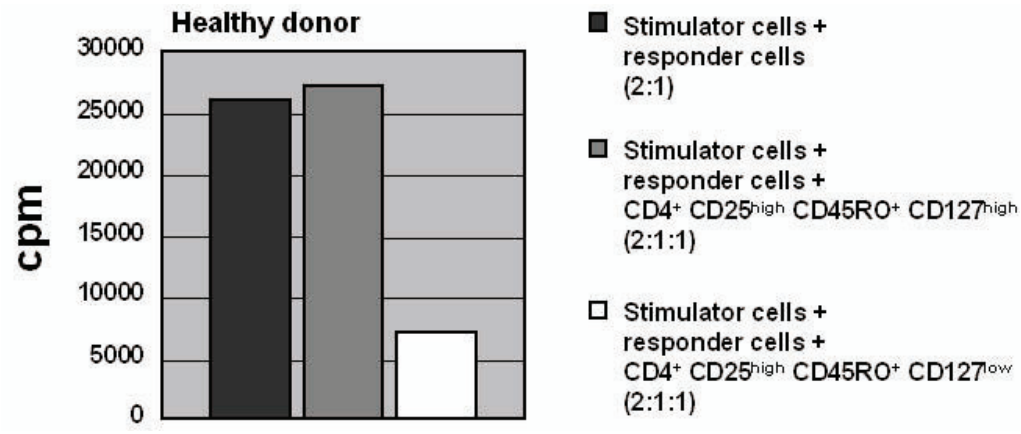



**Figure 30. Respective suppressive capacity of the $CD127^{high}$ and $CD127^{low}$ sub-populations of $CD4^+$ $CD25^{high}$ $CD45RO^+$ T cells, in one representative healthy volunteer.**

**The mean percentage of inhibition was 72.2% in this representative healthy volunteer.**

### *4.1.3.3. FoxP3*

Since the transcription factor FoxP3 is now widely accepted as a reliable marker of regulatory T cells, we were interested in assessing the relationship between the respective expression of CD127 and FoxP3 in the above-described $CD4^+$ $CD25^{high}$ T cells sub-populations in healthy volunteers. Of note, the staining of FoxP3 requires permeabilization and fixation of the cells, what precludes their future use in suppression assays for example. In co-stainings with CD4, CD25 and FoxP3, more than 90% of $FoxP3^+$ cells were found among $CD4^+$ T cells [**Figure 31A**]. In $CD4^+$ T cells, the expression level (mean fluorescence intensity, MFI) of FoxP3 was proportional to the one of CD25: the highest the CD25 MFI, the highest the FoxP3 MFI; however, a significant percentage of $FoxP3^+$ cells were found among $CD25^-$ cells [**Figure 31B**].

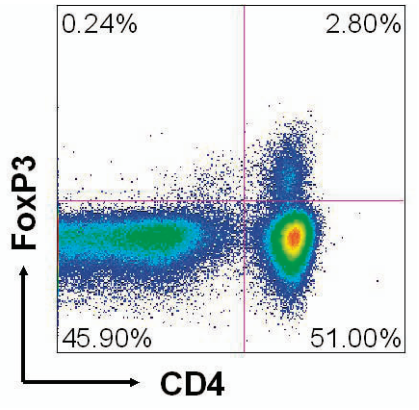



**Figure 31A. CD4 and FoxP3 staining, gated on lymphocytes.**

**In this healthy volunteer, the $CD4^+$ $FoxP3^+$ T cell population represents 2.80% of the total lymphocytes.**

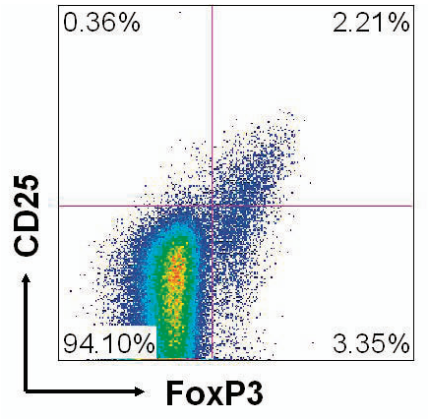



**Figure 31B. CD25 and FoxP3 staining, gated on $CD4^+$ T lymphocytes.**

**$FoxP3^+$ cells are found mainly among $CD25^+$ $CD4^+$ T cells.**

Co-staining with CD127 and FoxP3, in addition to CD4, CD25 and CD45RO, confirmed an inverse correlation between CD127 and FoxP3 expression in $CD4^+$ $CD25^{high}$ T cells ($P$=0.01); the $CD127^{low}$ cells were contained within the FoxP3 positive cell population, whereas $CD127^{high}$ cells were contained within the FoxP3 negative cell population [**Figure 32**].

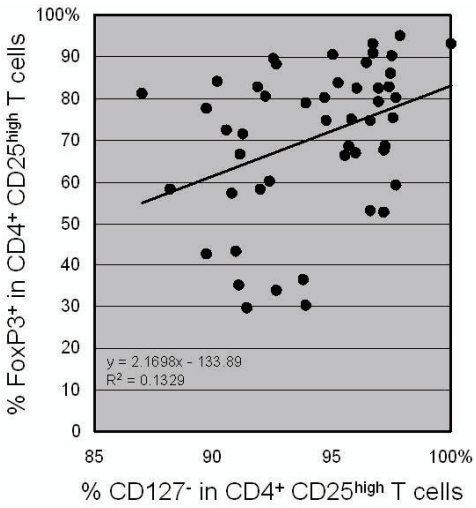



**Figure 32. Correlation between CD127 and FoxP3 expression in $CD4^+$ $CD25^{high}$ T cells, in 49 healthy volunteers.**

**The correlation was found to be statistically significant (*P*=0.01).**

Overall, among the 49 healthy volunteers tested, a majority of $CD4^+$ $CD25^{high}$ T cells were $FoxP3^+$ (mean ± std deviation: 70.86 ± 17.50%), whilst $FoxP3^-$ cells represented only 29.14% of $CD4^+$ $CD25^{high}$ T cells.

Collectively, these results indicate that CD127 is a valuable marker to discriminate between the regulatory and activated T cell sub-populations of $CD4^+$ $CD25^{high}$ T cells, in healthy donors, and are in total accordance with the above mentioned publications (Seddiki N *et al*., 2006; Liu W *et al*., 2006).

## 4.1.4. Discussion

Based on the results of our preliminary study on healthy volunteers, we next decided to perform the same kind of analyses in the clinical setting of solid organ transplantation. As mentioned in the Introduction part, there are evidences that regulatory T cells play a major role in the outcome of a graft. However, at the time we began this project, results in humans were quite controversial, probably due to the lack of reliable markers of regulatory T cells. Fortunately, as described in the healthy volunteers, new tools, such as FoxP3 and CD127, became available, and we decided to take advantage of them to study $CD4^+$ $CD25^{high}$ T cells sub-populations in solid organ transplant recipients, especially liver and kidney recipients.

## *4.2. $CD4^+$ $CD25^{high}$ T cells in solid organ transplant recipients*

Initial observations that regulatory $CD4^+$ $CD25^{high}$ T cells play a pivotal role in transplantation tolerance were performed in mice: Sakaguchi S *et al*. showed that removal of $CD4^+$ $CD25^{high}$ T cells from normal mice enhanced graft rejection (Sakaguchi S *et al*., 2001), and, conversely, they also showed that when regulatory $CD4^+$ $CD25^{high}$ T cells enriched from normal syngeneic mice were inoculated, together with naïve T cells, to syngeneic T-cell-deficient mice with allografts, the graft survival was significantly prolonged, and even permanent graft tolerance was established at large doses of these cells (Nishimura E *et al*., 2004). Studies in human organ transplantation have investigated the number and functional properties of regulatory $CD4^+$ $CD25^{high}$ T cells in relation to immunological quiescence (stable transplant recipients on immunosuppression), tolerance (stable transplant recipients off immunosuppression), and acute or chronic rejection. However, until recently, the obtained results were quite contradictory, most probably because of the lack of regulatory T cells specific markers. As mentioned above, FoxP3 and CD127 have been recently identified and became commercially available few years ago, prompting us to perform a complete study of the phenotype and function of $CD4^+$ $CD25^{high}$ T cells and their sub-populations in solid organ transplant recipients.

For this purpose, we decided to study a cohort of liver and kidney transplant recipients, treated at the Transplantation Centre at the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne, as well as at the Divisions of Nephrology and Transplantation at the Hôpitaux Universitaires de Genève (HUG) in Geneva.

Our initial results were published in the *Journal of Experimental Medicine* in July 2007 (Codarri L *et al*., 2007); a copy of this publication is enclosed in the following pages.

### 4.2.1. Expansion and tissue infiltration of an allospecific $CD4^+$ $CD25^+$ $CD45RO^+$ IL-7R$\alpha^{high}$ cell population in solid organ transplant recipients (*J Exp Med* 2007)

In this publication, we analyzed 45 healthy subjects, 7 patients under immunosuppressive drugs for various autoimmune diseases, as well as a cohort of 21 liver and 26 kidney transplant recipients, divided in groups based on their clinical status, as follows:

(a) 21 liver transplant recipients, who were all clinically stable, and had received a first cadaveric graft more than 12 months prior to the study;
(b) 11 kidney transplant recipients, who were clinically stable (as defined by a stable serum creatinine < 150 µmol/l, 24h-proteinuria < 0.5 g/day, no circulating anti-HLA antibodies), and had received a first graft (8 cadaveric, 3 living-donor) more than 12 months prior to the study;

(c) 4 kidney transplant recipients, who had a biopsy-proven diagnosis of chronic humoral rejection (based on the criteria of the Banff'05 classification (Solez K *et al*., 2007), presence of circulating donor-specific anti-HLA antibodies, capillary C4d deposits, deteriorating graft function), and had received a first graft (3 cadaveric, 1 living-donor) more than 12 months prior to the study;

(d) 11 kidney transplant recipients, who were enrolled in a prospective study conducted at the HUG: they all received a first living-donor graft, an induction regimen which was composed of a 3-4d course of thymoglobulin (1.5mg/kg) and a long-term steroid-free immunosuppression which was composed of tacrolimus and mycophenolate mofetil.

In summary, we could show that CD127 can also be used in liver and kidney transplant recipients as a discriminatory marker of $CD4^+$ $CD25^{high}$ regulatory ($CD127^{low}$) versus activated ($CD127^{high}$) T cells. According to a previous study (Demirkiran A *et al*., 2005), we found that the percentage of the $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{low}$ regulatory T cells was significantly decreased in transplant recipients as compared to healthy controls; furthermore, the percentage of the $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ activated T cells was concomitantly increased in stable transplant recipients, and the expansion of this sub-population was even greater among kidney transplant recipients with a biopsy-proven diagnosis of chronic humoral rejection. By functional experiments, we could demonstrate that the $CD127^{high}$ activated $CD4^+$ $CD25^{high}$ T cell sub-population contained alloreactive T cells, which secreted the pro-inflammatory cytokines IFNγ and TNFα upon polyclonal stimulation. Finally, we could demonstrate the presence of $CD4^+$ $CD127^+$ cells and of $CD25^+$ $CD127^+$ cells in the cellular infiltrate of renal biopsies with chronic humoral rejection. We also monitored prospectively the changes in these T cell populations during the first year after kidney transplantation; we observed that the percentage of the activated IL-7R$\alpha^{high}$ T cell population increased as soon as 1 month after transplantation and remained expanded during the year of observation. Our results suggest that monitoring the $CD4^+$ $CD25^{high}$ T cell sub-populations could become a useful tool in the long-term follow-up of transplant recipients.

Because the perspectives opened by these initial results were interesting, we decided to continue with an in-depth analysis of $CD4^+$ $CD25^{high}$ T cell sub-populations, in particular of the activated $CD127^{high}$ and regulatory $CD127^{low}$ sub-populations, in the clinical setting of kidney transplantation.

As demonstrated in our initial publication, the percentage of the activated T cell sub-population was increased in stable transplant recipients as compared to healthy subjects, and this increase was even greater in kidney recipients with chronic humoral rejection. Based on these observations, we decided to expand our study to various clinical situations. We opted to include more stable transplant recipients having received various induction and immunosuppressive regimens, more transplant recipients with chronic renal dysfunction, and transplant recipients on no or minimal immunosuppression. This part of the project was felt to

be important to assess the actual usefulness of monitoring this parameter in the routine follow-up of transplant recipients.

We were also interested in the phenotypic characteristics of these sub-populations, and in particular in the panel of homing receptors expressed; indeed, chemokines and their receptors are essential to control leucocyte migration and homing throughout the body in both physiological and pathological situations. In the context of the adaptive immune system, both chemokines and chemokine receptors orchestrate the immune response by providing the spatio-temporal guidance for T cell development, priming and effector functions (Viola A *et al*., 2008). In the context of renal transplantation, acute allograft rejection is characterized by the expression of chemokine receptors that direct the trafficking of allo-activated T cells into the graft in response to local production of chemokines, initially by resident cells; chemokines attract leucocytes bearing specific chemokine receptors (Stasikowska O *et al*., 2007). This part of the project is important to assess the migration profile of these sub-populations, in order to see whether they differ between the groups of patients based on their clinical conditions.

Overall, in addition to the initial 21 liver and 26 kidney transplant recipients already described in the above mentioned publication, we enrolled more than 100 additional kidney transplant recipients, at the Transplantation Centre at the CHUV in Lausanne as well as at the Divisions of Nephrology and Transplantation at the HUG in Geneva. The results of these parts of the project have not been published yet but a manuscript is in preparation; they are presented in the following sections, after the copy of our initial publication (which follows in the next pages).

# Expansion and tissue infiltration of an allospecific $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population in solid organ transplant recipients

Laura Codarri,[1] Laure Vallotton,[1] Donatella Ciuffreda,[1] Jean-Pierre Venetz,[2] Miguel Garcia,[1] Karine Hadaya,[4] Leo Buhler,[4] Samuel Rotman,[3] Manuel Pascual,[2] and Giuseppe Pantaleo[1]

[1]Laboratory of AIDS Immunopathogenesis, Division of Immunology and Allergy, Department of Medicine, [2]Transplantation Center, and [3]Institute of Pathology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, 1011 Lausanne, Switzerland
[4]Service of Nephrology and Transplantation, Hôpitaux Universitaires de Genève, University of Geneva, 1211 Geneva 14, Switzerland

**It has been recently shown (Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, et al. 2006. *J. Exp. Med.* 203:1693–1700.) that the expression of interleukin (IL) 7 receptor (R) α discriminates between two distinct CD4 T cell populations, both characterized by the expression of CD25, i.e. CD4 regulatory T (T reg) cells and activated CD4 T cells. T reg cells express low levels of IL-7Rα, whereas activated CD4 T cells are characterized by the expression of $IL\text{-}7R\alpha^{high}$. We have investigated the distribution of these two CD4 T cell populations in 36 subjects after liver and kidney transplantation and in 45 healthy subjects. According to a previous study (Demirkiran, A., A. Kok, J. Kwekkeboom, H.J. Metselaar, H.W. Tilanus, and L.J. van der Laan. 2005. *Transplant. Proc.* 37:1194–1196.), we observed that the T reg $CD25^+CD45RO^+IL\text{-}7R\alpha^{low}$ cell population was reduced in transplant recipients ($P < 0.00001$). Interestingly, the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population was significantly increased in stable transplant recipients compared with healthy subjects ($P < 0.00001$), and the expansion of this cell population was even greater in patients with documented humoral chronic rejection compared with stable transplant recipients ($P < 0.0001$). The expanded $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population contained allospecific CD4 T cells and secreted effector cytokines such as tumor necrosis factor α and interferon γ, thus potentially contributing to the mechanisms of chronic rejection. More importantly, $CD4^+IL\text{-}7R\alpha^+$ and $CD25^+IL\text{-}7R\alpha^+$ cells were part of the T cell population infiltrating the allograft of patients with a documented diagnosis of chronic humoral rejection. These results indicate that the $CD4^+CD25^+IL\text{-}7R\alpha^+$ cell population may represent a valuable, sensitive, and specific marker to monitor allospecific CD4 T cell responses both in blood and in tissues after organ transplantation.**

CORRESPONDENCE
Giuseppe Pantaleo:
giuseppe.pantaleo@chuv.ch
OR
Manuel Pascual:
manuel.pascual@chuv.ch

The antiallograft immune response remains an important cause of acute rejection and late graft dysfunction in solid organ transplantation. In addition to the clinical and pathological manifestations of graft dysfunction, various biological markers have been investigated to monitor graft rejection over the years. De novo donor-specific anti-HLA antibodies represent a valuable marker, as their development is generally associated with rejection (1). However, T cell immunity plays a pivotal role in graft rejection (2). In this regard, a series of biological markers related to the inflammatory response, such as cytokines, thromboxane B2, β2 microglobulin (3, 4), and other phenotypic and functional markers associated with T cell activation such as IL-2Rα (CD25) expression, CD69, HLA-DR (5–8), cell proliferation, cytokine secretion, and cytotoxicity (9–12), have been evaluated to monitor cell-mediated immunity. However, these markers are neither sensitive nor specific, and their

L. Codarri and L.Vallotton contributed equally this study.
The online version of this article contains supplemental material.

measurement may be technically complex and time-consuming. For these reasons, none of these markers is routinely used in the monitoring of transplant recipients.

It has been recently reported that regulatory CD4 T cells (i.e., T reg cells) express low levels of IL-7Rα compared with other CD4 T cell subsets (13–17), and it has been proposed that IL-7Rα can be used to discriminate between T reg cells and activated CD4 T cells within the $CD4^+CD25^+$ cell population (15, 17). The $CD4^+CD25^+IL\text{-}7R\alpha^{high}$ cell population contains activated T cells and is poorly represented (~5% within $CD4^+CD25^+$ T cells) in healthy subjects. Conversely, after transplantation, both activated allospecific CD4 T cells and T reg cells may play a critical role by either participating in allograft rejection or promoting tolerance (18–25).

In this study, we have searched for sensitive and highly specific cellular markers that may define the population of allospecific CD4 T cells to quantify this cell population after transplantation, to monitor possible changes, and to determine the pattern of the allospecific population in chronic graft rejection. We demonstrate that a CD4 T cell population defined by the $CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ phenotype contains allospecific cells and is expanded in the blood of stable transplant recipients and even further expanded in patients with a documented diagnosis of chronic rejection. In these latter patients, $CD4^+CD25^+IL\text{-}7R\alpha^+$ cells were a major component of T cells infiltrating the allograft.

## RESULTS AND DISCUSSION

### Distribution of $CD4^+CD25^+$ T cell populations defined by the expression of IL-7Rα (i.e., $IL\text{-}7R\alpha^{low}$ vs. $IL\text{-}7R\alpha^{high}$) in stable organ transplant recipients

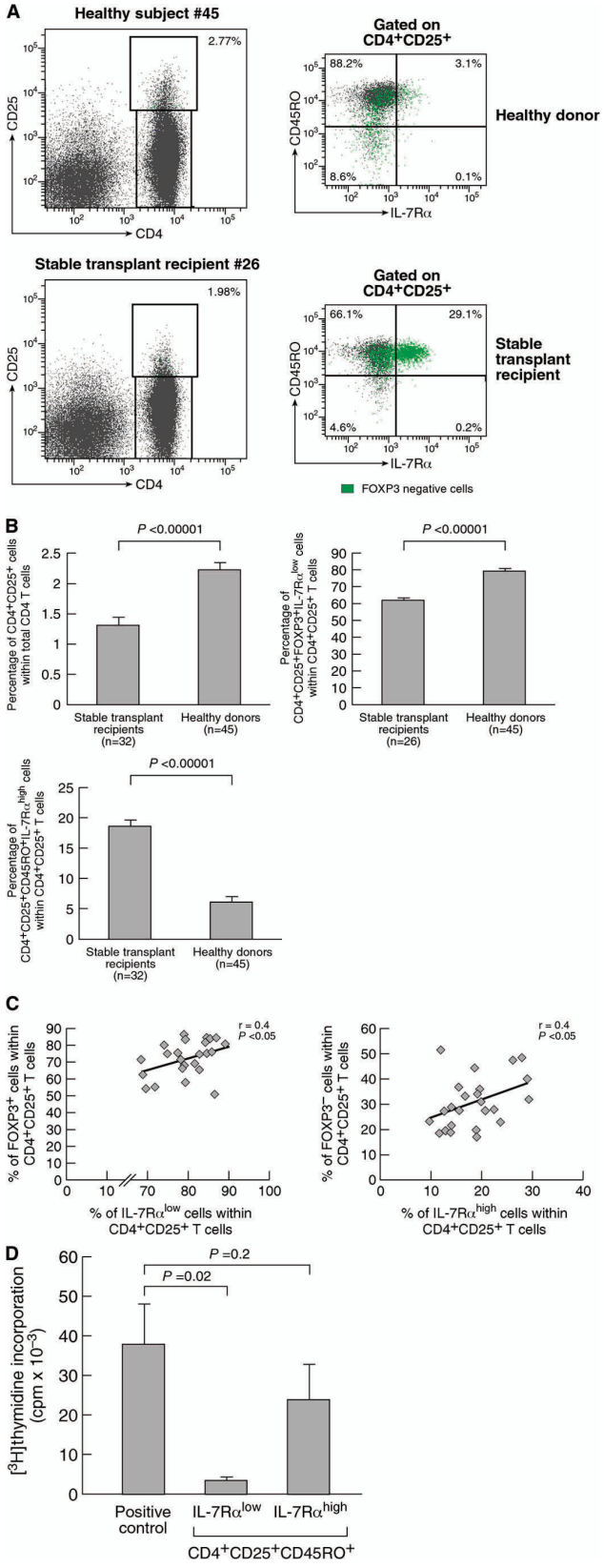
We have studied the distribution of T reg cells and activated CD4 T cells in blood mononuclear cells of 32 stable transplant recipients, 21 liver and 11 kidney transplant recipients, and 4 patients with documented chronic kidney rejection, as well as 45 healthy subjects.

In accordance with recent studies (15, 17), the majority (88.2%) of $CD4^+CD25^+$ T cells were $CD45RO^+FOXP3^+$ and $IL\text{-}7R\alpha^{low}$ in a representative healthy subject (1 out of 45), as shown in Fig. 1 A. This is the typical phenotype of T reg cells (26–28). Only a small percentage (3.1% in the example shown in Fig. 1 A) of $CD4^+CD25^+$ T cells expressed $IL\text{-}7R\alpha^{high}$, and these cells were FOXP3 negative. We then performed the same analysis in stable transplant recipients. In one representative stable liver transplant recipient (1 out of 32), the $CD4^+CD25^+CD45RO^+FOXP3^+IL\text{-}7R\alpha^{low}$ cell population was reduced (66.1%; Fig. 1 A) compared with the representative healthy subject. Interestingly, we observed a major increase (29.1%) in the $CD4^+CD25^+CD45RO^+FOXP3^-IL\text{-}7R\alpha^{high}$ cell population (Fig. 1 A). The differences in the distribution of $IL\text{-}7R\alpha^{low}$ and $IL\text{-}7R\alpha^{high}$ within the $CD4^+CD25^+CD45RO^+$ cell population between healthy subjects and stable transplant recipients were confirmed by the analysis of a larger number of subjects.

In agreement with a previous report (25), the percentage of total $CD4^+CD25^+$ T cells was significantly reduced ($P < 0.00001$) in stable transplant recipients receiving immunosuppressive therapy compared with healthy subjects. The reduction was in the range of 40%; the mean percentage of $CD4^+CD25^+$ T cells was 2.23 ± 0.12% in healthy subjects ($n = 45$) compared with 1.31 ± 0.14% in stable transplant recipients ($n = 32$; Fig. 1 B).

The analysis of IL-7Rα and FOXP3 expression allowed the discrimination between T reg cells (26–28) and activated CD4 T cells. This analysis showed that the percentage of T reg cells (the $IL\text{-}7R\alpha^{low}FOXP3^+$ cell population) was significantly lower ($P < 0.00001$) in stable transplant recipients (mean = 62 ± 1.54%) compared with healthy subjects (mean = 29.37 ± 2.33%; Fig. 1 B). Therefore, these results indicate that the reduction in T reg cells in stable transplant recipients is even greater than that estimated on the basis of the analysis of total $CD4^+CD25^+$ T cells. Furthermore, the population of activated CD4 T cells (i.e., the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population) was greatly expanded (mean = 18.38 ± 1.05%) in stable transplant recipients compared with healthy donors (mean = 6.16 ± 0.76%; Fig. 1 B). These differences were highly significant ($P < 0.00001$). On the basis of the present results and of recent studies (15, 17), we then analyzed the correlation between IL-7Rα and FOXP3 expression in $CD4^+CD25^+$ T cells in stable transplant recipients. This analysis confirmed a negative correlation between IL-7Rα and FOXP3 expression in $CD4^+CD25^+$ T cells (Fig. 1 C). The $IL\text{-}7R\alpha^{low}$ cells were contained within the FOXP3 positive cell population, whereas $IL\text{-}7R\alpha^{high}$ cells were within the FOXP3-negative cell population (Fig. 1 C). Collectively, these results indicated that IL-7Rα is a valuable marker to discriminate between T reg cells and activated CD4 T cells within the $CD4^+CD25^+$ T cell population in stable recipients of organ transplants.

Next, it was of interest to determine the suppressive activity of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{low}$ and $IL\text{-}7R\alpha^{high}$ cells. For these purposes, sorted purified $IL\text{-}7R\alpha^{low}$ and $IL\text{-}7R\alpha^{high}$ cell populations isolated from stable transplant recipients were assessed for their suppressive activity in a mixed lymphocyte reaction (MLR). The $IL\text{-}7R\alpha^{low}$ cell population (T reg cells) strongly suppressed the MLR (83% inhibition of proliferation; $P = 0.02$), whereas the $IL\text{-}7R\alpha^{high}$ cell population did not show any significant suppressive activity ($P = 0.2$; Fig. 1 D). These results obtained in stable transplant recipients are in agreement with those previously shown in healthy individuals (15, 17). Furthermore, the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population was also evaluated for the ability to secrete cytokines such as IFN-γ and TNF-α after polyclonal stimulation with anti-CD3 plus anti-CD28 antibodies. This analysis performed in blood mononuclear cells of 29 stable transplant recipients showed that a substantial percentage of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells secreted inflammatory cytokines, such as TNF-α (~15%) and IFN-γ (~4%), that may be involved in the mechanisms of chronic rejection (Fig. S1, http://www.jem.org/cgi/content/full/jem.20062120/DC1). Collectively, these results indicated that the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell




population does not contain T reg cells and suggested that the expansion of this cell population in transplant recipients is associated with the presence of the allograft.

### Larger expansion of the CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ cell population in transplant recipients with chronic rejection

To better support this hypothesis, we examined the presence of the CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ cell population in kidney transplant recipients with documented chronic humoral rejection. The total CD4$^+$CD25$^+$ T cell population was not significantly different in kidney recipients with documented chronic rejection compared with stable transplant recipients ($P = 0.21$; Fig. 2 A). The percentage of CD4$^+$CD25$^+$FOXP3$^+$IL-7R$\alpha^{low}$ cells (i.e., the T reg cells) within the total CD4$^+$CD25$^+$ cell population was slightly reduced in patients with chronic rejection (51.07 ± 5.8%) compared with stable transplant recipients (62.02 ± 1.54%; $P = 0.01$; Fig. 2 B). Interestingly, an almost doubled percentage of CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ cells within the total CD4$^+$CD25$^+$ cell population was observed in patients with documented chronic rejection as compared with stable transplant recipients (mean = 33.66 ± 3.43% vs. 18.38 ± 1.05%; Fig. 2 C). These differences were highly significant ($P < 0.0001$).

When the CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ population from patients with chronic rejection was compared with the one of stable kidney transplant recipients, the difference was also significant ($P = 0.0003$; unpublished data). Therefore,

**Figure 1. Expression of CD25, CD45RO, IL-7Rα, and FOXP3 on blood CD4 T cells of healthy subjects and stable transplant recipients.** Blood mononuclear cells from 45 healthy subjects and 32 stable organ transplant recipients (11 kidney and 21 liver transplant recipients) were analyzed for the surface expression of CD4, CD25, CD45RO, and IL-7Rα and for the intracellular expression of FOXP3. (A) Surface expression of CD45RO and IL-7Rα in CD4$^+$CD25$^+$ T cells. Representative flow cytometry profiles of one healthy donor (1 out of 45; top) and one stable liver transplant recipient (1 out of 32; bottom). The vast majority of CD4$^+$CD25$^+$ T cells are CD45RO$^+$IL-7R$\alpha^{low}$ and FOXP3$^+$ in the healthy donor, while a substantial percentage of CD4$^+$CD25$^+$ T cells are CD45RO$^+$IL-7R$\alpha^{high}$ and FOXP3$^-$ (green) in the stable transplant recipient. (B) Cumulative data on the proportion of CD4$^+$CD25$^+$ T cells (top left), CD4$^+$CD25$^+$FOXP3$^+$IL-7R$\alpha^{low}$ T cells (top right), and CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ T cells (bottom) in stable transplant recipients and healthy donors. (C) Correlation between the expression of FOXP3 and IL-7Rα within CD4$^+$CD25$^+$ T cells. The percentage of FOXP3$^+$ cells correlated with that of IL-7R$\alpha^{low}$ cells within CD4$^+$CD25$^+$ T cells, and the percentage of FOXP3$^-$ cells correlated with that of IL-7R$\alpha^{high}$ cells within CD4$^+$CD25$^+$ T cells. The analyses were performed in 26 stable transplant recipients. In both cases, these correlations were statistically significant ($P < 0.05$). (D) Suppressive activity of IL-7R$\alpha^{high}$ and IL-7R$\alpha^{low}$ CD4$^+$CD25$^+$CD45RO$^+$ T cell populations in stable transplant recipients. CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{high}$ and CD4$^+$CD25$^+$CD45RO$^+$IL-7R$\alpha^{low}$ cell populations were sorted from blood mononuclear cells of five stable liver transplant recipients, and their suppressive activity was evaluated in a MLR. The extent of cell proliferation in a MLR was assessed in the absence (positive control) or in presence of the sorted IL-7R$\alpha^{low}$ and IL-7R$\alpha^{high}$ cell populations. Cell proliferation was measured by [$^3$H]thymidine incorporation. The data shown are expressed as the mean ± SE and were obtained from five independent experiments.

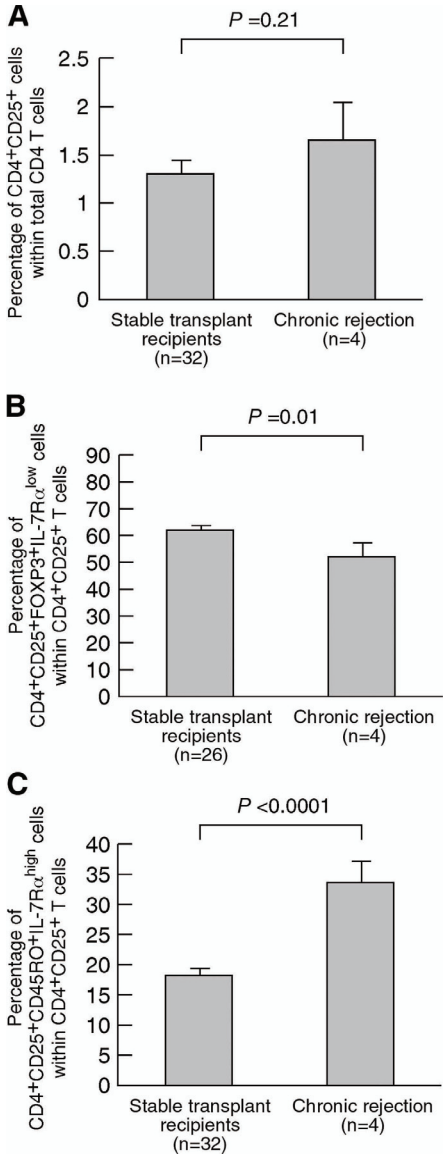



**Figure 2. Expression of CD25, CD45RO, IL-7Rα, and FOXP3 on blood CD4 T cells of stable transplant recipients and kidney transplant recipients with chronic rejection.** Blood mononuclear cells from 32 stable transplant recipients (11 kidney and 21 liver transplant recipients) and 4 kidney transplant recipients with biopsy-proven chronic rejection were analyzed for the surface expression of CD4, CD25, CD45RO, and IL-7Rα and for the intracellular expression of FOXP3. (A) Means ± SE of cumulative data on the proportion of $CD4^+CD25^+$ T cells in stable transplant recipients and transplant recipients with chronic rejection. The percentage of $CD4^+CD25^+$ T cells in total $CD4^+$ T cells was slightly increased in transplant recipients with chronic rejection compared with stable transplant recipients, but these differences were not significant ($P = 0.21$). (B) Means ± SE of cumulative data on the proportion of $CD4^+CD25^+FOXP3^+IL\text{-}7R\alpha^{low}$ T cells in stable transplant recipients and transplant recipients with chronic rejection. The proportion of $FOXP3^+IL\text{-}7R\alpha^{low}$ T cells within the $CD4^+CD25^+$ T cell population was slightly reduced in the patients with chronic rejection compared with stable transplant recipients, and these differences were significant ($P = 0.01$).(C) Means ± SE of cumulative data on the proportion of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cells in stable transplant recipients and transplant recipients with chronic rejection. The proportion of $CD45RO^+IL\text{-}7R\alpha^{high}$ T cells within the $CD4^+CD25^+$ T cell population was significantly increased in the chronic rejection group compared with the stable group ($P < 0.0001$).

although the expansion of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population in patients with chronic rejection was associated with a decrease of T reg cells, the reduction of the T reg cell population was not sufficient to explain the increase of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population, thus indicating an absolute increase in the number of this latter population. Indeed, the absolute number of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells was also significantly increased ($1.5 \pm 0.4 \times 10^3$ cells per 100 μl of blood) in patients with chronic rejection compared with stable transplant recipients ($0.8 \pm 0.1 \times 10^3$ cells per 100 μl of blood; $P = 0.04$). However, the absolute number of T reg cells did not significantly change between the stable transplant recipients ($3.8 \pm 0.5 \times 10^3$ cells per 100 μl of blood) and the patients with chronic rejection ($3 \pm 0.5 \times 10^3$ cells per 100 μl of blood; $P = 0.3$).

Collectively, these results indicate (a) an association between the presence of an expanded $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population and the occurrence of chronic allograft rejection, and that (b) the expansion of the activated $CD4^+CD25^+CD45RO^+$ cell population defined by the expression of IL-7Rα cannot be explained only by the result of the decrease of the T reg cell population defined by the expression of FOXP3. Therefore, monitoring IL-7Rα may be a more valuable tool than monitoring FOXP3 to assess the patient's immune status and/or the occurrence of allograft chronic rejection.

### The $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population contains allospecific CD4 T cells

To have insights on the possible involvement of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population in the allospecific response to the graft, we examined the allospecific response of the transplant recipient to the donor in a MLR. Indeed, we had the opportunity to address this issue in a case of documented kidney chronic humoral rejection in which blood mononuclear cells of the (living) donor were available. The $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population was largely expanded (~40% of the $CD4^+CD25^+$ T cells) at the time of the diagnosis of chronic humoral rejection (time point 1; Fig. 3). The MLR was performed using the transplant recipient–sorted $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells mixed with irradiated transplant donor or MHC unrelated blood mononuclear cells. Interestingly, we found that the proliferation of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population was substantially higher (stimulation index [SI] = 66) in the presence of irradiated transplant donor blood mononuclear cells compared with the proliferation in the presence of MHC unrelated blood mononuclear cells (SI = 13; Fig. 3). Because of the diagnosis of chronic humoral rejection, this

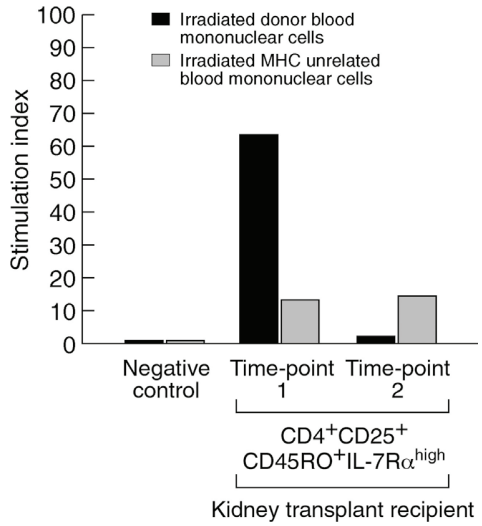



**Figure 3. Analysis of allospecific CD4 T cell responses.** Sorted purified $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cells ($7 \times 10^4$ cells) from two time points of a kidney transplant recipient with biopsy-proven chronic rejection were co-cultured with $10^5$ irradiated blood mononuclear cells (40 Gy) either from the organ donor or from an MHC unrelated subject. After 7 d of culture, cell proliferation was measured by [$^3$H]thymidine incorporation. Cell cultures containing only irradiated cells were used as negative controls.

patient was treated with high-dose (2 g/kg) i.v. Ig administration therapy. We had the opportunity to reassess the expansion of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population 15 mo after the first determination (time point 1) and 2 mo after treatment with i.v. Ig therapy. At this time (time point 2), the $IL\text{-}7R\alpha^{high}$ cell population had decreased to 20% of the $CD4^+CD25^+$ T cells, which corresponded to a reduction of ~50% compared with time point 1 (before the i.v. Ig therapy). At time point 2, we also analyzed the allospecific response mediated by the recipient-sorted $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells in a MLR with irradiated donor or MHC unrelated blood mononuclear cells. As shown at time point 2 in Fig. 3, there was no more evidence of an allospecific response against the transplant donor–specific cells (SI = 1.13), whereas the proliferative response against the MHC unrelated blood mononuclear cells remained unchanged compared with time point 1. Therefore, we observed an association between the magnitude of the expansion of the $IL\text{-}7R\alpha^{high}$–activated cell population and the ability to mediate allospecific response in vitro. Moreover, these results suggested that i.v. Ig administration may lead to a decrease (in percentage and function) in the $IL\text{-}7R\alpha^{high}$–activated cell population.

In support of the association between the magnitude of the $IL\text{-}7R\alpha^{high}$–activated cell population and allospecific function, we analyzed the allospecific response of the $IL\text{-}7R\alpha^{high}$ cell population in two additional cases of stable kidney transplant recipients in which blood mononuclear cells of the (living) donors were available. The percentage of the $IL\text{-}7R\alpha^{high}$ cell population was in the range of 20% (16.8 and 22.2%) in the two cases, and in agreement with the data shown for time point 2 in Fig. 3, allospecific responses were not detected (unpublished data).

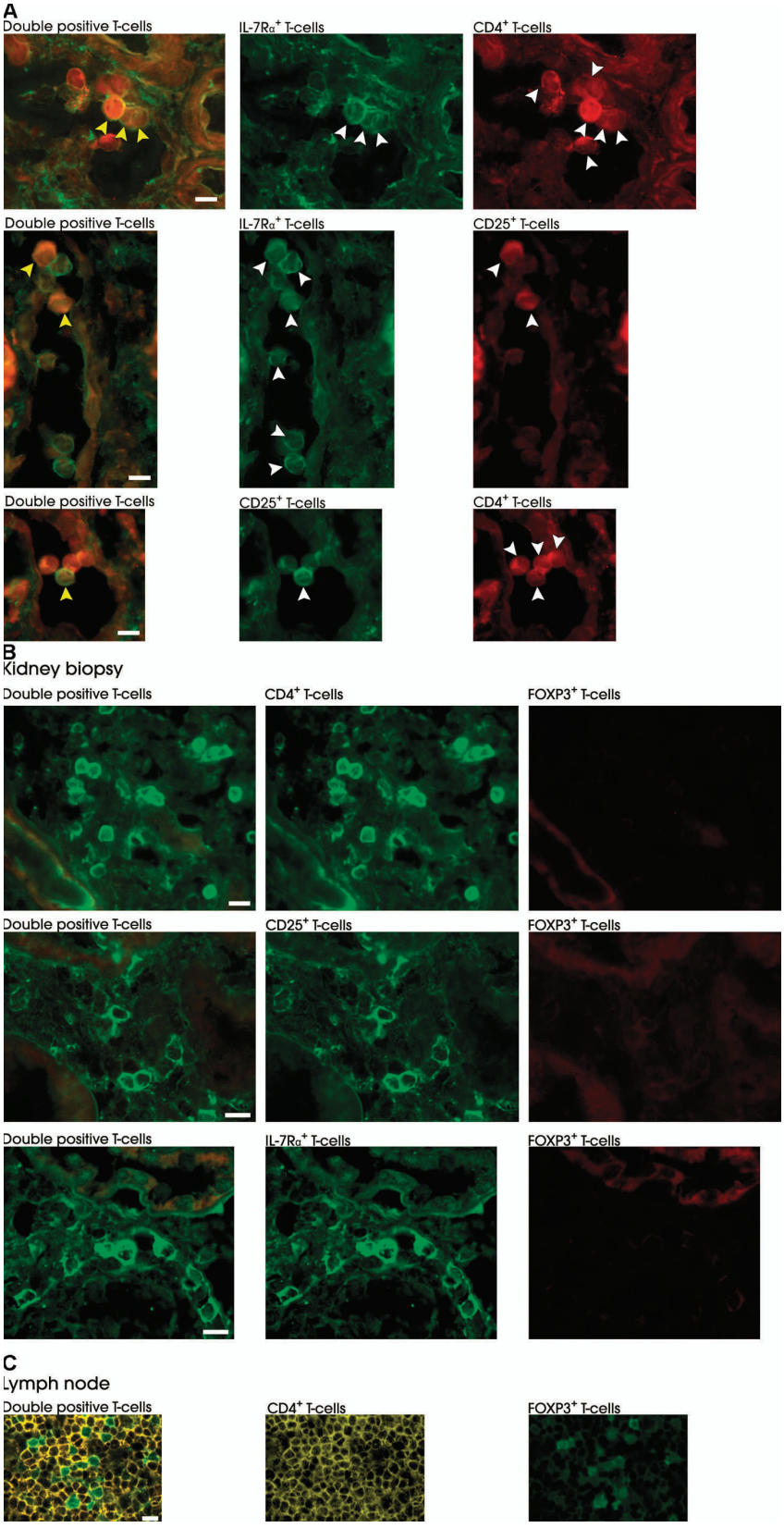
Furthermore, to better characterize the influence of immunosuppressive therapy on the $IL\text{-}7R\alpha^{high}$ cell population, we studied five additional transplant recipients and seven subjects with various autoimmune diseases receiving immunosuppressive therapy. An augmentation of immunosuppressive therapy in the transplant recipients was consistently associated with a significant subsequent reduction in the percentage of the $IL\text{-}7R\alpha^{high}$ cell population from 31.17 ± 6.33% to 17.33 ± 4.98% ($P = 0.002$). The percentage of this population in patients with autoimmune diseases ($n = 7$) receiving various types of immunosuppressive therapy was not significantly different from the one of healthy subjects ($P = 0.39$; unpublished data).

### $CD4^+CD25^+IL\text{-}7R\alpha^+$ cells infiltrate the allograft

We investigated the phenotype of the cells infiltrating the kidney tissue allograft in five patients with a documented diagnosis of chronic humoral rejection. For this purpose, frozen kidney biopsies were simultaneously stained with the following antibody combinations: CD4 plus CD25, CD4 plus IL-7Rα, CD25 plus IL-7Rα, CD4 plus FOXP3, IL-7Rα plus FOXP3, and CD25 plus FOXP3. Variable numbers of cells infiltrating the allograft were consistently found in all of the cases studied. Of interest, with regard to CD4 T cells, ~20% were $CD25^+$ and ~50% were $IL\text{-}7R\alpha^+$; all $CD25^+$ cells coexpressed IL-7Rα (Fig. 4 A). The estimates of the percentage of infiltrating $CD4^+CD25^+$ and $CD4^+$ $IL\text{-}7R\alpha^+$ cells have been generated from counting ~80 infiltrating CD4 T cells in 18 different microscopic fields of the five kidney biopsies. We also investigated the expression of FOXP3 in the CD4 T cells infiltrating the allograft, but $CD4^+FOXP3^+$ cells were not found in the five kidney biopsies examined (Fig. 4 B). However, $CD4^+FOXP3^+$ cells were consistently found in the lymph node biopsies used as control tissue (Fig. 4 C). It is also worth mentioning that cellular infiltrates were very scarce in the biopsy of patients with a kidney disease such as interstitial nephritis, and in this case, infiltrating CD4 T cells were $CD25^-$ and $IL\text{-}7R\alpha^-$ (unpublished data). Therefore, these results indicate that the predominant CD4 T cell population infiltrating the allograft in patients with chronic rejection is composed of $CD4^+CD25^+$ and $CD4^+IL\text{-}7R\alpha^+$cells.

### Importance of the analysis of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population in the clinical monitoring of transplant recipients

In this study, the results shown in Figs. 1 and 2 demonstrating the expansion in blood of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population were obtained from a cross-sectional analysis performed in 32 (21 liver and 11 kidney) organ transplant recipients. We recently had the opportunity to prospectively monitor the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population during the first year after transplantation in 11 kidney recipients receiving calcineurin-based immunosuppression with tacrolimus and mycophenolate mofetil (Fig. 5).


A
Double positive T-cells
IL-7Rα+ T-cells
CD4+ T-cells
Double positive T-cells
IL-7Rα+ T-cells
CD25+ T-cells
Double positive T-cells
CD25+ T-cells
CD4+ T-cells
B
Kidney biopsy
Double positive T-cells
CD4+ T-cells
FOXP3+ T-cells
Double positive T-cells
CD25+ T-cells
FOXP3+ T-cells
Double positive T-cells
IL-7Rα+ T-cells
FOXP3+ T-cells
C
Lymph node
Double positive T-cells
CD4+ T-cells
FOXP3+ T-cells

At study entry (before transplantation), there were no differences in the percentage of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells between the transplant recipients and living donors. The mean percentage in transplant recipients (5.04 ± 0.75%) and in living donors (4.65 ± 0.7%) was consistent with that observed in the large cohort of healthy subjects ($n$ = 45; 6.16 ± 0.76%; Fig. 5). Of interest, the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population was significantly increased (up to ~14% of the $CD4^+CD25^+$ T cells; $P < 0.005$) 1 mo after transplantation as compared with study entry, and the activated cell population remained expanded during the year of observation (Fig. 5). Interestingly, kidney allograft function in these recipients was stable during the study period, and there was no evidence of allograft rejection. These data further support the relationship between the expansion of the $IL\text{-}7R\alpha^{high}$ CD4 T cell population and the presence of the allograft and the usefulness of implementing the determination of this cellular marker in the immunologic monitoring of the transplant recipients.

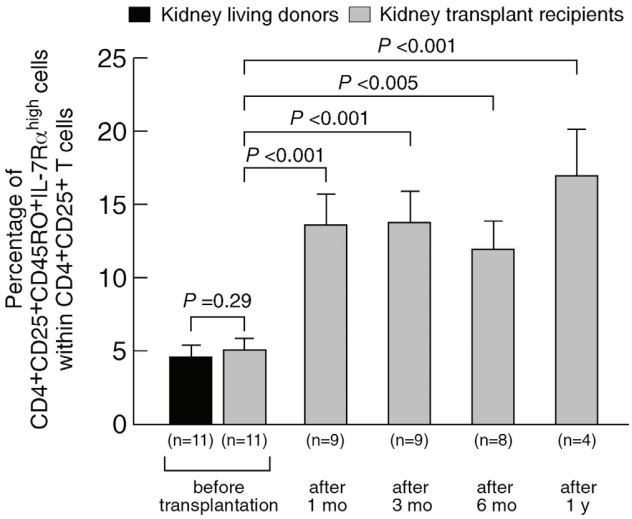



**Figure 5. Rapid expansion of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population after transplantation.** Means ± SE of cumulative data on the proportion of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cell population in transplant recipients before and up to 1 yr after transplantation. At the baseline (before transplantation), recipients and donors have a similar proportion of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cells. The $IL\text{-}7R\alpha^{high}$ CD4 T cell population is already significantly increased 1 mo after transplantation in the transplant recipients and remains expanded for up to 1 yr.

## CONCLUSIONS

We have identified the presence of a CD4 T cell population that is selectively expanded in patients after organ transplantation and is phenotypically defined by the expression of CD25, CD45RO, and high levels of IL-7Rα. Of interest, this cell population is expanded in stable transplant recipients in the absence of measurable circulating anti-HLA antibodies, thus indicating that it represents an allospecific cellular marker to monitor the immune response against the allograft. More importantly, this cell population contains allospecific CD4 T cells, it is substantially more expanded in patients with documented chronic humoral rejection, and ~50% of the CD4 T cells infiltrating the allograft (in the case of chronic rejection) are $IL\text{-}7R\alpha^+$. Collectively, these results suggest that the $CD4^+CD25^+IL\text{-}7R\alpha^+$ cell population represents a valuable, sensitive, and specific marker to monitor allospecific CD4 T cell responses in blood and tissue after organ transplantation. The precise contribution of this activated T cell population on the induction of effector mechanisms of late, chronic allograft rejection (e.g., production of alloantibodies with local complement activation) remains to be further studied. The clinical usefulness of this novel cellular marker in the monitoring of allograft recipients and its relationship with clinical outcomes need to be investigated in large prospective cohort studies of organ transplant recipients.

## MATERIALS AND METHODS

**Patients.** 21 liver and 15 kidney transplant recipients were studied at the Centre Hospitalier Universitaire Vaudois. All patients gave informed written consent before participating in the study, which was approved by the local institutional review board. 45 healthy volunteers were included as controls.

Peripheral blood was obtained from 21 clinically stable liver transplant recipients, all of whom had received a first cadaveric graft and were studied for >12 mo after transplantation. This group was composed of 15 males and 6 females, with a median age of 51.2 yr (range = 20–67 yr) at the time of the study and a median time from transplantation to the study of 7.6 yr (range = 1–19 yr). Their immunosuppressive therapy was based on cyclosporine or tacrolimus with prednisone ($n$ = 7) in combination with mycophenolate mofetil or azathioprine ($n$ = 2) .

Peripheral blood was also obtained from 15 kidney transplant recipients divided into two groups: (a) 11 patients with a stable graft function defined by a stable serum creatinine with values inferior to 150 μmol/liter and by a 24-h proteinuria inferior to 0.5 g/day, and (b) 4 patients with biopsy-proven chronic rejection. The stable kidney group was composed of seven males and four females, with a median age of 48.8 yr (range = 24–70 yr) at the time of the study and a median time from transplantation to the study of 8.6 yr (range = 1–19 yr). All patients had received a first graft: eight from a deceased donor, two from a

**Figure 4. $CD4^+IL\text{-}7R\alpha^+$ T cells infiltrate the tissue allograft.** Frozen kidney biopsies from transplant recipients with chronic rejection were stained with IL-7Rα and CD4 or CD25. (A, top) $IL\text{-}7R\alpha^+$ T cells (green) and $CD4^+$ T cells (red) are indicated by the arrowheads and are present in the interstitial compartment. Double-positive $CD4^+IL\text{-}7R\alpha^+$ T cells (yellow arrowheads) are colored in orange as a result of the merging of the two single-color images. (middle) $IL\text{-}7R\alpha^+$ T cells (green) and $CD25^+$ T cells (red) are also present in the interstitial compartment. Double-positive $CD25^+IL\text{-}7R\alpha^+$ T cells (yellow arrowheads) are colored in orange as a result of the merging of the two single-color images. (bottom) $CD25^+$ T cells (green) and $CD4^+$ T cells (red). Double-positive $CD4^+CD25^+$ T cells (yellow arrowheads) are colored in orange as a result of the merging of the two single-color images. (B, top) $CD4^+$ T cells (green) infiltrating the kidney of a patient with documented chronic rejection are FOXP3 negative (red). (middle and bottom) $CD25^+$ T cells (green) and $IL\text{-}7R\alpha^+$ T cells (green) are also negative for FOXP3 (red). (C) $CD4^+$ T cells (yellow) in the lymph node are FOXP3 positive (green). Bars, 10 μm.

living/related donor, and one from a living/unrelated donor. No patient in this group had circulating anti-HLA antibodies. The chronic rejection group was composed of two males and two females, with a median age of 42.8 yr (range = 26–56 yr) at the time of the study and a median time from transplantation to the study of 9 yr (range = 6–16 yr). Also in this group, patients had received a first graft: three from a deceased donor and one from a living/related donor (father to daughter). In the four patients with biopsy-proven chronic rejection (29, 30), circulating donor-specific anti-HLA antibodies (DSA) were detected in serum by ELISA (One Lambda, Inc.), and capillary C4d deposits were demonstrated in kidney graft biopsies. In three out of four biopsies, typical lamination with multilayering of the basement membrane of peritubular capillaries was also demonstrated by electron microscopy. In the living/donor recipient with chronic rejection, a repeat B cell cross match was positive (T and B cell cross matches were negative at the time of transplantation). In all four patients, circulating DSA were directed against defined class II antigens (anti–class II DSA). The median serum creatinine at the time of diagnosis of chronic rejection was 247 μmol/liter (range = 184–350 μmol/liter), whereas the median 24-h proteinuria was 2.1 g/day (range = 1–3.5 g/day).

A second group of 11 kidney transplant recipients was monitored prospectively at the Hôpitaux Universitaires de Genève and was composed of 7 males and 4 females, with a median age of 42.7 yr (range = 23–81 yr) at the time of the transplantation. The group of kidney donors (living donors) was composed of two males and nine females, with a median age of 52.8 yr (range = 32–73 yr) at the time of the donation. All of these 11 kidney transplant recipients received a 3–4-d thymoglobulin (1.5 mg/kg) course as induction therapy, with a calcineurin-based immunosuppressive regimen.

The group of patients with various autoimmune diseases studied at the Centre Hospitalier Universitaire Vaudois was composed of one male and six females, with a median age of 37.9 yr (range = 21–55 yr) at the time of the study. The patients received various regimens of immunosuppressive drugs.

**FACS analysis and sorting.** Peripheral blood mononuclear cells were isolated using standard Ficoll-Hypaque (GE Healthcare) gradient centrifugation. The antibodies used for flow cytometric analyses included PerCP, PerCP-Cy5.5, or PE-conjugated mouse anti–human CD4 (Becton Dickinson); allophycocyanin (APC)-conjugated mouse anti–human CD25 (BD Biosciences), FITC (BD Biosciences), or ECD (Beckman Coulter)-conjugated mouse anti–human CD45RO; and PE (Beckman Coulter)- or APC (R&D Systems)-conjugated mouse anti–human IL-7Rα. For intracellular FOXP3 analysis, cell preparations were fixed and permeabilized with fixation/permeabilization buffers (eBioscience) after staining of cell surface markers and stained with FITC-conjugated rat anti–human FOXP3 (eBioscience). For cell sorting experiments, $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{low}$, $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$, and $CD4^+CD25^-$ cell populations were isolated from the peripheral blood of transplant recipients. The grade of purity in all of the sorting experiments ranged between 92 and 98%. All flow cytometric analyses were performed on a FACSCalibur and LSRII, while cell sorting was performed on a FACSVantage SE and FACSAria (Becton Dickinson).

**Suppressive function assay.** The suppressive function of freshly sorted $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{low}$ and $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells isolated from the peripheral blood of liver transplant recipients was assessed in a MLR.

For these purposes, $10^5$ irradiated (40 Gy) allogeneic blood mononuclear cells (stimulator cells) and $5 \times 10^4$ $CD4^+CD25^-$ T cells isolated from liver transplant recipients (responder cells) were co-cultured either alone (positive control) or in the presence of $5 \times 10^4$ freshly sorted $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{low}$ or $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells. Proliferation was measured at day 7 by [$^3$H]thymidine incorporation.

**Allospecific T cell response.** Allospecific T cell proliferation of the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells isolated from one kidney recipient with documented chronic rejection was assessed by co-culturing $10^5$ irradiated (40 Gy) donor blood mononuclear cells with $7 \times 10^4$ sorted purified recipient $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells. Cultures were made in triplicates, and proliferation was measured at day 7 by [$^3$H]thymidine incorporation. As a control, the same sorted purified population was co-cultured with irradiated MHC unrelated blood mononuclear cells. The SI was calculated on the basis of the fold increase in the proliferation observed in the culture containing $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells and irradiated donor or MHC unrelated cells compared with the negative control, i.e., cultures containing irradiated cells alone.

**Cytokine production.** $2 \times 10^6$ cryopreserved blood mononuclear cells from liver and kidney transplant recipients were prestained with anti–IL-7Rα PE and mouse anti–human CD25 PE-Cy7 (BD Biosciences) antibodies and stimulated overnight with soluble 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies (BD Biosciences) in 1 ml of complete medium (RPMI plus 10% FBS) containing 1 μl/ml each of GolgiPlug and GolgiStop (BD Biosciences). At the end of the stimulation period, the cells were washed and stained with CD4 PerCP-Cy5.5 and CD45RO ECD antibodies. The cells were permeabilized with FACS Permeabilizing Solution 2 (BD Biosciences) and stained with mouse anti–human IFN-γ or TNF-α FITC and rat anti–human IL-2 APC (BD Biosciences). Data were acquired on an LSRII and analyzed using DiVa software (Becton Dickinson). Unstimulated cells were used as negative control for the production of cytokines. The background never exceeded 0.02%.

**Immunohistochemistry.** Frozen tissues from kidney biopsies were incubated at room temperature for 20 min with a blocking solution (5% normal goat serum) and immediately stained overnight at 4°C with a mix of primary antibodies: IgG1 mouse anti–human IL-7Rα (BD Biosciences) with IgG2a mouse anti–human CD4 (Beckman Coulter) or with IgG2b rat anti–human CD25 (Serotec) and mouse anti–human CD4 with rat anti–human CD25. The tissues were also stained with IgG2a rat anti–human FOXP3 (eBioscience) and IgG1 mouse anti–human CD4 (BD Biosciences) or IgG1 mouse anti–human CD25 (BD Biosciences) or mouse anti–human IL-7Rα. Secondary antibodies (goat IgG specific) were added after 16 h (after washing the unbound primary antibodies) and incubated for 1 h at room temperature in the dark. Goat anti–mouse IgG1 Alexa Fluor 488, goat anti–mouse IgG2a Alexa Fluor 594, goat anti–mouse IgG (H+L) Alexa Fluor 532, goat anti–rat IgG (H+L) FITC, biotinylated goat anti–rat IgG (H+L), streptavidin Texas red, and streptavidin FITC were all obtained from Invitrogen. The tissues were mounted with antifade reagent (ProLong Gold; Invitrogen) and observed on an imaging microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with epifluorescence using single-band excitation filters: Alexa Fluor 488, ex. 480/40 LP; Alexa Fluor 594, ex. 546/12; and Alexa Fluor 532, ex.535. Photos were taken using an AxioCam and AxioVision software (version 4.6; Carl Zeiss MicroImaging, Inc.). Each fluorochrome was imaged separately, and the resulting images were automatically pseudocolored and merged by the AxioVision software.

**Statistical analysis.** Statistical significance was calculated by the two-tailed *t* test and linear regression analysis. $P < 0.05$ was considered significant.

**Online supplemental material.** Fig. S1 shows that $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cells secrete inflammatory cytokines. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062120/DC1.

The authors would like to thank the Lausanne and Geneva transplant coordinators and Christiane Rotzetter for their precious help, as well as all the patients who agreed to take part in this study. They also thank Véronique Noguet and Nicole Prod'Hom for introducing L. Codarri to the techniques of immunohistochemistry, as well as Solange Moll for advice in the immunostaining techniques. Finally, they thank Yannick Krempp for graphical support and imaging advice.

The Centre Hospitalier Universitaire Vaudois Transplantation Center is supported by the 2004–2007 Strategic Plan of the Hospices–Centre Hospitalier Universitaire Vaudois, and M. Pascual received a grant from the Leenaards Foundation. The work of L. Vallotton is supported by a Swiss National Foundation grant (FN 323500-111275).

The authors have no conflicting financial interests.

**Submitted: 3 October 2006**
**Accepted: 25 May 2007**

## REFERENCES

1. McKenna, R.M., S.K. Takemoto, and P.I. Terasaki. 2000. Anti-HLA antibodies after solid organ transplantation. *Transplantation*. 69:319–326.
2. Sayegh, M.H., and L.A. Turka. 1998. The role of T-cell costimulatory activation pathways in transplant rejection. *N. Engl. J. Med.* 338:1813–1821.
3. Copin, M.C., C. Noel, M. Hazzan, A. Janin, F.R. Pruvot, J.P. Dessaint, G. Lelievre, and B. Gosselin. 1995. Diagnostic and predictive value of an immunohistochemical profile in asymptomatic acute rejection of renal allografts. *Transpl. Immunol.* 3:229–239.
4. Sabek, O., M.T. Dorak, M. Kotb, A.O. Gaber, and L. Gaber. 2002. Quantitative detection of T-cell activation markers by real-time PCR in renal transplant rejection and correlation with histopathologic evaluation. *Transplantation*. 74:701–707.
5. Reinke, P., E. Fietze, S. Ode-Hakim, S. Prosch, J. Lippert, R. Ewert, and H.D. Volk. 1994. Late-acute renal allograft rejection and symptomless cytomegalovirus infection. *Lancet*. 344:1737–1738.
6. Lun, A., M.Y. Cho, C. Muller, G. Staffa, W.O. Bechstein, C. Radke, P. Neuhaus, and H. Renz. 2002. Diagnostic value of peripheral blood T-cell activation and soluble IL-2 receptor for acute rejection in liver transplantation. *Clin. Chim. Acta*. 320:69–78.
7. Schowengerdt, K.O., F.J. Fricker, K.S. Bahjat, and S.T. Kuntz. 2000. Increased expression of the lymphocyte early activation marker CD69 in peripheral blood correlates with histologic evidence of cardiac allograft rejection. *Transplantation*. 69:2102–2107.
8. Creemers, P., J. Brink, H. Wainwright, K. Moore, E. Shephard, and D. Kahn. 2002. Evaluation of peripheral blood CD4 and CD8 lymphocyte subsets, CD69 expression and histologic rejection grade as diagnostic markers for the presence of cardiac allograft rejection. *Transpl. Immunol.* 10:285–292.
9. Chang, D.M., Y.A. Ding, S.Y. Kuo, M.L. Chang, and J. Wei. 1996. Cytokines and cell surface markers in prediction of cardiac allograft rejection. *Immunol. Invest.* 25:13–21.
10. Poggio, E.D., M. Roddy, J. Riley, M. Clemente, D.E. Hricik, R. Starling, J.B. Young, B. Gus, M.H. Yamani, and P.S. Heeger. 2005. Analysis of immune markers in human cardiac allograft recipients and association with coronary artery vasculopathy. *J. Heart Lung Transplant.* 24:1606–1613.
11. Poggio, E.D., M. Clemente, J. Riley, M. Roddy, N.S. Greenspan, C. Dejelo, N. Najafian, M.H. Sayegh, D.E. Hricik, and P.S. Heeger. 2004. Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. *J. Am. Soc. Nephrol.* 15:1952–1960.
12. Najafian, N., M.J. Albin, and K.A. Newell. 2006. How can we measure immunologic tolerance in humans? *J. Am. Soc. Nephrol.* 17:2652–2663.
13. Cozzo, C., J. Larkin III, and A.J. Caton. 2003. Cutting edge: self-peptides drive the peripheral expansion of CD4+CD25+ regulatory T cells. *J. Immunol.* 171:5678–5682.
14. Gavin, M.A., S.R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat. Immunol.* 3:33–41.
15. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203:1693–1700.
16. Holzinger, C., A. Schollhammer, M. Imhof, C. Reinwald, G. Kramer, A. Zuckermann, E. Wolner, and G. Steiner. 1995. Phenotypic patterns of mononuclear cells in dilated cardiomyopathy. *Circulation*. 92:2876–2885.
17. Liu, W., A.L. Putnam, Z. Xu-Yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B.F. de St. Groth, et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ T reg cells. *J. Exp. Med.* 203:1701–1711.
18. Graca, L., S.P. Cobbold, and H. Waldmann. 2002. Identification of regulatory T cells in tolerated allografts. *J. Exp. Med.* 195:1641–1646.
19. Kingsley, C.I., M. Karim, A.R. Bushell, and K.J. Wood. 2002. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J. Immunol.* 168:1080–1086.
20. Schenk, S., D.D. Kish, C. He, T. El-Sawy, E. Chiffoleau, C. Chen, Z. Wu, S. Sandner, A.V. Gorbachev, K. Fukamachi, et al. 2005. Alloreactive T cell responses and acute rejection of single class II MHC-disparate heart allografts are under strict regulation by CD4+ CD25+ T cells. *J. Immunol.* 174:3741–3748.
21. Benghiat, F.S., L. Graca, M.Y. Braun, S. Detienne, F. Moore, S. Buonocore, V. Flamand, H. Waldmann, M. Goldman, and A. Le Moine. 2005. Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression. *Transplantation*. 79:648–654.
22. Taylor, P.A., A. Panoskaltsis-Mortari, J.M. Swedin, P.J. Lucas, R.E. Gress, B.L. Levine, C.H. June, J.S. Serody, and B.R. Blazar. 2004. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood*. 104:3804–3812.
23. Lee, M.K., D.J. Moore, B.P. Jarrett, M.M. Lian, S. Deng, X. Huang, J.W. Markmann, M. Chiaccio, C.F. Barker, A.J. Caton, and J.F. Markmann. 2004. Promotion of allograft survival by CD4+CD25+ regulatory T cells: evidence for in vivo inhibition of effector cell proliferation. *J. Immunol.* 172:6539–6544.
24. Clark, F.J., R. Gregg, K. Piper, D. Dunnion, L. Freeman, M. Griffiths, G. Begum, P. Mahendra, C. Craddock, P. Moss, and R. Chakraverty. 2004. Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. *Blood*. 103:2410–2416.
25. Demirkiran, A., A. Kok, J. Kwekkeboom, H.J. Metselaar, H.W. Tilanus, and L.J. van der Laan. 2005. Decrease of CD4+CD25+ T cells in peripheral blood after liver transplantation: association with immunosuppression. *Transplant. Proc.* 37:1194–1196.
26. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 299:1057–1061.
27. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4:330–336.
28. Sakaguchi, S. 2003. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J. Clin. Invest.* 112:1310–1312.
29. Pascual, M., T. Theruvath, T. Kawai, N. Tolkoff-Rubin, and A.B. Cosimi. 2002. Strategies to improve long-term outcomes after renal transplantation. *N. Engl. J. Med.* 346:580–590.
30. Cardarelli, F., M. Pascual, N. Tolkoff-Rubin, F.L. Delmonico, W. Wong, D.A. Schoenfeld, H. Zhang, A.B. Cosimi, and S.L. Saidman. 2005. Prevalence and significance of anti-HLA and donor-specific antibodies long-term after renal transplantation. *Transpl. Int.* 18:532–540.

## 4.2.2. Demographic data of the patients

As mentioned above, we enrolled overall more than 100 new kidney transplant recipients in our study, in a retrospective as well as in a prospective way. We decided to classify them in different study groups, according to their clinical conditions, and we will use the same terminology throughout the following results and discussions.

For the **retrospective** part of our study, we could distinguish three different groups of patients, with a total of 91 patients:

(a) stable kidney transplant recipients (n=60): they are defined as patients with a stable serum creatinine level, a 24h-proteinuria inferior to 0.5 g/day, no circulating anti-HLA antibodies, and a standard immunosuppressive therapy (calcineurin inhibitor (n=54) or mTOR inhibitor (n=6) ± mycophenolate mofetil ± steroids);

(b) kidney transplant recipients with chronic humoral rejection (n=7): they are defined as patients with progressive deterioration of their graft function, presence of circulating donor-specific anti-HLA antibodies, and a biopsy-proven diagnosis of chronic humoral rejection (CHR) based on the criteria of the Banff'07 conference (Solez K *et al*., 2008; **Appendix I**);

(c) kidney transplant recipients on no or minimal immunosuppression (n=24): they are defined as patients with a stable serum creatinine level below 150 µmol/l, a 24h-proteinuria inferior to 0.5 g/day, no circulating anti-HLA antibodies, and no (n=1) or minimal immunosuppression (n=23; i.e. steroids alone, or mycophenolate mofetil ± steroids, or azathioprine ± steroids).

In addition to the above-described kidney transplant recipients, we also had the unique opportunity to analyze four liver transplant recipients on no or minimal immunosuppression: they are defined as patients with stable liver tests, and no (n=1) or minimal immunosuppression (n=3; i.e., steroids alone, or low-dose tacrolimus alone). All relevant demographic data of these patients are summarized in **Appendix II**.

For the **prospective** part of our study, patients were enrolled in two clinical protocols, differing by the induction and immunosuppressive regimens, with a total of 35 patients:

(a) group "THYMO" (n=19): induction by thymoglobulin (4 doses, 1.5 mg/kg/day, from days 0 to 3) and steroids (tapered in four days unless required), followed by maintenance immunosuppression with tacrolimus and mycophenolate mofetil, all patients received a first renal graft from a living-donor;

(b) group "BSX" (n=16): induction by basiliximab (20 mg at days 0 and 4), followed by maintenance immunosuppression with tacrolimus, mycophenolate mofetil and steroids, all patients received a first renal graft either from a living or from a cadaveric donor.

All relevant demographic data of these patients are summarized in **Appendix III**.

## 4.2.3. Analysis of functionally distinct subsets of $CD4^+$ $CD25^{high}$ T cells in solid organ transplant recipients: retrospective part of the study

Following the results of our initial publication, we performed the same kind of experiments (polychromatic flow cytometric phenotyping of peripheral blood mononuclear cells) in the above-mentioned 91 kidney transplant recipients and in the 4 liver transplant recipients. We used the same gating strategy as the one described in the chapter on healthy individuals [**Figures 23** and **24**]. Overall, our results were consistent with our publication.

### *4.2.3.1. Correlation with the clinical status of the kidney transplant recipients*

In order to be able to compare consistently the results between the different study groups, it is important to base our conclusions not only on percentages of sub-populations of cells relative to their parent or grand-parent population, but also on absolute numbers of cells, expressed as number of cells per µl) of blood for instance; indeed, the real functional properties of a cellular population depends critically on its numerical importance. For this purpose, we checked the leucocyte counts in the kidney transplant recipients (3 study groups). Overall, we found comparable leucocyte absolute counts in transplant recipients (mean ± standard error of the mean: 6.6 ± 0.2 G/L) compared to healthy individuals (6.8 ± 0.4 G/L; *P*=0.20) [**Figure 33A**]. When looking at the three study groups of kidney transplant recipients, we also found no statistical difference with healthy individuals and between each group: stable kidney transplant recipients had a mean leucocytes absolute count of 6.2 ± 0.2 G/L (*P*=0.09 as compared to healthy individuals), kidney transplant recipients with CHR 7.3 ± 0.8 G/L (*P*=0.32) and kidney transplant recipients on no or minimal immunosuppression 6.9 ± 0.4 G/L (*P*=0.43) [**Figure 33B**].

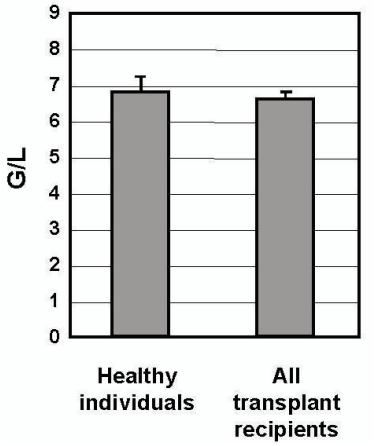



**Figure 33A. Leucocyte absolute counts in healthy individuals and transplant recipients.**

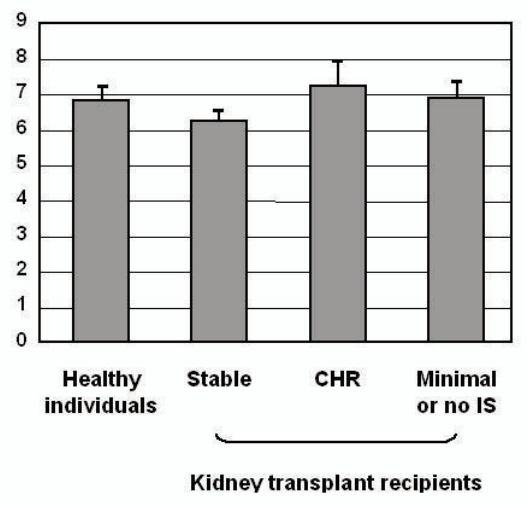



**Figure 33B. Leucocyte absolute counts in healthy individuals and the three study groups of kidney transplant recipients.**

#### 4.2.3.1.1. $CD4^+$ $CD25^{high}$ T cells

Then, we were interested in the percentage of $CD4^+$ $CD25^{high}$ T cells, to assess if differences between our three study groups were present. Overall, we found that the mean percentage of $CD25^{high}$ cells within $CD4^+$ T cells was 1.12% (range: 0.12-4.11%; median: 0.89%), which was significantly lower than the mean percentage found in healthy volunteers (1.62%; $P<0.005$) [**Figure 34A**]; this could be explained by the fact that most immunosuppressive treatments target intracellular signals involved in T cell activation after antigen presentation, which is, among others, characterized by the transient upregulation of the CD25 molecule on the cell surface. When looking at our three study groups, we found the lowest percentage of $CD4^+$ $CD25^{high}$ T cells in kidney transplant recipients with CHR ($0.82 \pm 0.15\%$; $P<0.05$ as compared to healthy volunteers), followed by stable transplant recipients ($1.06 \pm 0.11\%$; $P<0.005$) and kidney transplant recipients on no or minimal immunosuppression ($1.24 \pm 0.15\%$; $P<0.05$). These results are in accordance with recent publications (Louis S *et al*., 2006). Interestingly, among stable kidney transplant recipients, patients receiving sirolimus as immunosuppressive treatment displayed a $CD4^+$ $CD25^{high}$ T cells percentage ($1.72 \pm 0.46\%$) similar to the one found in healthy volunteers ($P=0.41$) [**Figure 34B**], in accordance with recent data (Segundo DS *et al*. (2006); Ruggenenti P *et al*. (2007); Noris M *et al*. (2007)); interestingly, as demonstrated by *in vitro* studies, the presence of sirolimus in culture experiments induces a selective expansion of murine and human regulatory T cells, whereas at the same time it kills effector T cells or at least prevents their expansion (Coenen JJ *et al*., 2007; Battaglia M *et al*., *J Immunol*, 2006; Strauss L *et al*., 2007; Keever-Taylor CA *et al*. 2007; May KF Jr *et al*., 2007).

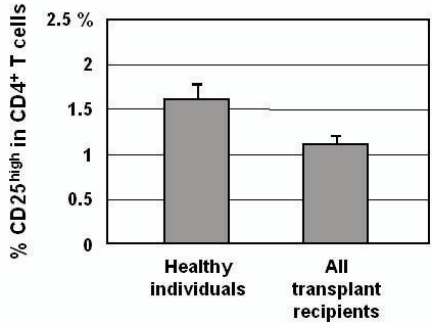



**Figure 34A. Percentage of $CD4^+$ $CD25^{high}$ T cells in 73 healthy individuals and 91 kidney transplant recipients.**

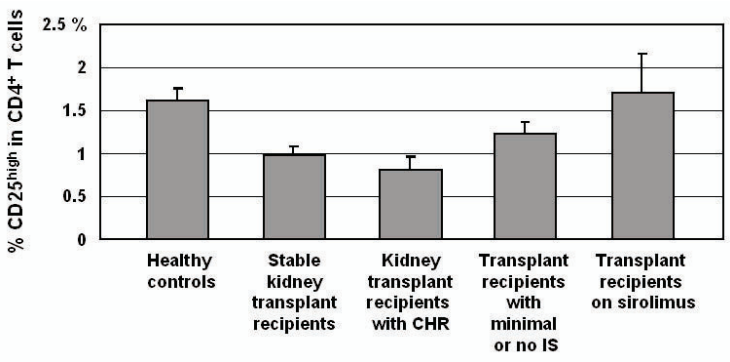



**Figure 34B. Percentage of $CD4^+$ $CD25^{high}$ T cells in kidney transplant recipients: 54 stable, 7 with CHR, 24 stable on no or minimal immunosuppression and 6 stable on sirolimus.**

However, since $CD4^+$ $CD25^{high}$ T cells contain both regulatory as well as activated T cells, it is important to discriminate these sub-populations and assess how they are influenced by the clinical status of the kidney transplant recipients. For this purpose, we performed the same staining protocol as described in the part on healthy individuals, including anti-CD4, CD25, CD45RO, CD127 and FoxP3 monoclonal antibodies, on our three kidney transplant recipients

study groups. In accordance with our publication, we found that the respective percentages of the activated ($CD4^+$ $CD25^{high}$ $CD127^{high}$ $FoxP3^-$) and regulatory ($CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$) T cell sub-populations were modified in stable kidney transplant recipients and in those with CHR as compared to healthy individuals. In addition, we could also observe significant changes in the newly enrolled group of patients on no or minimal immunosuppression.

#### 4.2.3.1.2. Activated T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ cells)

Overall, the activated T cell population was expanded in kidney transplant recipients ($13.51 \pm 0.90\%$ of $CD45RO^+$ $CD127^{high}$ cells within $CD4^+$ $CD25^{high}$ T cells) as compared to healthy individuals ($5.97 \pm 0.36\%$; $P<0.001$) [**Figure 35A**]. In stable transplant recipients, the percentage was found to be expanded to $14.57 \pm 0.97\%$ ($P<0.001$), and interestingly, an almost doubled percentage was observed in kidney transplant recipients with CHR ($25.32 \pm 4.66\%$; $P<0.001$). These results are in the range of those of our publication. However, it was very interesting to note that stable kidney transplant recipients on no or minimal immunosuppression did not exhibit a significant expansion of the activated T cell population ($7.40 \pm 0.72\%$; $P=0.08$). Furthermore, among stable transplant recipients, those on sirolimus-based immunosuppression exhibited even a lower activated T cell percentage as compared to the one found in healthy individuals ($3.41 \pm 0.74\%$; $P<0.05$) [**Figure 35B**]. In addition, in the four liver transplant recipients on no or minimal immunosuppression, the activated T cell population was not expanded ($9.05 \pm 0.93\%$), and this percentage was comparable to the one of their kidney recipients counterparts ($P=0.19$) [data not shown].

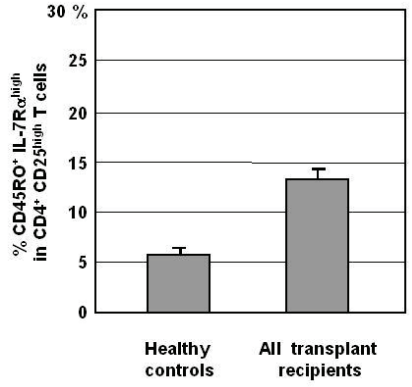



**Figure 35A. Percentage of the activated T cell sub-population in 73 healthy individuals and 91 kidney transplant recipients.**

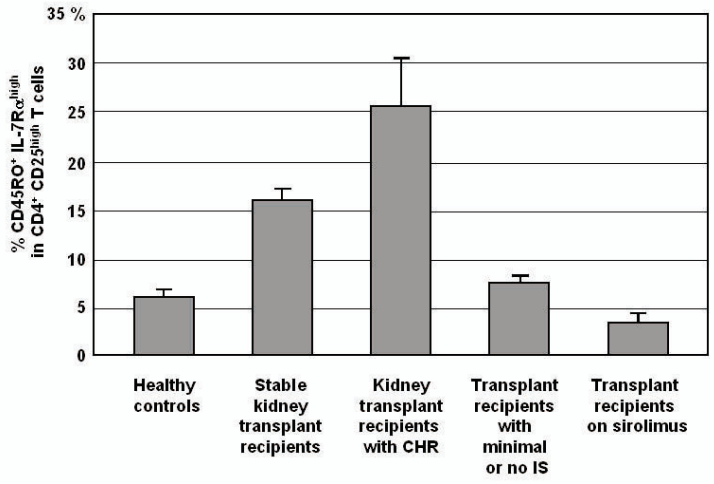



**Figure 35B. Percentage of the activated T cell sub-population in 73 healthy individuals, 54 stable kidney transplant recipients on CNI-based immunosuppression, 7 kidney transplant recipients with CHR, 24 kidney transplant recipients on no or minimal immunosuppression and 6 stable kidney transplant recipients on sirolimus-based immunosuppression.**

These results strongly suggest that the assessment of the activated T cell sub-population could be useful to help in the clinical evaluation of kidney transplant recipients. For example, it could be helpful to detect "(pseudo-)tolerant" patients who could benefit from immunosuppression minimization or even withdrawal. At the present time, this is usually done under close monitoring for rejection development, including clinical parameters such as therapeutic drug monitoring, assessment of serum creatinine and of development of circulating alloantibodies, and protocol renal biopsies. However, only prospective studies of immunosuppression weaning in a large controlled and randomized setting will enable proof of concept of this hypothesis. In this regard, monitoring activated T cells may be very useful.

We also assessed the mean absolute counts of this activated T cell sub-population; although values did not always reach statistical significance, the absolute counts displayed similar trends as the above-mentioned percentages. Briefly, the activated T cell population absolute count in stable kidney transplant recipients ($1.26 \pm 0.25$ cell per µl of blood; $P$=0.09) as well as in transplant recipients with CHR ($1.62 \pm 0.40$ cell/µl; $P$<0.01) tended to be, respectively was increased as compared to healthy individuals ($0.79 \pm 0.13$ cell/µl); conversely, in kidney transplant recipients on no or minimal immunosuppression ($0.55 \pm 0.11$ cell/µl; $P$=0.11) as well as in stable kidney transplant recipients on sirolimus ($0.33 \pm 0.16$ cell/µl; $P$=0.08), the activated T cell population absolute counts were comparable to the value found in healthy individuals [**Figure 35C**].

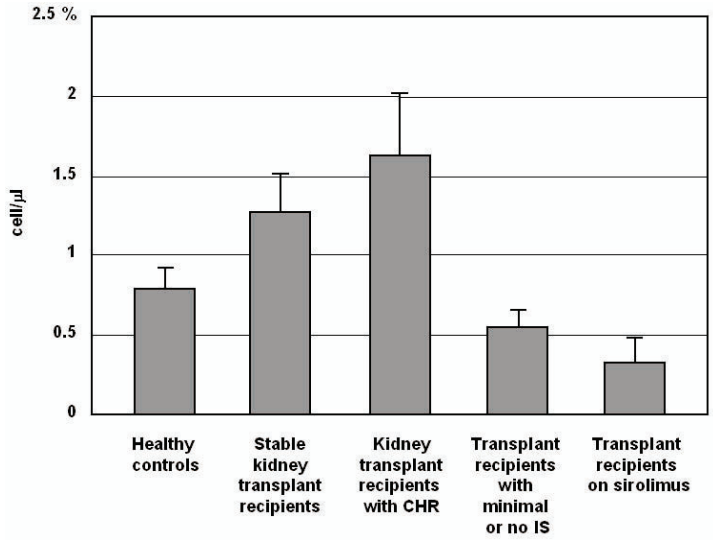



**Figure 35C. Mean absolute counts of the activated T cell sub-population in 73 healthy individuals, 54 stable kidney transplant recipients on CNI-based immunosuppression, 7 kidney transplant recipients with CHR, 24 kidney transplant recipients on no or minimal immunosuppression and 6 stable kidney transplant recipients on sirolimus-based immunosuppression.**

Thereafter, it was important to look at the changes of the regulatory T cell sub-population, defined as $CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ cells.

#### 4.2.3.1.3. Regulatory T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD127^{low}$ cells)

Overall, the regulatory T cell sub-population was found to be decreased in kidney transplant recipients (50.51 ± 1.98% of $CD127^{low}$ $FoxP3^+$ cells within $CD4^+$ $CD25^{high}$) as compared to healthy individuals (74.05 ± 2.01%; $P$<0.001) [**Figure 36A**]. The extent of this decrease was close to one third and comparable in the three study groups; in stable transplant recipients, the percentage of the regulatory T cell sub-population was decreased to 49.50 ± 2.27% ($P$<0.001 as compared to healthy individuals), in kidney transplant recipients with CHR to 50.50 ± 4.15% ($P$<0.001), and in kidney transplant recipients on no or minimal immunosuppression to 49.40 ± 8.71% ($P$<0.001). However, the regulatory T cell sub-population tended to be less decreased in the sub-group of stable kidney transplant recipients on sirolimus-based immunosuppression (62.48 ± 4.88%; $P$<0.05) and was statistically higher than in the stable kidney transplant recipients on CNI-based immunosuppression ($P$<0.05) [**Figure 36B**]. In addition, in the four liver transplant recipients on no or minimal immunosuppression, the regulatory T cell sub-population was decreased in the same extent (53.64 ± 2.02%; $P$=0.43) as in their kidney recipients counterparts [data not shown].

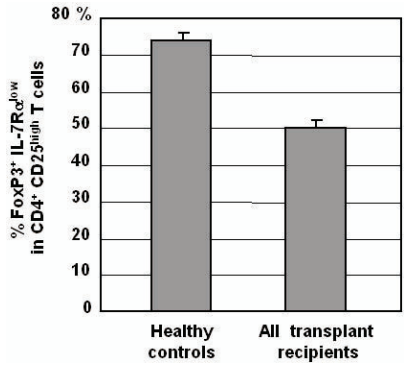



**Figure 36A. Percentage of the regulatory T cell sub-population in 73 healthy individuals and 91 kidney transplant recipients.**

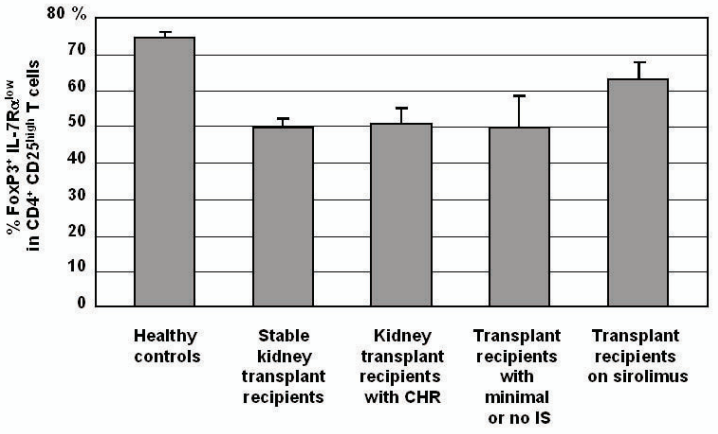



**Figure 36B. Percentage of the regulatory T cell sub-population in 73 healthy individuals, 54 stable kidney transplant recipients on CNI-based immunosuppression, 7 kidney transplant recipients with CHR, 24 kidney transplant recipients on no or minimal immunosuppression and 6 stable kidney transplant recipients on sirolimus-based immunosuppression.**

We also assessed the mean absolute count of this regulatory T cell sub-population; as compared to healthy individuals (7.54 ± 1.09 cell/μl), we found that the absolute number of regulatory T cells was significantly decreased in stable kidney transplant recipients (3.29 ± 0.50 cell/μl; $P$<0.001), whilst it tended to be decreased in kidney transplant recipients with CHR (2.92 ± 0.69 cell/μl; $P$=0.07), as well as in the stable recipients on sirolimus-based

immunosuppression (2.75 ± 0.71 cell/µl; *P*=0.06), but not in kidney transplant recipients on no or minimal immunosuppression (5.56 ± 0.42 cell/µl; *P*=0.28) [**Figure 36C**].

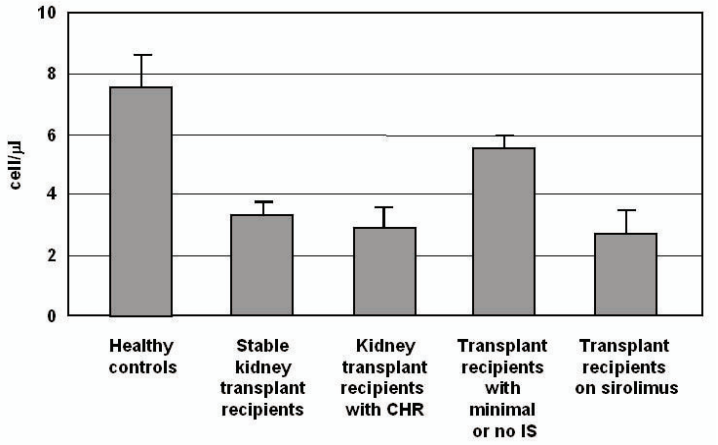



**Figure 36C. Mean absolute counts of the regulatory T cell sub-population in 73 healthy individuals, 54 stable kidney transplant recipients on CNI-based immunosuppression, 7 kidney transplant recipients with CHR, 24 kidney transplant recipients on no or minimal immunosuppression and 6 stable kidney transplant recipients on sirolimus-based immunosuppression.**

After integration of the results on the whole $CD4^+$ $CD25^{high}$ T cell population, as well as on the activated ($CD4^+$ $CD25^{high}$ $CD127^{high}$ $FoxP3^-$) and regulatory ($CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$) T cell sub-populations, the parameter that showed the better correlation with the clinical status of the patients was the percentage of the activated T cell sub-population. This conclusion is based on the respective percentages found in the different clinical study groups as well as on the results of statistical tests. The results of the Student's *t* test performed for the three parameters and for the clinical study groups are shown in **Table 3**.

The percentage of $CD4^+$ $CD25^{high}$ T cells was significantly reduced in all kidney transplant recipients except in the group on sirolimus-based immunosuppression, as compared to healthy individuals; however, the difference between stable kidney transplant recipients and those with CHR (*P*=0.27), between stable and those on no or minimal Immunosuppression (*P*=0.09), as well as between those with CHR and those on no or minimal immunosuppression (*P*=0.08), was not statistically significant.

Concerning the percentage of the regulatory $CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ T cell sub-population, it was significantly reduced in all transplant recipients as compared to healthy individuals. However, again, there was no sufficient statistical difference between the clinical study groups to permit a clear distinction.

On the other hand, the percentage of the activated $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ T cell sub-population displayed a clear and statistically significant differential repartition between healthy individuals and the different clinical study groups of kidney transplant recipients, and, more importantly, it permitted to discriminate between stable kidney transplant recipients and

kidney transplant recipients with CHR. However, the results of some individuals do not fit with the "expected" value based on their clinical status. We cannot exclude that other parameters play a role and modulate the percentage of the activated T cell sub-population. For example, it could be that concurrent infections might increase the percentage of this sub-population; to prevent this bias, we have carefully checked in all our volunteers before blood drawing that they did not have an overt concomitant infection, but the possibility of an occult infection still remains. This could explain why some stable kidney transplant recipients displayed a high percentage of the activated T cell sub-population. Another explanation could be that these patients were at the beginning of an ongoing chronic humoral rejection process without having developed clinical signs and symptoms yet. The only way to confirm this hypothesis would be to perform a renal biopsy, but most of the patients, as well as clinicians, are reluctant to this invasive procedure without any clear clinical justification. On the other hand, some stable kidney transplant recipients on standard CNI-based immunosuppression displayed lower percentages of the activated T cell sub-population and most of the time higher percentages of the regulatory T cell sub-population than expected; this particular blood phenotype could be the expression of a more tolerogenic state of these patients towards their grafts, and could indicate that they may benefit from a controlled tapering of their immunosuppression. Of course, further studies under strict clinical monitoring on selected patients are needed to prove this hypothesis.

**Percentage of $CD25^{high}$ cells within $CD4^{+}$ T cells**

| ***P*=** | **Stable** | **CHR** | **No or minimal IS** | **Sirolimus** | **All transplant recipients** |
|---|---|---|---|---|---|
| **Healthy individuals** | *** <0.001 | * <0.05 | * <0.05 | 0.41 | ** <0.01 |
| **Stable** | -- | 0.27 | 0.09 | * <0.05 | -- |
| **CHR** | -- | -- | 0.08 | * <0.05 | -- |
| **No or minimal IS** | -- | -- | -- | 0.11 | -- |

**Percentage of activated $CD45RO^{+}$ $CD127^{high}$ cells within $CD4^{+}$ $CD25^{high}$ T cells**

| ***P*=** | **Stable** | **CHR** | **No or minimal IS** | **Sirolimus** | **All transplant recipients** |
|---|---|---|---|---|---|
| **Healthy individuals** | *** <0.001 | *** <0.001 | 0.08 | * <0.05 | *** <0.001 |
| **Stable** | -- | ** <0.01 | *** <0.001 | *** <0.001 | -- |
| **CHR** | -- | -- | *** <0.001 | ** <0.01 | -- |
| **No or minimal IS** | -- | -- | -- | ** <0.01 | -- |

**Percentage of regulatory FoxP3$^{+}$ CD127$^{low}$ cells within CD4$^{+}$ CD25$^{high}$ T cells**

| *P*= | Stable | CHR | No or minimal IS | Sirolimus | All transplant recipients |
|---|---|---|---|---|---|
| **Healthy individuals** | *** <0.001 | *** <0.001 | *** <0.001 | ** <0.01 | *** <0.001 |
| **Stable** | -- | 0.45 | 0.49 | 0.14 | -- |
| **CHR** | -- | -- | 0.46 | 0.24 | -- |
| **No or minimal IS** | -- | -- | -- | 0.26 | -- |

**Table 3. Results of Student's *t* tests performed between healthy individuals, stable kidney transplant recipients, kidney transplant recipients with CHR, stable kidney transplant recipients on no or minimal immunosuppression, and stable kidney transplant recipients on sirolimus-based immunosuppression, for the CD4$^{+}$ CD25$^{high}$ T cell population, the activated CD4$^{+}$ CD25$^{high}$ CD45RO$^{+}$ CD127$^{high}$ and regulatory CD4$^{+}$ CD25$^{high}$ FoxP3$^{+}$ CD127$^{low}$ T cell sub-populations.**

**Statistically significant results are shown in red.**

***: significant; **: highly significant; ***: very highly significant.**

### *4.2.3.2. Correlation with the immunosuppressive treatments of the kidney transplant recipients*

We have already shown that sirolimus seems to have an impact on the repartition of CD4$^{+}$ CD25$^{high}$ T cells sub-populations different from CNIs, and we were interested in looking at the effect of other conventional immunosuppressive drugs. As already discussed in the Introduction part, most immunosuppressive drugs target the intracellular signals involved in T cell activation after antigen presentation. Conventional immunosuppressive drugs used in the maintenance phase of transplantation include several compounds, with different mechanisms of action: corticosteroids, azathioprine, CNIs, mTOR inhibitors (sirolimus and everolimus), and mycophenolate mofetil (MMF). We will briefly review their mechanisms of action.

Corticosteroids (prednisone, prednisolone and methylprednisolone) inhibit transcription factors such as NF-κB and AP-1, which are involved in transcription of cytokine and chemokine genes, including IL-2, TNFα and IFNγ.

Azathioprine was the first immunosuppressive agent to be used extensively in solid organ transplantation. It is thought to act by releasing 6-mercaptopurine, which interferes with a specific step of the cell cycle, namely DNA synthesis.

Calcineurin inhibitors (CNIs), including cyclosporine A and tacrolimus, engage respectively cyclophilin and FK506-binding protein 12, which in turn inhibit calcineurin, a potent phosphatase of the T cell activation transduction pathway. Both drugs limit the production of cytokines, including IL-2, by blocking the activation of the transcription factor NFAT.

The mTOR inhibitors (sirolimus and everolimus) exert their effect at the level of the mammalian target of rapamycin (mTOR), a key protein in the transduction pathway from IL-2 receptor to cell cycle activation.

Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid, which inhibits inosine monophosphate dehydrogenase, a key enzyme in purine synthesis. T cells in particular are dependent on this pathway for cell division. It suppresses the proliferation of antigen-stimulated T cells.

To analyze the impact of the immunosuppressive agents, we compared the results of the $CD4^{+}$ $CD25^{high}$ T cells sub-populations among stable transplant recipients with different immunosuppressive drugs; we also tested one individual who had his immunosuppressive regimen changed, at two different timepoints.

Overall, among stable kidney transplant recipients on CNI-based immunosuppression, 47 received tacrolimus (TAC) whilst 11 received cyclosporine A (CsA); the various immunosuppressive regimens are shown in **Table 4**. The sub-group of stable patients on sirolimus has already been discussed in the preceding section.

| **Immunosuppression** | **N=** |
|---|---|
| ***Tacrolimus*** | 47 |
| *triple immunosuppression* | 17 |
| TAC + MMF + pred | 16 |
| TAC + aza + pred | 1 |
| *double immunosuppression* | 30 |
| TAC + MMF | 27 |
| TAC + aza | 2 |
| TAC + pred | 1 |
| ***Cyclosporine*** | 11 |
| *triple immunosuppression* | 3 |
| CsA + MMF + pred | 3 |
| *double immunosuppression* | 3 |
| CsA + MMF | 2 |
| CsA + pred | 1 |
| *single immunosuppression* | 5 |
| CsA | 5 |

**Table 4. Immunosuppressive regimens of 58 stable kidney transplant recipients on CNI-based immunosuppression.**

Concerning the percentage of the activated $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ T cell sub-population, we did not find any statistical difference between patients receiving TAC (15.56 ± 1.01%) and those receiving CsA (12.94 ± 1.65%; *P*=0.13), regardless of the use of other immunosuppressive drugs (e.g. MMF). In patients who did not receive MMF, there was no statistical difference between those receiving CsA (15.87 ± 2.03%) and those receiving TAC (18.54 ± 1.48%; *P*=0.32). Looking at the number of immunosuppressive agents that were prescribed, we did not find any statistical difference between patients receiving one, two, or three immunosuppressants, regardless of the CNI that was used.

Concerning the percentage of the regulatory $CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ T cell sub-population, there was no significant difference between stable kidney transplant recipients receiving CsA (55.48 ± 4.87%) as compared to those receiving TAC (46.64 ± 2.68%; *P*=0.08), regardless of the use of other immunosuppressive drugs. In this case, the concomitant administration of MMF did not affect the percentage of the regulatory T cell sub-population (CsA: 45.98 ± 7.99%; TAC: 46.08 ± 2.87%; *P*=0.50). These results are in accordance with previously published data showing that MMF-exposed murine regulatory T cells maintain expression of FoxP3 and suppressive function *in vitro* (Zeiser R. *et al*., 2006).

We had the opportunity to test a stable kidney transplant recipient (RN25) who had his immunosuppressive regimen changed for medical reasons: his treatment consisted of mycophenolate mofetil (360 mg/day) and tacrolimus (2x2 mg/day; trough level: 7.7 µg/l), and was subsequently changed for sirolimus (1 mg/day; trough level: 4.2 µg/l) and mycophenolate mofetil (180 mg/day). Interestingly, when he was on a CNI-based immunosuppression, the percentage of $CD45RO^+$ $CD127^{high}$ T cells among $CD4^+$ $CD25^{high}$ cells was 20.45%; this percentage subsequently dropped to 2.34% nine months after tacrolimus to sirolimus switch, without any change in his clinical status. This result indicates a striking impact of sirolimus on the percentage of activated T cells, at least in stable kidney transplant recipients.

In conclusion, apart from the significant results of kidney transplant recipients on sirolimus-based immunosuppression shown in the preceding section, there was no striking effect of the other immunosuppressive agents on the percentage of the activated T cell sub-population, or on the percentage of the regulatory T cell sub-population.

### *4.2.3.3. Homing properties of $CD4^+$ $CD25^{high}$ T cell sub-populations in kidney transplantation*

One aspect that should be taken into account is that immune cells continuously travel through different lymphoid compartments (bloodstream, secondary lymphoid organs), and, after activation (e.g. rejection in the case of transplantation), through the different tissues they serve. The specific migration of immune cells to sites of inflammation is under the control of a family of chemoattractant cytokines known as chemokines, which induce directed chemotaxis in responsive cells such as leucocytes, monocytes, neutrophils and other effector cells. Chemokines can be released by many different types of cells, including monocytes, macrophages, neutrophils, mast cells, endothelial cells, platelets, keratinocytes, and fibroblasts, among others. They guide cells involved in innate immunity as well as cells involved in adaptive immunity, but some chemokines also function in lymphocyte development and angiogenesis. Responsive cells bear chemokine receptors on their cell surface, allowing them to respond to the recruitment signal provided by the ligation of a chemokine to its specific receptor. The panel of chemokine receptors expressed by immune cells can provide helpful information on their origin and function: for instance, chemokine receptors CCR3, CCR4 and CCR8 are typically expressed by $T_H2$ cells and CCR2, CCR5, CxCR3 and CxCR6 by $T_H1$ cells.

In the literature, it was shown that regulatory T cells are heterogeneous in their expression of tissue-specific and inflammatory homing receptors, which allows them to migrate in a wide range of tissues and to suppress functionally distinct T cell subsets. The efficient suppression of immune responses requires the expression of a diverse array of adhesion and chemokine receptors by regulatory T cells; depending on the cytokine milieu in which they are activated, they might differentiate to have distinct chemokine receptor expression profiles. Indeed, antigen-specific regulatory T cells upregulate their expression of many nonlymphoid tissue-homing receptors, that overlap those of follicular $T_H$ cells (CxCR5), $T_H1$ cells (CxCR3, CCR5, CxCR6), $T_H2$ cells (CCR3, CCR4, CCR8), and $T_H17$ cells (CCR2, CCR4, CCR6, CxCR3) (Lee JH *et al*., 2007; Hirahara K *et al*., 2006; Lim HW *et al*., 2008). On the other hand, transplantation, and especially allograft rejection, which is characterized by the infiltration of mononuclear cells within renal tissue, can be a source of inflammation, requiring expression of diverse chemokines by the affected tissue and of diverse chemokine receptors by the responsive cells. Experimental studies on human renal tissue in patients with renal allograft rejection indicate that MCP-1, MIP-1α (CCL3), MIP-1β (CCL4), RANTES, and IL-8 (CxCL8) play a major role in the resolution or progression of the inflammatory process; renal cells and inflammatory cells also express chemokine receptors, especially CCR5, CCR1, CCR2, and CxCR3 (Stasikowska O *et al*., 2007). It was shown that during acute renal rejection CCR1 mRNA was detectable whilst CCR3 and CCR8 were absent, and that leucocytes in diffuse interstitial infiltrates were mainly CCR5 positive, and those in

nodular aggregates mainly expressed CxCR4, indicating that renal allograft rejection is primarily the result of a $T_H1$-type immune response (Segerer S *et al*., 2001). As demonstrated by Waldmann H *et al*. (2008), the ability of a graft to recruit regulatory T cells will depend on these cells having the right homing and chemokine receptors, and the graft being able to generate the appropriate ligands to attract them.

| Receptor | Ligand(s) | Expression pattern | Function(s) |
|---|---|---|---|
| **CCR1** | CCL3, CCL4, CCL5, CCL6, CCL14, CCL15, CCL16, CCL23 | $T_H1$ cells | Migration to inflamed tissues |
| **CCR3** | CCL3, CCL8, CCL16 | $T_H2$ cells | Migration to sites of allergic inflammation |
| **CCR4** | CCL3, CCL5, CCL17, CCL22 | Skin-tropic $CD4^+$ cells<br>$T_H2$ cells<br>$T_H17$ cells<br>Treg cells | Migration to normal and inflamed skin<br>Migration to asthmatic airways<br>Migration to sites of inflammation |
| **CCR5** | CCL2, CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16 | Naïve $CD8^+$ cells<br>$T_H1$ cells<br>CTLs<br>Some Treg cells | Guided encounters with "helped" DCs in lymph nodes<br>Migration to inflamed tissues<br>Migration to inflamed tissues |
| **CCR6** | CCL20 | $T_H17$ cells | Migration to inflamed tissues |
| **CCR7** | CCL19, CCL21 | Naïve cells<br>$T_{CM}$ cells<br>Some Treg cells | Migration to resting lymph nodes<br>Interstitial motility in lymph nodes<br>Migration to resting lymph nodes |
| **CCR9** | CCL25 | Gut-tropic $CD4^+$ and $CD8^+$ cells | Migration to lamina propria and GALT |
| **CxCR1** | CxCL6-CxCL8 | CTLs | Migration to inflamed tissues |
| **CxCR2** | CxCL1-CxCL8 | CTLs | Migration to inflamed tissues |
| **CxCR3** | CxCL9, CxCL10, CxCL11 | $T_H1$ cells<br>CTLs<br>$T_H17$ cells<br>NK T cells<br>Some Treg cells | Migration to inflamed tissues<br>Migration to inflamed lymph nodes and tissue<br>Migration to inflamed tissues<br>Migration to inflamed tissues |
| **CxCR4** | CxCL12 | $T_{CM}$ cells<br>Naïve cells | Migration to resting lymph nodes |
| **CxCR5** | CxCL13 | Follicular helper $CD4^+$ cells<br>Some Treg cells | Migration to T cell–B cell border and GC in lymph nodes |

**Table 5. Chemokine receptors on T cell subsets (adapted from Bromley SK *et al*., 2008).**

**$T_{CM}$: central memory T cells; GC: germinal centre; GALT: gut-associated lymphoid tissue.**

In the context of our study, it was interesting to analyze the panel of chemokine receptors expressed by the activated and regulatory circulating $CD4^+$ $CD25^{high}$ T cell sub-populations, to compare them with $CD4^+$ $CD25^{neg}$ T cells, and to try to see if patients with different clinical conditions (stable, CHR, minimal immunosuppression) display a differential expression of chemokine receptors. The methods used in this part of the study are described in the "Materials and methods" chapter. For technical reasons, we could not test all 91 kidney transplant recipients of our retrospective study; we decided to select representative subjects and controls: we tested 5 stable kidney transplant recipients, 4 kidney transplant recipients with CHR, 5 kidney transplant recipients on minimal immunosuppression, and 5 healthy individuals. We have also made a selection of the chemokine receptors we wanted to test, on the base of the available literature. They are briefly described in **Table 5** (adapted from Bromley SK *et al*., 2008).

#### 4.2.3.3.1. CCR1 / CCR3

No expression of CCR1 or CCR3 could be detected on circulating T cells in any group of patients or in healthy individuals. We can explain these results by the fact that in the absence of an acute inflammatory episode, as it was the case in our patients, there is no reason to find CCR1 or CCR3 positive T cells in the peripheral blood. On the contrary, it would be interesting to test patients with ongoing acute rejection; we can speculate that in this particular situation CCR1 and/or CCR3 positive circulating T cells would be detectable, in the peripheral blood as well as in renal biopsies.

#### 4.2.3.3.2. CCR4 / CCR5 / CCR6

CCR4, CCR5 and CCR6 displayed a comparable expression pattern in our three kidney transplant recipients groups and in healthy individuals. In circulating $CD3^+$ $CD4^+$ T cells, only approximately 7% of circulating T cells were CCR4 positive, 2% were CCR5 positive, and 13% were CCR6 positive; these results also apply to $CD4^+$ $CD25^{neg}$ T cells since most $CD3^+$ $CD4^+$ T cells are CD25 negative. By contrast, significantly more $CD4^+$ $CD25^{high}$ T cells were positive for CCR4 ($29.1 \pm 3.5\%$; $P<0.001$ as compared to $CD4^+$ $CD25^{neg}$ cells), CCR5 ($5.0 \pm 1.2\%$; $P<0.05$) and CCR6 ($24.8 \pm 3.0\%$; $P<0.05$). When comparing the expression profile of the regulatory and activated $CD4^+$ $CD25^{high}$ T cell sub-populations, we found that a significantly higher percentage of activated T cells expressed CCR6 ($57.8 \pm 3.7\%$; $P<0.001$) as compared to regulatory T cells ($29.6 \pm 2.9\%$); on the other hand, regulatory T cells expressed significantly more CCR4 ($43.8 \pm 3.1\%$; $P<0.05$) than activated T cells ($33.9 \pm 3.2\%$). Expression of CCR5 was low and comparable in both sub-populations (activated T cells: $5.7 \pm 1.2\%$; regulatory T cells: $4.9 \pm 1.1\%$; $P=0.33$). Interestingly, naïve $CD4^+$ $CD25^{high}$ T cells (defined as CD45RO negative) did virtually not express CCR4, CCR5 or CCR6. These observations confirm that CCR4, CCR5 and CCR6 are mainly expressed by memory T cells, especially memory $CD4^+$ T cells. They also show that these chemokine

receptors are up-regulated in $CD4^+$ $CD25^{high}$ T cells, as compared to $CD4^+$ $CD25^{neg}$ T cells, which is consistent with their role in organizing the migration of T cells to sites of inflammation. Interestingly, we found that a higher percentage of regulatory T cells expressed CCR4 as compared to activated T cells, whilst on the contrary a higher percentage of activated T cells were found to express CCR6. However, we did not find a significant difference between kidney transplant recipients and healthy individuals on one hand, and stable kidney transplant recipients and transplant recipients with CHR on the other hand, as we could have expected. One explanation could be the same as the one given for CCR1 and CCR3, namely that none of our patients was experiencing acute rejection at the time of this study.

#### 4.2.3.3.3. CCR7

The chemokine receptor CCR7, which typically controls the migration of T cells to secondary lymphoid organs such as lymph nodes, was found to be expressed by approximately 70% of $CD3^+$ $CD4^+$ T cells. However, there were significant differences between kidney transplant recipients and healthy individuals. As compared to healthy individuals ($74.1 \pm 3.0\%$ of $CD3^+$ $CD4^+$ cells were CCR7 positive), the percentage of CCR7 positive cells among $CD3^+$ $CD4^+$ cells was significantly decreased in stable kidney transplant recipients ($48.2 \pm 1.7\%$; $P<0.005$) and in kidney transplant recipients with CHR ($61.2 \pm 5.0\%$; $P<0.05$); by contrast, it was comparable to healthy individuals in kidney transplant recipients on minimal immunosuppression ($72.7 \pm 8.0\%$; $P=0.44$). Again, the same observations could be applied to $CD4^+$ $CD25^{neg}$ T cells, but not to $CD4^+$ $CD25^{high}$ T cells. In this cell population, a smaller proportion of cells expressed CCR7, and percentages found in healthy individuals ($35.0 \pm 5.5\%$), stable kidney transplant recipients ($19.8 \pm 0.6\%$), kidney transplant recipients with CHR ($41.1 \pm 6.0\%$) and those on minimal immunosuppression ($49.3 \pm 5.8\%$) were not statistically different. When we compared regulatory and activated $CD4^+$ $CD25^{high}$ T cell sub-populations, we found a trend, however not statistically significant, in a higher percentage of CCR7 positive cells among activated T cells (approximately 40%) as compared to regulatory T cells (approximately 30%). By contrast, a very high proportion of naïve $CD4^+$ $CD25^{high}$ T cells (defined as CD45RO negative) expressed CCR7 (approximately 80%). These results confirm that CCR7 is mainly expressed by naïve cells, and also by a significant proportion of memory T cells, which are called "central memory" ($T_{CM}$). This subset of memory T cells is functionally important, since central memory T cells are in the first line in lymph nodes for the presentation of antigens; they have limited effector function, but they proliferate and become effector cells upon secondary antigenic stimulation. By contrast, another subset of memory T cells, called "effector memory" ($T_{EM}$), does not express CCR7 and consequently does not home to lymph nodes but to peripheral tissues, where it can rapidly produce effector cytokines (e.g. IFNγ); however, its proliferative capacity is limited. $T_{CM}$ are involved in secondary responses and long-term function, whilst $T_{EM}$ provide immediate

function. In our case, the majority of $CD4^+$ $CD25^{neg}$ T cells appear to be $T_{CM}$, although we observed significant differences between kidney transplant recipients and healthy individuals; this could be explained by the presence of the antigenic stimulus provided by the allograft, promoting the preferential differentiation of $T_{EM}$ over $T_{CM}$ except in transplant recipients on minimal immunosuppression who may display a more "tolerogenic" state. In $CD4^+$ $CD25^{high}$ T cells, the majority of cells were CCR7 negative $T_{EM}$, and percentages found in healthy individuals and kidney transplant recipients were comparable. These results further support the idea that both regulatory and activated T cells are functionally heterogeneous populations from the point of view of their migratory properties.

#### 4.2.3.3.4. CCR9

No CCR9 expression could be detected on $CD3^+$ $CD4^+$ T cells, which could be expected, CCR9 being expressed by T cells homing to the gut.

#### 4.2.3.3.5. CxCR1 / CxCR2

CxCR1 and CxCR2 were not detected in $CD3^+$ $CD4^+$ T cells, they seem to be specifically expressed on cytotoxic $CD8^+$ T lymphocytes.

#### 4.2.3.3.6. CxCR3

CxCR3 was found to be expressed by a small proportion of $CD3^+$ $CD4^+$ T cells as well as $CD4^+$ $CD25^{neg}$ T cells ($5.8 \pm 0.7\%$), but no difference was found between the kidney transplant recipients and healthy individuals. However, we observed that significantly more activated $CD4^+$ $CD25^{high}$ T cells expressed CxCR3 ($9.3 \pm 1.8\%$) as compared to the regulatory T cell sub-population ($4.8 \pm 0.8\%$; $P<0.05$), although these percentages remain small. Furthermore, naïve $CD4^+$ $CD25^{high}$ T cells expressed virtually no CxCR3.

#### 4.2.3.3.7. CxCR4

Almost all $CD3^+$ $CD4^+$ T cells expressed CxCR4 ($92.5 \pm 1.3\%$), and no difference was found between the different groups of patients and the healthy individuals, or between $CD4^+$ $CD25^{high}$ T cells ($91.2 \pm 1.3\%$) and $CD4^+$ $CD25^{neg}$ T cells ($92.5 \pm 1.3\%$; $P=0.24$). As found for CCR7, we also observed a trend to a higher percentage of activated T cells expressing CxCR4 ($90.9 \pm 1.4\%$) as compared to regulatory T cells ($87.5 \pm 1.6\%$; $P=0.07$), and a significant increase of CxCR4 positive cells in the naïve population of $CD4^+$ $CD25^{high}$ cells ($97.4 \pm 0.4\%$; $P<0.001$). This is in accordance with the fact that CxCR4 is mainly expressed by $T_{CM}$ and naïve T cells.

#### 4.2.3.3.8. CxCR5

A small percentage of $CD3^+$ $CD4^+$ T cells were found to express CxCR5 ($11.7 \pm 1.0\%$), and no difference was found between the different groups of patients and the healthy individuals, or between $CD4^+$ $CD25^{high}$ T cells ($12.2 \pm 2.0\%$) and $CD4^+$ $CD25^{neg}$ T cells ($11.2 \pm 1.0\%$;

*P*=0.36). Comparing activated and regulatory T cell sub-populations, we observed a small increase in the percentage of activated T cells expressing CxCR5 (14.0 ± 2.8%) as compared to regulatory T cells (8.5 ± 1.2%; P=0.04), and naïve $CD4^+$ $CD25^{high}$ T cells expressed even more CxCR5 (22.8 ± 5.1%).

Based on these results, the main conclusion is that T cell populations that seem to share the same function are in fact highly heterogeneous from the point of view of their migration and homing properties. The overall $CD3^+$ $CD4^+$ T cell population expressed mainly CCR7 and CxCR4 (both lymph node homing receptors) and a small percentage also expressed CxCR5 (homing to lymph nodes) and CCR4, CCR5, CCR6, CxCR3 (homing to inflamed tissues); obviously, the same phenotype was found among $CD4^+$ $CD25^{neg}$ T cells. This indicates that $CD4^+$ $CD25^{neg}$ T cells mainly contain central memory and naïve cells that have not yet been activated, recirculating in the bloodstream between secondary lymphoid organs, such as lymph nodes. On the other hand, $CD4^+$ $CD25^{high}$ T cells expressed more or less the same chemokine receptors, but in significantly different proportions as compared to $CD4^+$ $CD25^{neg}$ T cells. We found that CCR7 was less expressed, whilst CCR4 and CCR6 were more expressed, suggesting that $CD4^+$ $CD25^{high}$ T cells contained less cells homing to lymph nodes and were enriched in cells homing to sites of inflammation. This could be expected since $CD4^+$ $CD25^{high}$ T cells contain recently activated T cells, which have upregulated CD25 expression on their cell surface. When we compared the chemokine receptor expression profile of activated and regulatory $CD4^+$ $CD25^{high}$ T cell sub-populations, we found some differences, but they were not striking. It is not surprising that these populations studied in the peripheral blood are heterogeneous from the point of view of their homing characteristics; both must have the capacity to migrate to secondary lymphoid organs as well as to peripheral tissues to exert their function where it is required. The picture would have certainly been completely different if we had looked into renal biopsies of patients with acute or chronic rejection; we would have expected to find cells expressing chemokine receptors specific for peripheral tissues and inflammation. Another point that would be interesting to look at is the case of transplant recipients with ongoing acute rejection; we can speculate that a majority of circulating T cells would express chemokine receptors controlling their migration to the site of inflammation.

## 4.2.4. Discussion of the retrospective part

In this retrospective part of our study, we have extended the results of our initial results published in *The Journal of Experimental Medicine* in July 2007. Furthermore, we have increased our knowledge of how $CD4^+$ $CD25^{high}$ T cells and the activated, respectively regulatory, T cell sub-populations are influenced by the clinical status of the kidney transplant recipients as well as by the prescribed immunosuppressive regimens.

We have shown that the overall percentage of $CD4^+$ $CD25^{high}$ T cells was significantly decreased in all transplant recipients, with the exception of stable transplant recipients receiving sirolimus as immunosuppressive therapy. This reduction is probably due to the direct inhibitory effect of immunosuppressive drugs on the activation of T cells, limiting upregulation of the CD25 molecule at the T cell surface. The extent of the reduction of the percentage of $CD4^+$ $CD25^{high}$ T cells was similar in all study groups (except in the already mentioned group of stable patients on sirolimus), thus limiting the usefulness of this parameter to help in clinical diagnosis.

The percentage of the sub-population of regulatory T cells, defined as $CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD127^{low}$ cells, was also found to be significantly decreased in all transplant recipients, as compared to healthy individuals. The lowest percentage was found in patients receiving a CNI, whilst the use of sirolimus seemed to preserve regulatory T cells; the use of MMF did not affect the percentage of the sub-population. However, the extent of the reduction of regulatory T cells was similar in all study groups, again limiting the practical usefulness of this parameter in a clinical setting.

By contrast, we found that there was a very good correlation between the percentage of the activated T cell sub-population, defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ cells, and the clinical status of the patients. The highest percentage was found in kidney transplant recipients suffering from chronic humoral rejection, whilst clinically stable recipients on no or minimal immunosuppression displayed a percentage comparable to the one of healthy individuals; in clinically stable patients receiving standard CNI-based immunosuppression, the percentage of the activated T cell sub-population was intermediate.

Finally, we have analyzed the chemokine receptor profile of $CD3^+$ $CD4^+$ T cells, of $CD4^+$ $CD25^{high}$ T cells and of the activated and regulatory $CD4^+$ $CD25^{high}$ T cell sub-populations. We could observe that these populations are very heterogeneous from the point of view of their migratory and homing properties, and that the proportion of cells expressing secondary lymph node homing or peripheral tissue homing chemokine receptors was significantly different between the populations we studied.

These results could reflect the fact that the level of activation of T cells is different depending on the degree of graft acceptance by the transplant recipient. They also indicate that stable transplant recipients, despite apparently efficient immunosuppressive therapy, have a significant percentage of peripherally circulating activated T cells, which in this situation can be named "alloreactive". On the other hand, stable transplant recipients on no or minimal immunosuppression seem to be in a more tolerogenic state towards their graft, since the percentage of circulating alloreactive T cells is comparable to the one of healthy individuals. Of note, the small amount (approximately 5-6%) of "activated T cells" found in healthy

individuals is likely due to the continuous challenge of our immune system by foreign antigens.

The quality of this retrospective part of our study could have been improved by considering at least two additional points. First, we have been focusing on the phenotype of peripherally circulating T lymphocytes subsets; however, it is obvious that the situation observed in the peripheral circulating blood may not reflect exactly what happens in the draining lymph nodes and/or in the organ targeted by the potentially detrimental immune response, namely the allograft. In this regard, it could be interesting to compare the phenotype of T lymphocytes in the draining lymph nodes as well as in the allograft in our different study groups; however, as already mentioned, this implies the use of invasive techniques that may be not ethically acceptable in otherwise healthy individuals. The second important limitation of our study is that we did not consider the functional aspect of the $CD4^+$ $CD25^{high}$ T cells sub-populations; it would be very interesting to examine the respective functionality of these subsets. On one hand, the suppressive capacity of the regulatory T cell subset should be assessed in our study groups, and when possible, it should include a specific donor antigens stimulation as compared to a third-party antigens stimulation of responder cells. On the other hand, the proliferative response of the activated T cell subset (alloimmune response) should also be assessed; again, when possible (availability of cells of the organ donor), it should include the proliferative response toward donor antigens, the proliferative response toward third-party antigens (fully HLA-A, -B, and -DR mismatched with recipient and donor), as well as the non-specific proliferative response toward common mitogenic agents. In addition, the proliferative response could also be examined with regard to the depletion of regulatory T cells.

To complete our study, we decided to add a prospective view of the evolution of $CD4^+$ $CD25^{high}$ T cells and their sub-populations. This part of the study was implemented to monitor the above-mentioned parameters at defined timepoints over the first year after transplantation, in close correlation with clinical data. It would allow us to compare two routinely used induction regimens, which have distinct mechanisms of action. Preliminary results have been described in our publication, in the part *"Importance of the analysis of the $CD4^+$ $CD25^{high}$ $CD45RO^+$ IL-7R$\alpha^{high}$ cell population in the clinical monitoring of transplant recipients"*.

Results of this prospective part of the study are shown in the next section.

## 4.2.5. Analysis of functionally distinct subsets of $CD4^+$ $CD25^{high}$ T cells in solid organ transplant recipients: prospective part of the study

We performed the same kind of immunological experiments (polychromatic flow cytometric phenotyping of peripheral blood mononuclear cells), using the same gating strategy as the one described in the chapter on healthy individuals [**Figures 23** and **24**].

### *4.2.5.1. Patient characteristics*

Thirty-five patients were studied prospectively, all of them having received a first kidney graft from a cadaveric or living donor. Nineteen of them received thymoglobulin as induction regimen, followed by a standard, but steroid-free, maintenance immunosuppressive therapy (tacrolimus and mycophenolate mofetil) ("THYMO group"); on the other hand, 16 received basiliximab as induction, followed by tacrolimus, mycophenolate mofetil and prednisone ("BSX group"). Relevant demographic data of these patients are summarized in **Appendix III**. The two study groups did not differ in terms of gender, age at the time of transplantation, and HLA mismatches.

### *4.2.5.2. Thymoglobulin and basiliximab induction regimens*

The two induction regimens that were compared in this part of the study are individually based on different modes of action and thus may exert different effects on various lymphocyte sub-populations. They will be discussed hereafter, focusing on $CD4^+$ $CD25^+$ T cells.

Thymoglobulin (Thymoglobulin®; Genzyme Transplant) has been in use for more than 30 years. It consists of a purified polyclonal anti-lymphocyte preparation obtained by immunization of rabbits with human thymocytes. It contains cytotoxic antibodies directed against antigens expressed on human T lymphocytes (such as CD2, CD3, CD4, CD8, CD11a, CD18, CD25, CD44, CD45, HLA-DR, HLA class I and $\alpha_2$-microglobulin), and results in rapid depletion of T lymphocytes, primarily through complement-dependent cell lysis in the blood compartment and apoptotic cell death in lymphoid tissues. Its half-life is about 2-3 days, but in 80% of patients thymoglobulin can still be detected in the serum 2 months after the end of its administration. Besides T cell depletion, possible mechanisms by which thymoglobulin may induce immunosuppression *in vivo* include: T cell clearance from the circulation, and modulation of T cell activation, trafficking and cytotoxic activities. Treatment with thymoglobulin has been shown to be associated with both short- and long-term changes in T cell populations, in particular characterized by expansion of specific T cell subsets exhibiting regulatory properties (Mueller TF, 2007). Recent studies suggest that thymoglobulin relatively spares $CD4^+$ $CD25^{high}$ regulatory T cells, compared with $CD8^+$ T cells, $CD4^+$ naïve T cells and central memory T cells (Lopez M *et al.*, 2006; Pearl JP *et al.*, 2005), and thus could be beneficial in the optic of transplantation tolerance development.

Basiliximab (Simulect®; Novartis Pharma) is a chimeric monoclonal antibody (half-life: 7 days) that specifically binds to and blocks the interleukin-2 receptor α chain (IL-2Rα, CD25), which is expressed on the surface of activated as well as regulatory T cells. The complete and constant blockade of the IL-2Rα is maintained as long as the serum level of basiliximab is higher than 0.2 μg/ml. This specific high affinity binding to CD25 competitively inhibits IL-2-mediated activation of lymphocytes, a critical pathway in the cell-mediated immune response involved in allograft rejection. However, IL-2 is also critical for the generation and survival of regulatory T cells, which produce only negligible amounts of IL-2 on their own and depend on other T cell subsets for IL-2 providing. Therefore, the possible tolerance promoting effect of basiliximab, through elimination of alloreactive activated $CD4^+$ $CD25^+$ T cells, could be offset by the prevention of regulatory cell function. Two studies, among others, have addressed these concerns. Game DS *et al*. (2005) showed in *in vitro* experiments that human $CD4^+$ $CD25^+$ cells cultured in presence of basiliximab retained suppressive capacity on the proliferation and IFNγ secretion by $CD4^+$ $CD25^{neg}$ cells responding to allogeneic and other polyclonal stimuli. On the other hand, in renal transplant recipients receiving basiliximab as induction, Bluestone JA *et al*. (2008) reported that basiliximab caused a profound, but transient, reduction of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, within 7 days of treatment and with a progressive recovery 90 days after treatment, but that their function was maintained during the treatment; by contrast, basiliximab did not have a long-term effect on the percentage or on the functionality of circulating $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, as compared to pre-transplant levels. However, basiliximab did also lead to a dramatic decrease in $CD4^+$ $CD25^+$ $FoxP3^-$ activated cells, which contain potential alloreactive effector T cells; thus, the authors concluded that the net result was a shift toward regulation.

From a clinical point of view, several studies have compared the effectiveness and safety of thymoglobulin versus the anti-IL-2R monoclonal antibodies (among others, Brennan DC *et al*., 1999; Al Najjar A *et al*., 2006). Taken together, these two studies suggest that for the lower immunological risk patients basiliximab may be a safer choice (lower incidence of side effects) while for the higher immunological risk patients (retransplants, patients with panel-reactive antibodies greater than 20%, 6 HLA antigen mismatches, patients of African descent) thymoglobulin is likely to be a more effective therapy (lower incidence of biopsy-proven acute rejection).

In our prospective study, all kidney transplant recipients could be classified as low immunological risk patients, allowing us to perform consistent comparisons between the two study groups.

### *4.2.5.3. Clinical outcome of the patients*

The major concern in the first months after kidney transplantation is the development of a rejection episode. Nowadays, the incidence of hyperacute rejection has become insignificant thanks to the routine detection of pre-existing anti-donor antibodies. Likewise, acute rejections have become rare events (incidence of approximately 10-15%) with the improvement of induction regimens and the availability of more potent immunosuppressive drugs capable of preventing or treating them.

In our cohort of patients, as expected no hyperacute rejection occurred; the incidence of acute rejection (within the first 6 months after transplantation) was 10.5% (2/19 patients) in the THYMO group, and 12.5% (2/16 patients) in the BSX group (not statistically significant). In the THYMO group, both acute rejection episodes were cell-mediated and severe, whilst in the BSX group there were one humoral and one cellular rejection episodes, both of moderate severity. All four rejection episodes resolved successfully with a short course of high-dose corticosteroids and the recipients' serum creatinine levels at 6 months after transplantation were similar (THYMO group: 129.5 μmol/l; BSX group: 125.5 μmol/l) and comparable to the one of the rejection-free patients.

The overall serum creatinine levels were not statistically different at any timepoint between the two study groups [**Table 6**].

| | **Serum creatinine (μmol/l)** | | *P* value |
|---|---|---|---|
| | ***THYMO group*** | ***BSX group*** | |
| Before transplantation | 654.1 ± 68.3 | 544.9 ± 57.1 | *0.13* |
| 3 months post-transplantation | 124.9 ± 7.7 | 114.0 ± 5.5 | *0.15* |
| 6 months post-transplantation | 124.6 ± 7.9 | 130.6 ± 10.0 | *0.33* |
| 12 months post-transplantation | 126.9 ± 8.2 | 132.9 ± 11.2 | *0.34* |

**Table 6. Respective serum creatinine levels in the THYMO and BSX groups, before and 3, 6 and 12 months after kidney transplantation.**

### *4.2.5.4. Changes in leucocyte and lymphocyte absolute counts during the first year after kidney transplantation*

As expected based on the differing modes of action of the two induction regimens studied, the leucocyte and lymphocyte counts varied differently in the THYMO and in the BSX groups.

In both study groups, the pre-transplant leucocyte count (normal range: 4.0-10.0 G/l) was comparable to the one of healthy individuals (THYMO group: 7.1 ± 0.5 G/L; BSX group:

7.0 ± 0.5 G/L; healthy individuals: 6.8 ± 0.4 G/L). Similarly, the pre-transplant relative percentage of lymphocytes among leucocytes (normal range: 25-40%) was also comparable in the THYMO group (26.0 ± 2.4%), in the BSX group (22.1 ± 2.3%) and in healthy individuals (24.3 ± 1.0%).

In the THYMO group, the mean absolute leucocyte count dropped of approximately 25% by 1 month after transplantation (5.3 ± 0.6 G/L), continued to drop at 3 months (4.2 ± 0.6 G/L) and 6 months (3.7 ± 0.4 G/L), and then began to recover partially at 12 months (4.5 ± 0.4 G/L), but without reaching the pre-transplant count or the count found in healthy volunteers [**Figure 37**]. This long-lasting leucocyte depleting effect of thymoglobulin (in patients receiving an immunosuppressive regimen composed of CNIs and MMF) is well known and has been shown to persist until at least 2 years after transplantation (Hardinger KL *et al*., 2004; Toso C *et al*., 2008); moreover, as already mentioned, it is accompanied by a redistribution of T cell sub-populations, that will be discussed later.

On the other hand, basiliximab did not have the depleting effect of thymoglobulin; in the BSX group, the mean absolute leucocyte count remained stable and in a normal range throughout the 12 months after transplantation (3 months after transplantation: 7.8 ± 0.8 G/l; 6 months: 7.0 ± 0.6 G/l; 12 months: 7.3 ± 0.8 G/l) [**Figure 37**].

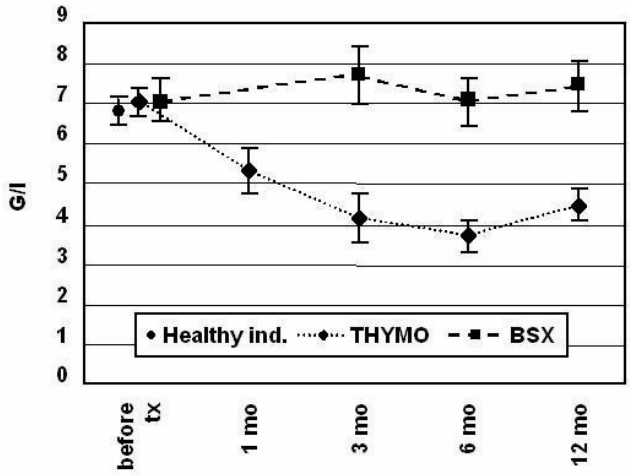



**Figure 37. Mean absolute leucocyte count in the THYMO and in the BSX groups, before and during the 12 months after transplantation.**

Looking at the lymphocyte population among leucocytes, we found that basiliximab did not induce a change in the proportion of the whole lymphocyte population: approximately 20-25% (1.5-2 G/l) of leucocytes belonged to the lymphocyte lineage, a percentage that remained stable throughout the 12 months after transplantation. By contrast, in addition to its above-mentioned depleting effect on the total leucocyte population absolute count, thymoglobulin preferentially affected lymphocytes; at 1 month after transplantation, the

percentage of lymphocytes dropped to 6.35%, and then slowly increased during the next 12 months. The mean absolute lymphocyte count followed approximately the same kinetic [**Figures 38A** and **38B**].

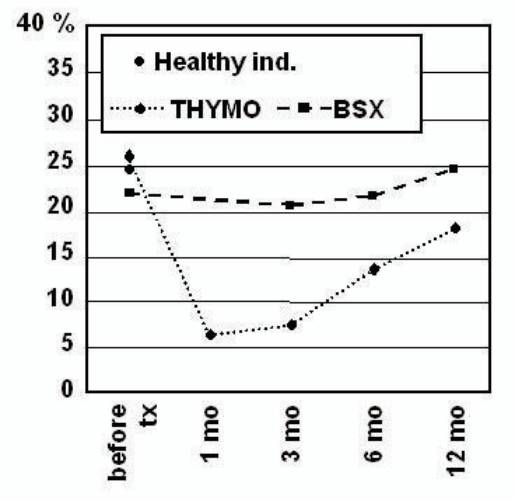



**Figure 38A. Mean percentage of lymphocytes among leucocytes, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

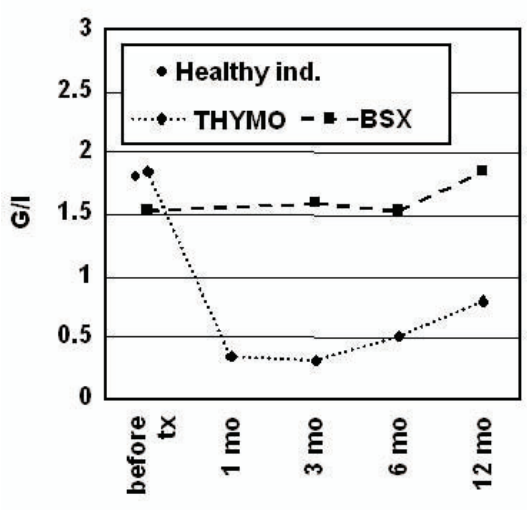



**Figure 38B. Mean lymphocyte absolute count, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

Because of the depleting effect of thymoglobulin, it will be important for us to base our results not only on relative percentages of T cell sub-populations, but also on absolute numbers.

### *4.2.5.5. Changes in T cell sub-populations during the first year after kidney transplantation*

In line with the analyses performed in the retrospective part of our study, we were interested in studying the evolution of CD4 T cells, and in particular of $CD4^+$ $CD25^{high}$ T cells and their sub-populations, during the first year after kidney transplantation, and in comparing the respective impact of the two different regimens described above (thymoglobulin and basiliximab).

#### 4.2.5.5.1. $CD4^+$ T cells

Before transplantation, the mean proportion of $CD4^+$ T cells among the whole lymphocyte population in both THYMO and BSX groups was slightly but significantly decreased as compared to healthy individuals (THYMO: $41.73 \pm 2.75\%$; BSX group: $39.28 \pm 2.26\%$; healthy individuals: $50.04 \pm 1.74\%$; $P<0.05$ and $P<0.005$ respectively) [**Figure 39A**]; this result is in accordance with Litjens NHR *et al*. (2006), who showed that end-stage renal disease is associated with lymphocytopenia, especially in the $CD4^+$ T cell and B cell compartments.

At 1 month after transplantation, the mean percentage of $CD4^+$ T cells among total lymphocytes in the THYMO group was found to be decreased by approximately 50% (19.99 ± 2.72%; $P$<0.001) as compared to pre-transplant value. Although we had not access to blood samples of the immediate post-operative period, we can speculate that this percentage was even more dramatically reduced in the days during and immediately after thymoglobulin treatment. A correlate of this observation is that thymoglobulin induces a significant change in the CD4/CD8 ratio, which is consistent with previous studies (Mueller TF, 2003). These changes stably persisted during the next 12 months of observation (3 months: 23.38 ± 2.10%; 6 months: 24.16 ± 2.41%; 12 months: 26.58 ± 2.83%) [**Figure 39A**]. This indicates that thymoglobulin induces a profound change in lymphocyte sub-populations, with a preferential depletion of $CD4^+$ T cells, as could be expected from its binding properties and its regeneration characteristics promoting an earlier homeostatic response of $CD8^+$ T cells.

By contrast, basiliximab did not decrease the CD4/CD8 ratio in the BSX group. By 3 months after transplantation, the proportion of $CD4^+$ T cells among lymphocytes had gone back to normal values (48.87 ± 2.89%; $P$=0.34 as compared to healthy individuals), and remained stable until the end of the observation period (6 months: 50.36 ± 2.57%, $P$=0.48; 12 months: 50.08 ± 3.13%, $P$=0.44) [**Figure 39A**].

Considering the $CD4^+$ T cell absolute count, the results are consistent with the changes observed in $CD4^+$ T cell and lymphocyte percentages. In the THYMO study group, thymoglobulin induced a drop in CD4 T cells that persisted during the 12 months of observation. On the other hand, basiliximab did not decrease the absolute number of $CD4^+$ T cells [**Figure 39B**].

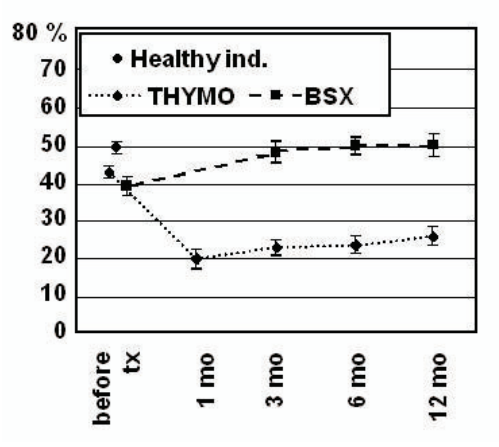



**Figure 39A. Mean percentage of $CD4^+$ T cells among lymphocytes, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

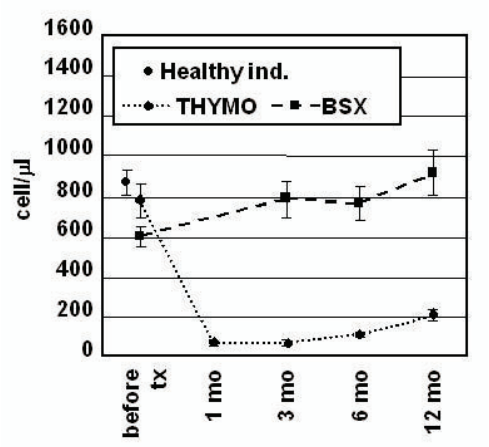



**Figure 39B. Mean $CD4^+$ T cell absolute count, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

#### 4.2.5.5.2. $CD4^+$ $CD25^{high}$ T cells

The mean percentage of $CD25^{high}$ cells within $CD4^+$ T cells before transplantation was comparable in the THYMO group (1.48 ± 0.23%; *P*=0.30), in the BSX group (1.54 ± 0.24%; *P*=0.39) and in healthy individuals (1.62 ± 0.16%) [**Figure 40A**].

In the THYMO group, after transplantation, the proportion of $CD25^{high}$ cells among $CD4^+$ T cells followed a bimodal kinetic: after an initial significant increase at 1 month (2.94 ± 0.52%; *P*<0.01 as compared to pre-transplant value), it slowly decreased at 3 months (2.21 ± 0.37%; *P*=0.06) and was comparable to pre-transplant value at 6 months (1.67 ± 0.37%; *P*=0.40) and 12 months (1.65 ± 0.42%; *P*=0.36) [**Figure 40A**]. However, when considering the mean absolute count of $CD4^+$ $CD25^{high}$ T cells, the strong depleting effect of thymoglobulin on lymphocytes, and especially on $CD4^+$ T cells, has to be taken into account. Indeed, a sharp drop in the absolute $CD4^+$ $CD25^{high}$ T cell count was observed as soon as 1 month after transplantation (1.51 ± 0.22 cell/µl; *P*<0.001 as compared to pre-transplant value and healthy individuals), that persisted throughout the next 12 months [**Figure 40B**].

In the BSX study group, the evolution of the proportion of $CD25^{high}$ cells among $CD4^+$ T cells followed a different kinetic; a progressive decrease could be observed after transplantation (3 months: 0.74 ± 0.16%; 6 months: 0.43 ± 0.06%; 12 months: 0.21 ± 0.02%; *P*<0.01 as compared to pre-transplant value, to healthy individuals, and to patients of the THYMO group) [**Figure 40A**]. It would have been interesting to assess the percentage of $CD4^+$ $CD25^{high}$ T cells in the immediate post-operative period (first month), when basiliximab exerted its full anti-IL-2R effect, but unfortunately we had not access to blood samples of this period; however, based on our observations and on previous publications (Toso C *et al.*, 2008), we can hypothesize that the expression of CD25 would have been hardly detectable. Since basiliximab had almost no effect on lymphocyte and $CD4^+$ T cell absolute counts, it was not a surprise to observe that the $CD4^+$ $CD25^{high}$ T cell absolute count also constantly decreased after transplantation [**Figure 40B**]. However, it should be noted that the decrease of $CD4^+$ $CD25^{high}$ T cell percentage and absolute count observed in the BSX group after transplantation is probably also due to the effect of the maintenance immunosuppressive therapy.

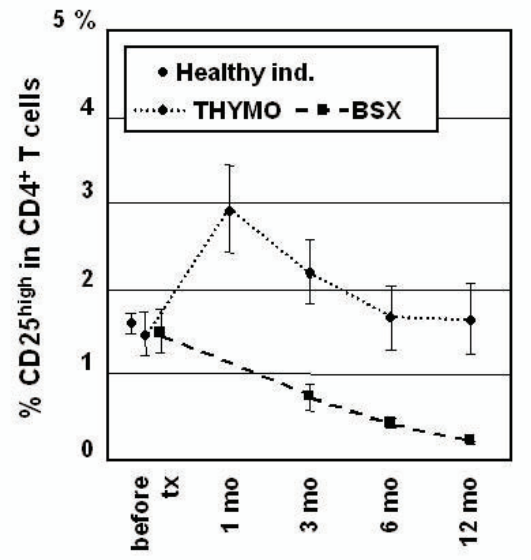



**Figure 40A. Mean percentage of $CD25^{high}$ cells among $CD4^+$ T cells, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

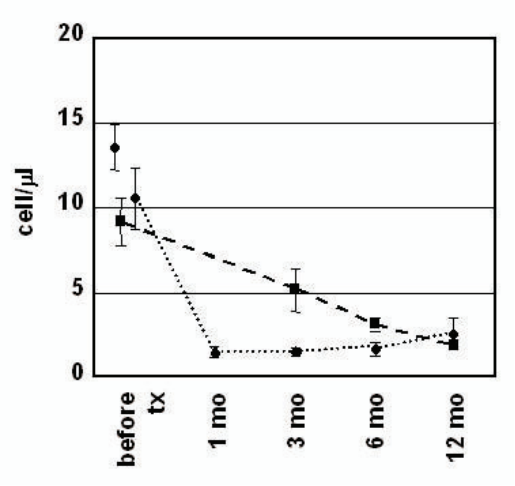



**Figure 40B. Mean $CD4^+$ $CD25^{high}$ T cell absolute count, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

Although, as shown above, thymoglobulin and basiliximab had a completely different impact on lymphocyte, $CD4^+$, and $CD4^+$ $CD25^{high}$ T cell absolute counts, it was very interesting to note that at 12 months after transplantation the absolute count of $CD4^+$ $CD25^{high}$ T cells was comparable in the THYMO ($2.68 \pm 0.51$ cell/µl) and in the BSX ($2.10 \pm 0.45$ cell/µl) study groups ($P$=0.23). We can hypothesize that both induction regimens exert their effect in the first one or two months after transplantation, whilst the changes observed afterwards are likely due to the maintenance immunosuppressive drugs (e.g. CNIs and MMF).

As already emphasized in the retrospective part of our study, $CD4^+$ $CD25^{high}$ T cells contain two functionally distinct subsets of cells, namely regulatory and activated T cells, which may differently impact on the clinical outcome of the transplantation. Their respective evolutions during the first year after transplantation and the effect of thymoglobulin versus basiliximab will be discussed in the next two parts.

#### 4.2.5.5.3. Regulatory T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD127^{low}$ cells)

Before transplantation, the percentage of the regulatory T cell sub-population (defined as the percentage of $FoxP3^+$ $CD127^{low}$ cells within $CD4^+$ $CD25^{high}$ T cells) was found to be comparable between the THYMO group ($74.29 \pm 4.26\%$) and the BSX group ($73.48 \pm 3.42\%$), and as compared to healthy individuals ($74.05 \pm 2.01\%$; $P$=0.27 and $P$=0.21 respectively).

In the THYMO study group, the percentage of regulatory T cells tended to slowly decrease until 6 months after transplantation (1 month: $62.28 \pm 4.41\%$; 3 months: $48.31 \pm 4.43\%$;

6 months: 43.01 ± 3.55%); data at 12 months were not available. These results reached statistical significance, as compared to pre-transplant value or healthy individuals, at 3 months ($P$<0.005 and $P$=0.06 respectively) and 6 months after transplantation ($P$<0.001 and $P$<0.05) [**Figure 41**].

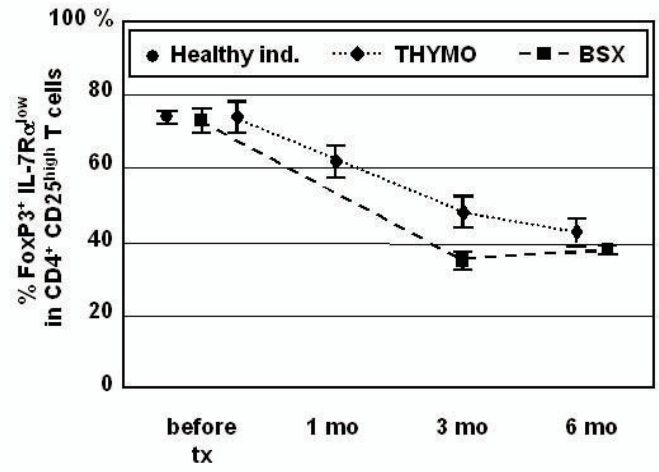



**Figure 41. Evolution of the percentage of the regulatory T cell sub-population, before and 1, 3 and 6 months after transplantation, in the THYMO and BSX groups.**

The evolution of regulatory T cells in the BSX study group was comparable to the one in the THYMO group. This percentage was found to be significantly decreased at 3 months (35.06 ± 1.90%) and 6 months (38.45 ± 1.33%) after transplantation, as compared to pre-transplant value and healthy individuals [**Figure 41**]. The respective percentages of regulatory T cells found at 3 months and 6 months after transplantation were statistically comparable between the THYMO group and the BSX group ($P$=0.07 and $P$=0.36).

Of note, similar to the results found in stable kidney transplant recipients of the retrospective part of this study, the percentages of regulatory T cells were found to be decreased in these patients, probably as an effect of maintenance immunosuppressive therapy.

We also calculated the absolute regulatory T cell count, thus taking into account the observed depleting effects of thymoglobulin and basiliximab. The long-lasting lymphocyte depleting effect of thymoglobulin induced a sharp drop of the regulatory T cell absolute count in the THYMO group after transplantation, which persisted during the 6 months of observation (before transplantation: 8.96 ± 1.28 cell/μl; 1 month: 1.03 ± 0.14 cell/μl; 3 months: 0.94 ± 0.23 cell/μl; 6 months: 0.91 ± 0.11 cell/μl) [**Figure 42**]. All these results were statistically significant as compared to both pre-transplant value and healthy individuals.

On the other hand, the specific $CD4^+$ $CD25^{high}$ T cell depleting effect of basiliximab did also affect the absolute count of regulatory T cells in the BSX study group; it was found to be significantly decreased at 3 months (1.80 ± 0.19 cell/μl) and 6 months (2.51 ± 0.11 cell/μl)

after transplantation, as compared to pre-transplant value (6.85 ± 1.05 cell/µl) and healthy individuals [**Figure 42**].

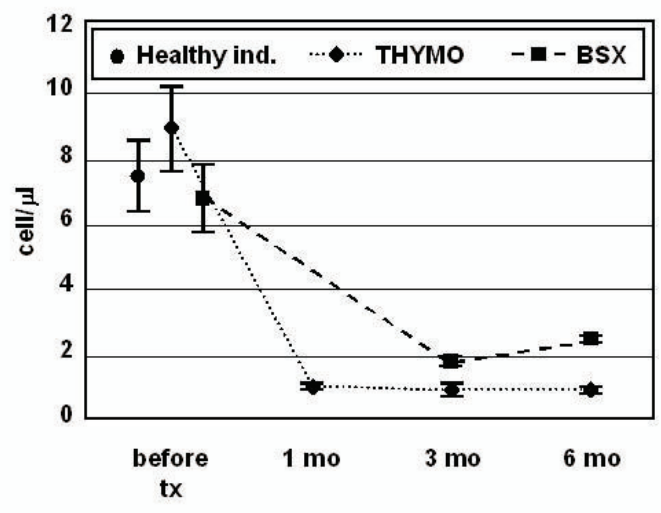



**Figure 42. Evolution of the mean absolute count of the regulatory T cell sub-population, before and 1, 3 and 6 months after transplantation, in the THYMO and BSX groups.**

#### 4.2.5.5.4. Activated T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ cells)

Before transplantation, the percentage of the activated T cell sub-population (defined as the percentage of $CD45RO^+$ $CD127^{high}$ cells within $CD4^+$ $CD25^{high}$ T cells) was comparable in the THYMO (5.94 ± 0.79%) and in the BSX (7.66 ± 0.91%) study groups ($P$=0.10), and both were comparable to healthy individuals (5.97 ± 0.36%).

Thereafter, this population was found to be expanded as soon as 1 month after transplantation in the THYMO group (18.72 ± 3.25%; $P$<0.001 as compared to pre-transplant value). This expansion persisted at 3 months after transplantation (15.27 ± 1.51%; $P$<0.001), at 6 months (13.01 ± 1.54%; $P$<0.001) and 12 months (15.50 ± 1.87%; $P$<0.001) [**Figure 43**].

Similarly, at 3 months after transplantation, the activated T cell sub-population was found to be expanded (19.39 ± 1.70%; $P$<0.001 as compared to pre-transplant value) in the BSX study group, even to a significantly higher percentage as compared to the THYMO group ($P$<0.05). At 6 and 12 months after transplantation, this expansion also persisted (6 months: 14.41 ± 1.42%, $P$<0.005 as compared to pre-transplant value; 12 months: 13.69 ± 2.34%, $P$<0.05), but values were not statistically different from the percentages found in the THYMO study group (6 months: $P$=0.20; 12 months: $P$=0.28) [**Figure 43**].

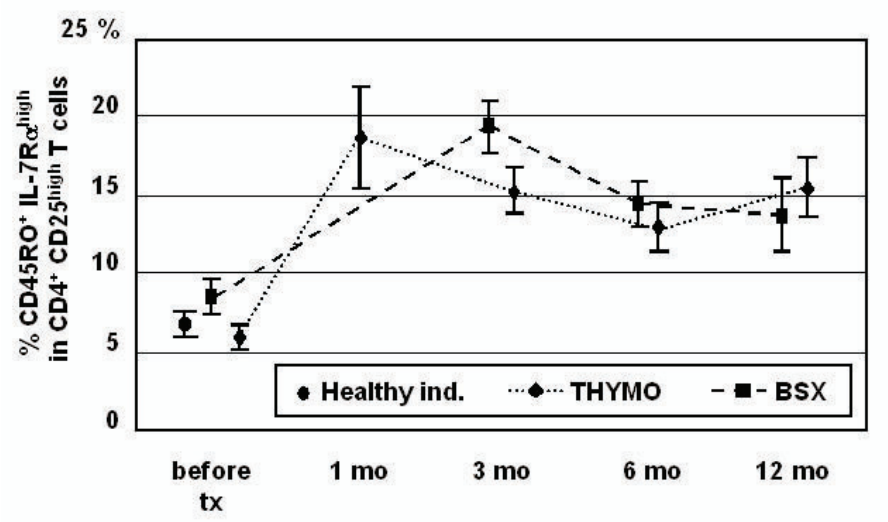



**Figure 43. Evolution of the percentage of the activated T cell sub-population, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

Of note, activated T cell sub-population percentages found in both THYMO and BSX study groups at 12 months after transplantation were comparable to those found in the stable kidney transplant recipients (14.57 ± 0.97%) of the retrospective part of this study. These results indicate that, despite adequate induction and maintenance immunosuppression, there is a rapid expansion of a population of activated $CD4^+$ T cells after kidney transplantation, but with no apparent clinical relevance for the patients (no rejection), at least during the limited period of observation. However, it was also necessary to take into account the lymphocyte depleting effect of thymoglobulin and the $CD4^+$ $CD25^{high}$ T cells depleting effect of basiliximab, and to adjust these results to absolute counts.

As expected on the base of the profound and sustained $CD4^+$ lymphocyte depleting effect of thymoglobulin, the mean absolute count of activated T cells dropped after transplantation in the THYMO group, remaining significantly below pre-transplant value and control value during the 12 months of observation (before transplantation: 0.60 ± 0.10 cell/µl; 1 month: 0.29 ± 0.06 cell/µl; 3 months: 0.25 ± 0.07 cell/µl; 6 months: 0.27 ± 0.10 cell/µl; 12 months: 0.38 ± 0.07 cell/µl) [**Figure 44**].

Conversely, in the BSX study group, the mean absolute count of activated T cells was found to remain stable at 3 months after transplantation (0.70 ± 0.11 cell/µl), as compared to pre-transplant value (0.66 ± 0.11 cell/µl; *P*=0.30) and healthy individuals (0.79 ± 0.13 cell/µl; *P*=0.24). However, thereafter, the activated T cells absolute count continuously decreased (6 months: 0.43 ± 0.06 cell/µl; 12 months: 0.28 ± 0.06 cell/µl) to values comparable to the one found in the THYMO group at the same timepoints, significantly below pre-transplant and control values [**Figure 44**].

This apparent different evolution of the activated T cells absolute count during the first three months after transplantation could reflect the persisting effect of the induction agents, which subsequently disappears and is replaced by the effect of the maintenance immunosuppressive treatments.

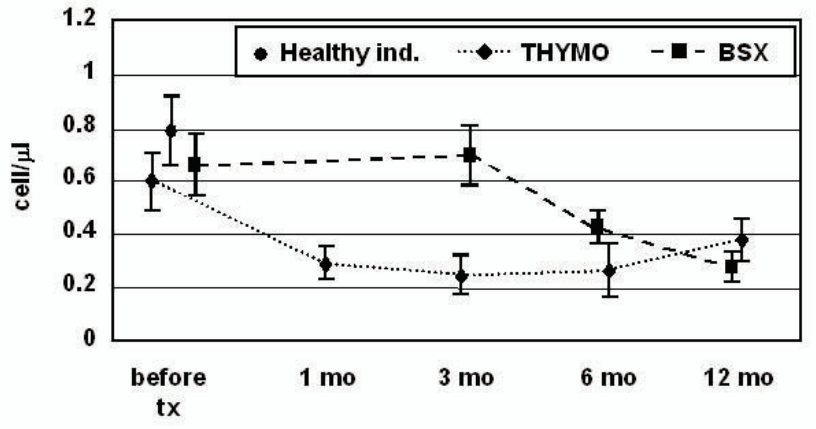



**Figure 44. Evolution of the mean absolute count of the activated T cell sub-population, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

Of note, in the THYMO group, there was a patient (GER-R13; see **Appendix III**) who experienced a severe and diffuse acute cellular rejection at day 40 after transplantation. Interestingly, the percentage of the activated T cell sub-population was highly increased (60.18% of $CD45RO^{+}$ $CD127^{high}$ cells within $CD4^{+}$ $CD25^{high}$ T cells), as was its absolute count (1.02 cell/µl) at that timepoint.

Taken together, both thymoglobulin and basiliximab induction regimens resulted in a significant decrease in activated T cells absolute count, what could be considered as a potential beneficial effect. However, both led to an even more dramatic decrease in regulatory T cells, what definitely cannot be beneficial for the outcome of a graft. As a consequence, the net result is the relative selective increase in activated (effector) T cells within the $CD4^{+}$ $CD25^{high}$ T cell population. Another way to analyze the relative evolution of these $CD4^{+}$ $CD25^{high}$ T cell sub-populations and to compare the two study groups could be to consider the ratio between the absolute counts of the activated and the regulatory subsets, rather than the absolute counts themselves. Applying this strategy, it becomes evident that in both study groups there is a relative increase of activated T cells as compared to their regulatory counterparts [**Figure 45**]. However, the kinetic in the THYMO and in the BSX study groups seems to be different. In the THYMO group, there is a significant increase of the activated/regulatory ratio of approximately four-fold, as soon as 1 month after transplantation, lasting until 6 months; at 12 months, the activated/regulatory ratio tended to decrease. In the BSX study group, the activated/regulatory ratio was also found to be increased at 3 months (approximately four-fold), and drastically decreased at 6 months (approximately two-fold);

unfortunately, no blood sample was collected at 1 month after transplantation, but based on our knowledge of the depleting effect exerted by basiliximab on $CD4^+$ $CD25^{high}$ T cells (which should be stronger at 1 month than at 3 months), we can speculate that the activated/regulatory ratio would have been even more increased at 1 month after transplantation. Conversely, at 12 months after transplantation, we can hypothesize that the activated/regulatory ratio could have returned to "normal" or nearly "normal" values. Although it might be tempting to conclude that thymoglobulin is safer in the immediate post-transplantation period and that basiliximab is better for the long-term, these conclusions have to be taken with caution and be tested in larger studies with more frequent assessment of activated and regulatory T cell sub-populations, as well as with longer observation periods.

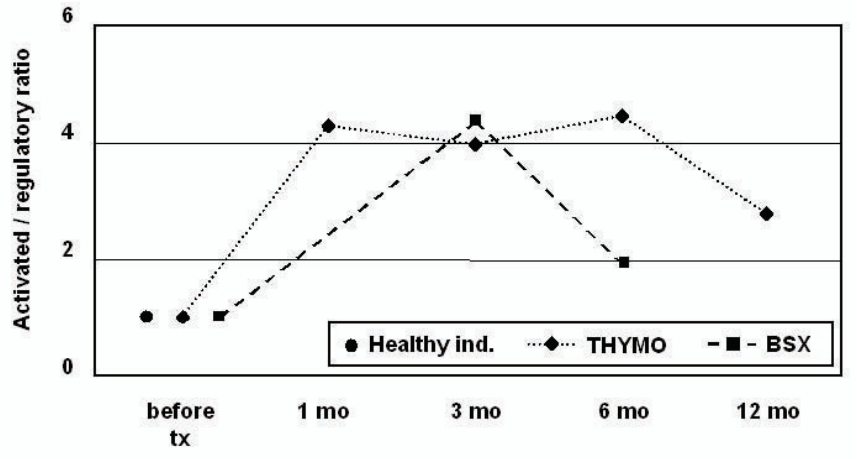



**Figure 45. Ratio between activated and regulatory T cell sub-populations, before and 1, 3, 6 and 12 months after transplantation, in the THYMO and BSX groups.**

**All values were normalized according to the values found before transplantation.**

## 4.2.6. Discussion of the prospective part

In this prospective part of our study, we have analyzed the evolution of $CD4^+$ $CD25^{high}$ T cells and their subsets (activated and regulatory) during the first 12 months after kidney transplantation, and we have compared the respective impact of two different induction regimens (thymoglobulin and basiliximab) on the percentages and absolute counts of the above-mentioned cell populations.

Briefly, we have confirmed that thymoglobulin had a profound and persistent CD4 T cell depleting effect, whilst basiliximab induced depletion of $CD4^+$ $CD25^{high}$ T cells, as expected based on their respective mechanisms of action. We have observed that in both THYMO and BSX study groups there was a significant increase of the percentage of the activated T cell sub-population, as soon as 1 month after transplantation, and a concomitant significant decrease of the percentage of the regulatory T cell sub-population. However, when looking at T cell sub-population absolute counts, the CD4 T lymphocyte depleting effect of

thymoglobulin resulted in a net decrease of both activated and regulatory T cell sub-populations in the THYMO group. By contrast, basiliximab caused the drop of regulatory T cells after transplantation, whilst activated T cells initially remained stable (at 3 months) and subsequently decreased.

To avoid the bias of depletion, we decided to analyze these results by considering the ratio between activated and regulatory T cell absolute counts. The net result was an increase of this ratio in favor of the activated T cells, in both study groups, especially in the immediate post-transplantation period. This observation could explain, at least in part, why the first year after transplantation is well-known to be at risk for the development of acute rejection episodes. The balance between activated and regulatory T cells is shifted towards activated T cells; relatively expanded activated T cells (which contain alloreactive, potentially detrimental T cells) may subsequently escape the control of regulatory T cells in decreased numbers, although most of the time alloreactive T cells are kept in check thanks to potent immunosuppression. After this critical period, and with the disappearance of the negative impact of induction treatments on regulatory T cells, accommodation may occur, with the possible expansion of a regulatory T cell pool able to regulate anti-donor immune reactivity in an antigen-specific way. However, larger studies with a longer observation period are needed, looking at these parameters in order to correlate them with the long-term clinical outcome of transplant recipients.

This prospective part of our study confirmed the results obtained in the retrospective part and permitted to make the link between the pre-transplant status and the "chronic" stable phase after transplantation.

However, this prospective part of our study suffers from the same limitations as the one that have been already discussed in our retrospective part. Briefly, we examined only the phenotype of peripherally circulating T lymphocytes subsets, which may not exactly reflect the immunological events occurring in the renal allograft or its draining lymph nodes; again, there are many controversies among transplantation centers on the usefulness and ethical acceptability of performing protocol renal biopsies in otherwise healthy patients. On the other hand, as was performed for the phenotype, the functionality of the $CD4^{+}$ $CD25^{high}$ T cells sub-populations should also be examined prospectively; in particular, the suppressive capacity of the regulatory T cell subset should examined in the THYMO and BSX study groups, at the different timepoints during the first year after renal transplantation and for specific regulation on donor as well as third party antigens.

# 5. GENERAL CONCLUSIONS AND PERSPECTIVES

Transplantation is now widely used to replace damaged or failing organs. In recent years, major advances have been made in the surgical techniques as well as in the understanding of the mechanisms implicated in the acceptance or the rejection of the transplanted organ. In this regard, the study of immunology, in particular of the immune responses elicited by a graft, represents a major challenge and, in the future, it will allow a better management of the transplant recipients.

A role of $CD4^+$ $CD25^{high}$ T cells in organ transplantation has been established more than ten years ago. Since then, the interest for the biology of this particular subset of immune cells has been growing steadily, and it has become clear now that the $CD4^+$ $CD25^{high}$ regulatory T cells may be used as means for immune intervention strategies aiming at the development of transplantation tolerance, or, at least, as an additional tool for the long-term follow-up of transplant recipients.

In this regard, we were interested in dissecting the phenotype and function of $CD4^+$ $CD25^{high}$ T cells, first in healthy individuals, and second in solid organ transplant recipients. When this project started five years ago, there were a lot of controversies on these subjects – and some of them still remain today. For example, in humans, there was no established distinctive marker to distinguish $CD4^+$ $CD25^{high}$ regulatory T cells from conventional activated T cells; moreover, the mechanism of action of regulatory T cells (i.e., the mechanism by which they exert their suppressive effect on other immune cells) was still under debate. And the data available for transplant recipients were even more controversial. In this context, it was of great interest to perform a comprehensive study, in healthy individuals and in solid organ transplant recipients.

Our initial results permitted to demonstrate that:

- a novel phenotypic surface marker, the α chain of the IL-7 receptor (CD127), allowed a clear discrimination between regulatory and activated $CD4^+$ $CD25^+$ T cells, in healthy individuals (these results have been confirmed by two publications (Liu W *et al*., 2006; Seddiki N *et al*., 2006));
- this marker could also be used in patients after solid organ transplantation (liver and kidney);
- the regulatory and activated T cell populations, defined on the base of CD127 expression in $CD4^+$ $CD25^{high}$ T cells, were differentially distributed in healthy individuals as compared to transplant recipients.

This last observation prompted us to also focus our interest on the activated sub-population of $CD4^+$ $CD25^{high}$ T cells. We hypothesized that these activated T cells could play an important role in the immune response directed towards the graft in the context of transplantation.

Our subsequent results, published in the Journal of Experimental Medicine in 2007 (Codarri L *et al*., 2007), showed that:

- the proportion of the activated T cell sub-population among $CD4^+$ $CD25^{high}$ T cells was significantly increased in stable liver and kidney transplant recipients as compared to healthy individuals, and even more in kidney transplant recipients with a biopsy-proven diagnosis of chronic humoral rejection;
- cells with the typical activated phenotype were found among the cells infiltrating renal allografts of patients with chronic humoral rejection;
- the activated T cell sub-population contained cells with an antigenic specificity for organ donor antigens ("alloreactive T cells").

To achieve a better understanding of the role and function of the $CD4^+$ $CD25^{high}$ T cell sub-populations, we decided to increase the number of studied kidney transplant recipients, as well as the panel of clinical conditions, in terms of immunological status, maintenance immunosuppression, and induction regimens (retrospective part of the study, patients already being transplanted).

We could demonstrate that:

- the proportion of the activated T cell sub-population among $CD4^+$ $CD25^{high}$ T cells was significantly increased in the vast majority of stable kidney transplant recipients, as compared to healthy individuals, whilst the proportion of the regulatory T cell sub-population was found to be decreased;
- the activated T cell sub-population was significantly more expanded in kidney transplant recipients with chronic humoral rejection, whilst the proportion of the regulatory T cell sub-population was significantly more decreased.

In addition, we obtained potentially clinically important data indicating that:

- stable kidney transplant recipients on minimal immunosuppression ("pseudo-tolerant" recipients) were comparable to healthy individuals in terms of distribution of the activated and regulatory T cell sub-populations among $CD4^+$ $CD25^{high}$ T cells (i.e., "normal" profile);
- stable kidney transplant recipients on sirolimus-based immunosuppression also displayed a $CD4^+$ $CD25^{high}$ T cell sub-populations distribution profile which was comparable to healthy individuals;
- the effect of cyclosporine A, tacrolimus and mycophenolate mofetil on the distribution of the activated and regulatory T cell sub-populations among $CD4^+$ $CD25^{high}$ T cells was similar;
- $CD4^+$ $CD25^{high}$ T cell sub-populations expressed different chemokine homing receptors as compared to conventional $CD4^+$ $CD25^{neg}$ T cells and $CD3^+$ $CD4^+$ cells.

In addition to these retrospective results, we conducted a prospective study, to analyze the evolution of $CD4^+$ $CD25^{high}$ T cell subsets during the first year after renal transplantation, and the potential influence of two different induction agents (thymoglobulin versus basiliximab). This prospective study could demonstrate that:

- with either induction agent, the proportion of activated T cells increased rapidly after transplantation, whilst the proportion of regulatory T cells decreased progressively over the first 12 months after transplantation;
- thymoglobulin had a strong and long-lasting depleting effect on T cells; as a consequence, both activated and regulatory T cells absolute counts were decreased after transplantation, but in different proportions, with the net result of a relative selective increase in activated T cells;
- basiliximab did not deplete T cells but reduced the proportion of $CD25^{high}$ cells among $CD4^+$ T cells; the activated T cells absolute count remained stable for the first three months after transplantation and subsequently decreased, whilst the regulatory T cell absolute count decreased immediately after transplantation; similar to thymoglobulin, the net result was a relative selective increase in activated T cells.

Our study has allowed to have a better knowledge of $CD4^+$ $CD25^{high}$ T cells in healthy individuals, and, more importantly, in solid organ transplant recipients. In particular, we have shown that this cell population is heterogeneous in terms of phenotype and function: by contrast to what was believed until recently, $CD4^+$ $CD25^{high}$ T cells do not contain only regulatory T cells, but they also contain a small, but significant, proportion of activated T cells. This observation is perhaps not so important in healthy individuals, where approximately 95% of $CD4^+$ $CD25^{high}$ T cells are regulatory T cells; by contrast, it is necessary to take this observation into account when studying conditions where the proportion of activated T cells can be expected to be increased, e.g. all clinical situations with inflammatory syndromes. The fact that researchers used only the CD4 and CD25 markers until recently may explain why studies on the role of regulatory T cells in inflammatory pathologies, including transplantation and autoimmunity, have led to controversial results. Indeed, significant changes in the relative and/or absolute distribution of regulatory and/or activated T cells within $CD4^+$ $CD25^{high}$ T cells may have a significant impact on the observed frequency of $CD4^+$ $CD25^{high}$ T cells as well as on their suppressive capacity. We have overcome this difficulty by using additional markers, namely the α chain of the IL-7 receptor (CD127) and FoxP3. However, we must acknowledge that the study of regulatory T cells phenotypic markers is probably not at the end: other markers will probably be identified in the future, which will further help in the isolation of a purer population of regulatory T cells and in the characterization of their properties.

Our observation of an increased proportion of activated T cells within $CD4^+$ $CD25^{high}$ T cells in the blood of solid organ transplant recipients was a completely new finding (Codarri L *et*

*al*., 2007). Furthermore, the fact that this population could also be detected in renal biopsies of patients with chronic humoral rejection and that it contained alloreactive cells was a strong stimulus for us to continue to study the presence of this population in solid organ transplant recipients, besides regulatory T cells. Overall, our retrospective and prospective results provide a confirmation of our initial data, but most importantly they provide also new evidence on the clinical usefulness of monitoring these activated T cells in transplant recipients. Measuring these parameters provides for the first time a direct, and easy to use, assessment of the anti-donor immunological activity related to the presence of a renal allograft. This may be helpful in the routine follow-up of transplant recipients, as a complement to existing diagnostic tools (e.g., serum creatinine measurement, anti-HLA antibodies measurement, renal biopsy) for the assessment of graft function as well as for the individualization of the immunosuppressive treatment. However, it will be necessary to complete our understanding of the implicated mechanisms, e.g. in order to eliminate possible confounding factors such as concomitant infections or inflammatory pathologies. This goal may be achieved by conducting well-designed prospective clinical protocols, that will correlate clinical, biological and histopathological findings with the parameters of interest, as well as by designing immunosuppression minimization protocols.

Finally, our results on kidney transplant recipients presented in this work are further supported by results obtained by colleagues in our laboratory, who studied activated and regulatory T cells in the context of orthotopic liver transplantation, and the impact of hepatitis C chronic infection. This study has resulted in a manuscript which is currently submitted to the *American Journal of Transplantation* and which is reproduced in **Appendix IV**.

# 6. REFERENCES

**A**

Al Najjar A, Etienne I, Le Pogamp P, Bridoux F, Le Meur Y, Toupance O, Mousson C, Caillard S, Hurault de Ligny B, Marlière JF, Lebranchu Y. Long-term results of monoclonal anti-IL-2-receptor antibody versus polyclonal antilymphocyte antibodies as induction therapy in renal transplantation. *Transplant Proc*. 2006; 7: 2298-2299.

Ali S, Malik G, Burns A, Robertson H, Kirby JA. Renal transplantation: examination of the regulation of chemokine binding during acute rejection. *Transplantation*. 2005; 79: 672-679.

Al-Lamki RS, Wang J, Skepper JN, Thiru S, Pober JS, Bradley JR. Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab Invest*. 2001; 81: 1503-1515.

Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, Ziegler SF, Roncarolo MG, Levings MK. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest*. 2005; 115: 3276-3284.

Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol*. 2004; 5: 266-271.

Ashton-Chess J, Giral M, Soulillou JP, Brouard S. Can immune monitoring help to minimize immunosuppression in kidney transplantation? *Transpl Int*. 2009; 22: 110-119.

**B**

Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, Balk AH, Weimar W. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation*. 2005; 80: 110-117.

Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, Perroni L, Dagna-Bricarelli F, Sartirana C, Matthes-Martin S, Lawitschka A, Azzari C, Ziegler SF, Levings MK, Roncarolo MG. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J Clin Invest*. 2006; 116: 1713-1722.

Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+ CD25high regulatory cells in human peripheral blood. *J Immunol*. 2001; 167: 1245-1253.

Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. *J Immunol*. 2001; 167: 7199-7206.

Baldwin WM 3rd, Larsen CP, Fairchild RL. Innate immune responses to transplants: a significant variable with cadaver donors. *Immunity*. 2001; 14: 369-376.

Baron U, Floess S, Wieczorek G, Baumann K, Grützkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, Türbachova I, Hamann A, Olek S, Huehn J. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol*. 2007; 9: 2378-2389.

Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med*. 2002; 95: 603-616.

Battaglia M, Stabilini A, Draghici E, Migliavacca B, Gregori S, Bonifacio E, Roncarolo MG. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes*. 2006; 55: 40-49.

Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+ CD25+ FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol*. 2006; 177: 8338-8347.

Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+ CD25+ FoxP3+ regulatory T cells. *Blood*. 2005; 105: 4743-4748.

Becker YT, Samaniego-Picota M, Sollinger HW. The emerging role of rituximab in organ transplantation. *Transpl Int*. 2006; 19: 621-628.

Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol*. 1999; 162: 352-358.

Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001; 27: 20-21.

Berke G. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. *Annu Rev Immunol*. 1994; 12: 735-773.

Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A*. 2005; 102: 5138-5143.

Billingham RE, Brent L, Medawar PB, Sparrow EM. Quantitative studies on tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. *Proc R Soc Lond B Biol Sci*. 1954; 143: 43-58.

Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature*. 1953; 172: 603-606.

Billingham RE, Brent L, Medawar PB. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc R Soc Lond B Biol Sci*. 1954; 143: 58-80.

Bluestone JA, Liu W, Yabu JM, Laszik ZG, Putnam A, Belingheri M, Gross DM, Townsend RM, Vincenti F. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. *Am J Transplant*. 2008; 8: 2086-2096.

Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med*. 1998; 187: 129-134.

Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'acqua ML, Rossini PM, Battistini L, Rotzschke O, Falk K. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 2007; 110: 1225-1232.

Brändle D, Joergensen J, Zenke G, Bürki K, Hof RP. Contribution of donor-specific antibodies to acute allograft rejection: evidence from B cell-deficient mice. *Transplantation*. 1998; 65: 1489-1493.

Brauer RB, Baldwin WM 3rd, Ibrahim S, Sanfilippo F. The contribution of terminal complement components to acute and hyperacute allograft rejection in the rat. *Transplantation*. 1995; 59: 288-293.

Brennan DC, Daller JA, Lake KD, Cibrik D, Del Castillo D; Thymoglobulin Induction Study Group. Rabbit antithymocyte globulin versus basiliximab in renal transplantation. *N Engl J Med*. 2006; 355: 1967-1977.

Brennan DC, Flavin K, Lowell JA, Howard TK, Shenoy S, Burgess S, Dolan S, Kano JM, Mahon M, Schnitzler MA, Woodward R, Irish W, Singer GG. A randomized, double-blinded comparison of Thymoglobulin versus Atgam for induction immunosuppressive therapy in adult renal transplant recipients. *Transplantation*. 1999; 67: 1011-1018.

Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nature Immunol*. 2008; 9: 970-980.

Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K, von Boehmer H, Buer J, Hansen W. Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol*. 2004; 34: 623-630.

Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001; 27: 68-73.

Bryder D, Rossi DJ, Weissman IL. Hematopoietic stem cells: The paradigmatic tissue specific stem cell. *Am J Pathol*. 2006; 169: 338-346.

**C**

Calne R. Cyclosporine as a milestone in transplantation. *Transplant Proc*. 2004; 36: 13S-15S.

Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nat Rev Immunol*. 2007; 7: 305-310.

Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, Ley TJ. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity*. 2007; 27: 635-646.

Cecka JM. The UNOS Renal Transplant Registry. *Clin Transpl*. 2002:1-20.

Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai AI, Piazza F, Lederman S, Colonna M, Cortesini R, Dalla-Favera R, Suciu-Foca N. Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat Immunol*. 2002; 3: 237-243.

Chapman JR, Valantine H, Albanell J, Arns WA, Campistol JM, Eisen H, Frigerio M, Lehmkuhl H, Marcen R, Morris R, Nashan B, Pascual J, Pohanka E, Segovia J, Zuckermann A. Proliferation signal inhibitors in transplantation: questions at the cutting edge of everolimus therapy. *Transplant Proc*. 2007; 39: 2937-2950.

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+ CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003; 198: 1875-1886.

Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science*. 1994; 265: 1237-1240.

Cheng CH, Chang HR, Chiang CW, Shu KH, Chou MC. Possible mechanism by which rapamycin increases cyclosporine nephrotoxicity. *Transplant Proc*. 2008; 40: 2373-2375.

Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature*. 1992; 357: 695-697.

Cockwell P, Chakravorty SJ, Girdlestone J, Savage CO. Fractalkine expression in human renal inflammation. *J Pathol*. 2002; 196: 85-90.

Codarri L, Vallotton L, Ciuffreda D, Venetz JP, Garcia M, Hadaya K, Buhler L, Rotman S, Pascual M, Pantaleo G. Expansion and tissue infiltration of an allospecific $CD4^+$ $CD25^+$ $CD45RO^+$ IL-7R$\alpha^{high}$ cell population in solid organ transplant recipients. *J Exp Med*. 2007; 204: 1533-1541.

Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Rapamycin, and not cyclosporine A, preserves the highly suppressive CD27+ subset of human CD4+ CD25+ regulatory T cells. *Blood*. 2006; 107: 1018-1023.

Coenen JJ, Koenen HJ, van Rijssen E, Kasran A, Boon L, Hilbrands LB, Joosten I. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant*. 2007; 39: 537-545.

Collins AB, Chicano SL, Cornell LD, Tolkoff-Rubin N, Goes NB, Saidman SL, Farrell ML, Cosimi AB, Colvin RB. Putative antibody-mediated rejection with C4d deposition in HLA-identical, ABO-compatible renal allografts. *Transplant Proc*. 2006; 38: 3427-3429.

Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature.* 2007; 450: 566-569.

Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol*. 2007; 18: 1046-1056.

Cornell LD, Smith RN, Colvin RB. Kidney transplantation: mechanisms of rejection and acceptance. *Annu Rev Pathol*. 2008; 3: 189-220.

Cosio FG, Pelletier RP, Falkenhain ME, Henry ML, Elkhammas EA, Davies EA, Bumgardner GL, Ferguson RM. Impact of acute rejection and early allograft function on renal allograft survival. *Transplantation*. 1997; 63: 1611-1615.

Cottrez F, Groux H. Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol*. 2001; 167: 773-778.

**D**

D'Ambrosio D, Iellem A, Bonecchi R, Mazzeo D, Sozzani S, Mantovani A, Sinigaglia F. Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol*. 1998; 161: 5111-5115.

Davenport A, Younie ME, Parsons JE, Klouda PT. Development of cytotoxic antibodies following renal allograft transplantation is associated with reduced graft survival due to chronic vascular rejection. *Nephrol Dial Transplant*. 1994; 9: 1315-1319.

Davies JD, Leong LY, Mellor A, Cobbold SP, Waldmann H. T cell suppression in transplantation tolerance through linked recognition. *J Immunol*. 1996; 156: 3602-3607.

Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007; 204: 1257-1265.

Demirkiran A, Hendrikx TK, Baan CC, van der Laan LJW. Impact of immunosuppressive drugs on CD4+ CD25+ FOXP3+ regulatory T cells: does in vitro evidence translate to clinical setting? *Transplantation*. 2008; 85: 783-789.

Demirkiran A, Kok A, Kwekkeboom J, Kusters JG, Metselaar HJ, Tilanus HW, van der Laan LJ. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. *Liver Transpl*. 2006; 12: 277-284.

Demirkiran A, Kok A, Kwekkeboom J, Metselaar HJ, Tilanus HW, van der Laan LJ. Decrease of CD4+ CD25+ T cells in peripheral blood after liver transplantation: association with immunosuppression. *Transplant Proc*. 2005; 37: 1194-1196.

DeSilva DR, Urdahl KB, Jenkins MK. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol*. 1991; 147: 3261-3267.

Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of $CD4^+$ $CD25^+$ T cells with regulatory properties from human blood. *J Exp Med.* 2001; 193: 1303-1310.

Dijke IE, Weimar W, Baan CC. The control of anti-donor immune responses by regulatory T cells in organ transplant patients. *Transplant Proc*. 2008; 40: 1249-1252.

Dragun D, Müller DN, Bräsen JH, Fritsche L, Nieminen-Kelhä M, Dechend R, Kintscher U, Rudolph B, Hoebeke J, Eckert D, Mazak I, Plehm R, Schönemann C, Unger T, Budde K, Neumayer HH, Luft FC, Wallukat G. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. *N Engl J Med*. 2005; 352: 558-569.

**F**

Fahim T, Böhmig GA, Exner M, Huttary N, Kerschner H, Kandutsch S, Kerjaschki D, Bramböck A, Nagy-Bojarszky K, Regele H. The cellular lesion of humoral rejection: predominant recruitment of monocytes to peritubular and glomerular capillaries. *Am J Transplant*. 2007; 7: 385-393.

Fangmann J, Dalchau R, Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J Exp Med*. 1992; 175: 1521-1529.

Faria AM, Weiner HL. Oral tolerance. *Immunol Rev*. 2005; 206: 232-259.

Fernandez-Miranda C, Morales JM, Porres A, Gomez-Gerique J, Guijarro C, Aranda JL, Andres A, Rodicio JL, Del Palacio A. Increased lipoproteins and fibrinogen in chronic renal allograft dysfunction. *Am J Nephrol*. 1997; 17: 445-449.

Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmüller G, Land W, Albert E. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int*. 1993; 43: 1333-1338.

Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Bopp T, Schmitt E, Klein-Hessling S, Serfling E, Hamann A, Huehn J. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 2007; 5: e38.

Fontenot JD, Dooley JL, Farr AG, Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. *J Exp Med*. 2005; 202: 901-906.

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+$ $CD25^+$ regulatory T cells. *Nat Immunol*. 2003; 4: 330-336.

Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005; 6: 1142-1151.

Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 2005; 22: 329-341.

Fuggle SV, Sanderson JB, Gray DW, Richardson A, Morris PJ. Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys – correlation with intragraft events. *Transplantation*. 1993; 55: 117-123.

**G**

Gajiwala KS, Burley SK. Winged helix proteins. *Curr Opin Struct Biol*. 2000; 10: 110-116.

Game DS, Hernandez-Fuentes MP, Lechler RI. Everolimus and basiliximab permit suppression by human $CD4^+$ $CD25^+$ cells in vitro. *Am J Transplant*. 2005; 5: 454-464.

Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant*. 2007; 7: 1722-1732.

Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A*. 2006; 103: 6659-6664.

Gebauer BS, Hricik DE, Atallah A, Bryan K, Riley J, Tary-Lehmann M, Greenspan NS, Dejelo C, Boehm BO, Hering BJ, Heeger PS. Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. *Am J Transplant*. 2002; 2: 857-866.

George TC, Bilsborough J, Viney JL, Norment AM. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur J Immunol*. 2003; 33: 502-511.

Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*. 1970; 18: 723–735.

Gibson T, Medawar PB. The fate of skin homografts in man. *J Anat*. 1943; 77 (Pt 4): 299-310.

Gloor JM, DeGoey SR, Pineda AA, Moore SB, Prieto M, Nyberg SL, Larson TS, Griffin MD, Textor SC, Velosa JA, Schwab TR, Fix LA, Stegall MD. Overcoming a positive crossmatch in living-donor kidney transplantation. *Am J Transplant*. 2003; 3: 1017-1023.

Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol*. 1993; 150: 4244-4252.

Godfrey VL, Wilkinson JE, Russell LB. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol*. 1991; 138: 1379-1387.

Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. *J Exp Med*. 2002; 195: 1641-1646.

Graca L, Honey K, Adams E, Cobbold SP, Waldmann H. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol*. 2000; 165: 4783-4786.

Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005; 23: 515-548.

Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory T cells induced by 1α,25-dihydroxyvitamin $D_3$ and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol*. 2001; 167: 1945-1953.

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*. 1997; 389: 737-742.

**H**

Hall BM, Pearce NW, Gurley KE, Dorsch SE. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J Exp Med*. 1990; 171: 141-157.

Halloran PF, Melk A, Barth C. Rethinking chronic allograft nephropathy: the concept of accelerated senescence. *J Am Soc Nephrol*. 1999; 10: 167-181.

Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med*. 2004; 351: 2715-2729.

Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol*. 2001; 166: 3789-3796.

Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 1992**;** 356**:** 607-609.

Hardinger KL, Schnitzler MA, Miller B, Lowell JA, Shenoy S, Koch MJ, Enkvetchakul D, Ceriotti C, Brennan DC. Five-year follow up of thymoglobulin versus ATGAM induction in adult renal transplantation. *Transplantation*. 2004; 1: 136-141.

Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, Hipkiss EL, Getnet D, Goldberg MV, Maris CH, Housseau F, Yu H, Pardoll DM, Drake CG. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J Immunol*. 2007; 179: 4313-4317.

Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. *J Immunol Methods*. 2007; 319: 41-52.

Hashimoto S, Kobayashi A, Kooguchi K, Kitamura Y, Onodera H, Nakajima H. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2000; 161: 237-243.

Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M, Taniguchi T. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA. *Science*. 1989; 244: 551-556.

Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol*. 2005; 5: 271-283.

Hernandez-Fuentes MP, Warrens AN, Lechler RI. Immunologic monitoring. *Immunol Rev*. 2003; 196: 247-264.

Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The majority of human peripheral blood $CD4^+$ $CD25^{high}$ $FoxP3^+$ regulatory T cells bear functional skin-homing receptors. *J Immunol*. 2006; 177: 4488-4494.

Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+) CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. 2002; 196: 389-399.

Holzinger C, Schöllhammer A, Imhof M, Reinwald C, Kramer G, Zuckermann A, Wolner E, Steiner G. Phenotypic patterns of mononuclear cells in dilated cardiomyopathy. *Circulation*. 1995; 92: 2876-2885.

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003; 299: 1057-1061.

Hourmant M, Cesbron-Gautier A, Terasaki PI, Mizutani K, Moreau A, Meurette A, Dantal J, Giral M, Blancho G, Cantarovich D, Karam G, Follea G, Soulillou JP, Bignon JD. Frequency and clinical implications of development of donor-specific and non-donor-specific HLA antibodies after kidney transplantation. *J Am Soc Nephrol*. 2005; 16: 2804-2812.

Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, Dejelo C, Schulak JA, Heeger PS. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant*. 2003; 3: 878-884.

Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity*. 2004; 21: 267-277.

Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA. Role of LAG-3 in regulatory T cells. *Immunity*. 2004; 21: 503-513.

Humar A, Gillingham KJ, Payne WD, Dunn DL, Sutherland DE, Matas AJ. Association between cytomegalovirus disease and chronic rejection in kidney transplant recipients. *Transplantation*. 1999; 68: 1879-1883.

**I**

Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature*. 1984; 307: 168-170.

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006; 126: 1121-1133.

Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev*. 2006; 212: 256-271.

**J**

Jabs WJ, Sedlmeyer A, Ramassar V, Hidalgo LG, Urmson J, Afrouzian M, Zhu LF, Halloran PF. Heterogeneity in the evolution and mechanisms of the lesions of kidney allograft rejection in mice. *Am J Transplant*. 2003; 3: 1501-1509.

Janeway CA, Travers P, Walport M, Schlomchik MJ. Autoimmunity and Transplantation. *Immunobiology: the immune system in health and disease*. 2001; 5: 523-524. Garland Publishing, New York.

Janson PC, Winerdal ME, Marits P, Thörn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS ONE*. 2008; 3: e1612.

Janssens W, Carlier V, Wu B, VanderElst L, Jacquemin MG, Saint-Remy JM. CD4+ CD25+ T cells lyse antigen-presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner. *J Immunol*. 2003; 171: 4604-4612.

Jenkins MK, Taylor PS, Norton SD, Urdahl KB. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol*. 1991; 147: 2461-2466.

Jiang S, Lechler RI, He XS, Huang JF. Regulatory T cells and transplantation tolerance. *Human Immunol*. 2006; 67: 765-776.

Joffre O, Gorsse N, Romagnoli P, Hudrisier D, van Meerwijk JP. Induction of antigen-specific tolerance to bone marrow allografts with CD4+ CD25+ T lymphocytes. *Blood*. 2004; 103: 4216-4221.

Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human $CD4^+$ $CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J Exp Med.* 2001; 193: 1285-1294.

Ju ST, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. *Proc Natl Acad Sci USA*. 1994; 91: 4185-4189.

Judge TA, Wu Z, Zheng XG, Sharpe AH, Sayegh MH, Turka LA. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J Immunol*. 1999; 162: 1947-1951.

**K**

Kahan BD. Cyclosporine. *N Engl J Med*. 1989; 321: 1725-1738.

Kang SM, Tang Q, Bluestone JA. CD4+ CD25+ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant*. 2007; 7: 1457-1463.

Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 1996; 4: 313-319.

Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*. 1996; 382: 174-177.

Karimova A, Pinsky DJ. The endothelial response to oxygen deprivation: biology and clinical implications. *Intensive Care Med*. 2001; 27: 19-31.

Kasiske BL. Clinical correlates to chronic renal allograft rejection. *Kidney Int Suppl*. 1997; 63: S71-74.

Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med*. 2000; 6: 114.

Keever-Taylor CA, Browning MB, Johnson BD, Truitt RL, Bredeson CN, Behn B, Tsao A. Rapamycin enriches for CD4(+) CD25(+) CD27(+) Foxp3(+) regulatory T cells in ex vivo-expanded CD25-enriched products from healthy donors and patients with multiple sclerosis. *Cytotherapy*. 2007; 9: 144-157.

Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+ CD25+ T regulatory cells. *Nat Immunol*. 2003; 4: 337-342.

Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH Jr, Knechtle SJ. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A*. 1997; 94: 8789-8794.

Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol*. 2006; 177: 566-573.

Koo DD, Welsh KI, McLaren AJ, Roake JA, Morris PJ, Fuggle SV. Cadaver versus living donor kidneys: impact of donor factors on antigen induction before transplantation. *Kidney Int*. 1999; 56: 1551-1559.

Kreijveld E, Koenen HJ, Klasen IS, Hilbrands LB, Joosten I. Following anti-CD25 treatment, a functional CD4+ CD25+ regulatory T-cell pool is present in renal transplant recipients. *Am J Transplant*. 2007; 7: 249-255.

Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol*. 2005; 23: 877-900.

**L**

Lakkis FG, Arakelov A, Konieczny BT, Inoue Y. Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat Med*. 2000; 6: 686-688.

Langan LL, Park LP, Hughes TL, Irish A, Luxton G, Witt CS, Christiansen FT. Post-transplant HLA class II antibodies and high soluble CD30 levels are independently associated with poor kidney graft survival. *Am J Transplant*. 2007; 7: 847-856.

Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation – friend or foe? *Immunity*. 2001; 14: 357-368.

Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J Exp Med*. 1982; 155: 31-41.

Lee JH, Kang SG, Kim CH. FoxP3$^{+}$ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol*. 2007; 178: 301-311.

Lee MK 4th, Moore DJ, Jarrett BP, Lian MM, Deng S, Huang X, Markmann JW, Chiaccio M, Barker CF, Caton AJ, Markmann JF. Promotion of allograft survival by CD4+ CD25+ regulatory T cells: evidence for *in vivo* inhibition of effector cell proliferation. *J Immunol*. 2004; 172: 6539-6544.

Lee PC, Terasaki PI, Takemoto SK, Lee PH, Hung CJ, Chen YL, Tsai A, Lei HY. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation*. 2002; 74: 1192-1194.

Levings MK, Sangregorio R, Roncarolo MG. Human $CD25^+$ $CD4^+$ T cells suppress naive and memory T-cell proliferation and can be expanded *in vitro* without loss of suppressor function. *J Exp Med.* 2001; 193: 1295-1302.

Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD, Nguyen BV, Gadina M, Sher A, Paul WE, O'Shea JJ. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A*. 2001; 98: 15137-15142.

Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and $FoxP3^+$ regulatory T cells. *J Immunol*. 2008; 180: 122-129.

Litjens NHR, van Druninger CJ, Betjes MGH. Progressive loss of renal function is associated with activation and depletion of naïve T lymphocytes. *Clin Immunol*. 2006; 118: 83-91.

Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. *Immunology*. 2007; 122: 149-156.

Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, de St Groth BF, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ Treg cells. *J Exp Med*. 2006; 203: 1701-1711.

Liu Z, Braunstein NS, Suciu-Foca N. T cell recognition of allopeptides in context of syngeneic MHC. *J Immunol*. 1992; 148: 35-40.

Liu Z, Fang Y, Wang X, Wang P, Yun P, Xu H. Upregulation of molecules associated with T-regulatory function by thymoglobulin pretreatment of human CD4+ cells. *Transplantation*. 2008; 86: 1419-1426.

Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, Dayer JM. CCR5 is characteristic of Th1 lymphocytes. *Nature*. 1998; 391: 344-345.

Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. *Science*. 1994; 264: 1587-1589.

Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells. *J Am Soc Nephrol*. 2006; 17: 2844-2853.

Louis S, Braudeau C, Giral M, Dupont A, Moizant F, Robillard N, Moreau A, Soulillou JP, Brouard S. Contrasting $CD25^{hi}$ $CD4^+$ T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation*. 2006; 81: 398-407.

**M**

Madden DR. The three-dimensional structure of peptide-MHC complexes. *Annu. Rev. Immunol*. 1995; 13: 587-622.

Mansoor M, Melendez AJ. Recent trials for FTY720 (fingolimod): a new generation of immunomodulators structurally similar to sphingosine. *Rev Recent Clin Trials*. 2008; 3: 62-69.

Markmann JF, Odorico JS, Bassiri H, Desai N, Kim JI, Barker CF. Deletion of donor-reactive T lymphocytes in adult mice after intrathymic inoculation with lymphoid cells. *Transplantation*. 1993; 55: 871-876.

Martin L, Guignier F, Bocrie O, D'Athis P, Rageot D, Rifle G, Justrabo E, Mousson C. Detection of anti-HLA antibodies with flow cytometry in needle core biopsies of renal transplants recipients with chronic allograft nephropathy. *Transplantation*. 2005; 79: 1459-1461.

Martínez-Llordella M, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, Lerut J, Latinne D, Margarit C, Bilbao I, Brouard S, Hernández-Fuentes M, Soulillou JP, Sánchez-Fueyo A. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant*. 2007; 7: 309-319.

Mauiyyedi S, Pelle PD, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin NE, Williams WW, Cosimi AA, Schneeberger EE, Colvin RB. Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J Am Soc Nephrol*. 2001; 12: 574-582.

May KF Jr, Chang X, Zhang H, Lute KD, Zhou P, Kocak E, Zheng P, Liu Y. B7-deficient autoreactive T cells are highly susceptible to suppression by CD4(+)CD25(+) regulatory T cells. *J Immunol*. 2007; 178: 1542-1552.

Meloni F, Vitulo P, Bianco AM, Paschetto E, Morosini M, Cascina A, Mazzucchelli I, Ciardelli L, Oggionni T, Fietta AM, Pozzi E, Viganò M. Regulatory CD4+ CD25+ T cells in the peripheral blood of lung transplant recipients: correlation with transplant outcome. *Transplantation*. 2004; 77: 762-766.

Minami Y, Kono T, Miyazaki T, Taniguchi T. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol*. 1993; 11: 245-268.

Minguet S, Huber M, Rosenkranz L, Schamel WW, Reth M, Brummer T. Adenosine and cAMP are potent inhibitors of the NF-kappa B pathway downstream of immunoreceptors. *Eur J Immunol*. 2005; 35: 31-41.

Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med.* 2007; 13: 108-116.

Moger V, Ravishankar MS, Sakhuja V, Kohli HS, Sud K, Gupta KL, Jha V. Intravenous immunoglobulin: A safe option for treatment of steroid-resistant rejection in the presence of infection. *Transplantation*. 2004; 77: 1455-1456.

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992; 68: 869-877.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986; 136: 2348-2357.

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989; 7: 145-173.

Mueller TF. Mechanisms of action of thymoglobulin. *Transplantation*. 2007; 84: S5-S10.

Mueller TF. Phenotypic changes with immunosuppression in human recipients. *Front Biosci*. 2003; 8: d1254.

Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med*. 2003; 198: 1951-1957.

Mustelin T, Tasken K. Positive and negative regulation of T-cell activation through kinases and phosphatases. *Biochem J*. 2003; 371: 15-27.

**N**

Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+) CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med*. 2001; 194: 629-644.

Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Chapman JR, Allen RD. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. *Transplantation*. 2004; 78: 557-565.

Nankivell BJ, Fenton-Lee CA, Kuypers DR, Cheung E, Allen RD, O'Connell PJ, Chapman JR. Effect of histological damage on long-term kidney transplant outcome. *Transplantation*. 2001; 71: 515-523.

Natarajan K, Li H, Mariuzza RA, Margulies DH. MHC class I molecules, structure and function. *Rev Immunogenet*. 1999; 1: 32-46.

Nishimura E, Sakihama T, Setoguchi R, Tanaka K, Sakaguchi S. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+ CD25+ CD4+ regulatory T cells. *Int Immunol*. 2004; 16: 1189-1201.

Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, Migliorati G, Riccardi C. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc Natl Acad Sci U S A*. 1997; 94: 6216-6221.

Nocera A, Tagliamacco A, De Palma R, Del Galdo F, Ferrante A, Fontana I, Barocci S, Ginevri F, Rolla D, Ravetti JL, Valente U. Cytokine mRNA expression in chronically rejected human renal allografts. *Clin Transplant*. 2004; 18: 564-570.

Noris M, Casiraghi F, Todeschini M, Cravedi P, Cugini D, Monteferrante G, Aiello S, Cassis L, Gotti E, Gaspari F, Cattaneo D, Perico N, Remuzzi G. Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol*. 2007; 18: 1007-1018.

Norton SD, Hovinen DE, Jenkins MK. IL-2 secretion and T cell clonal anergy are induced by distinct biochemical pathways. *J Immunol*. 1991; 146: 1125-1129.

**O**

Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, Miyachi Y, Tsukada T, Sakaguchi S. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*. 2007; 446: 685-689.

Opelz G. Efficacy of rejection prophylaxis with OKT3 in renal transplantation. Collaborative Transplant Study. *Transplantation*. 1995; 60: 1220-1224.

O'Shea GM, Sun AM. Encapsulation of rat islets of Langerhans prolongs xenograft survival in diabetic mice. *Diabetes*. 1986; 35: 943-946.

**P**

Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci U S A*. 2004; 101: 1993-1998.

Palm NW, Medzhitov R. Antifungal defense turns 17. *Nat Immunol*. 2007; 8: 549-551.

Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+ CD25+ Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol*. 2007; 8: 1353-1362.

Parham P, Lawlor DA, Salter RD, Lomen CE, Bjorkman PJ, Ennis PD. HLA -A, B, C: Pattern of polymorphism in peptide-binding proteins. *Immunobiology of HLA: Immunogenetics and Histocompatibility*. 1987; 2 (B. DuPont, ed.): 28-34. Springer-Verlag, New York.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005; 11: 1133-1141.

Paul LC, Benediktsson H. Post-transplant hypertension and chronic renal allograft failure. *Kidney Int Suppl*. 1995; 52: S34-37.

Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell*. 1994; 76: 241-251.

Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A*. 2004; 101: 10398-10403.

Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, Swanson SJ, Mannon RB, Roederer M, Kirk AD. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant*. 2005; 3: 465-474.

Peter ME, Krammer PH. Mechanisms of CD95 (Apo-1/Fas)-mediated apoptosis. *Curr Opin Immunol*. 1998; 10: 545-551.

Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. *J Clin Invest*. 2000; 106: 1003-1010.

Pilmore HL, Painter DM, Bishop GA, McCaughan GW, Eris JM. Early up-regulation of macrophages and myofibroblasts: a new marker for development of chronic renal allograft rejection. *Transplantation*. 2000; 69: 2658-2662.

Poggio ED, Clemente M, Riley J, Roddy M, Greenspan NS, Dejelo C, Najafian N, Sayegh MH, Hricik DE, Heeger PS. Alloreactivity in renal transplant recipients with and without chronic allograft nephropathy. *J Am Soc Nephrol*. 2004; 15: 1952-1960.

**Q**

Qin S, Cobbold SP, Pope H, Elliott J, Kioussis D, Davies J, Waldmann H. "Infectious" transplantation tolerance. *Science*. 1993; 259: 974-977.

**R**

Raimondi G, Shufesky WJ, Tokita D, Morelli AE, Thomson AW. Regulated compartmentalization of programmed cell death-1 discriminates CD4+ CD25+ resting regulatory T cells from activated T cells. *J Immunol*. 2006; 176: 2808-2816.

Ramsdell F. FoxP3 and natural regulatory T cells: key to a cell lineage? *Immunity*. 2003; 19: 165-168.

Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, Sharpe AH, Powrie F. Blockade of CTLA-4 on CD4+ CD25+ regulatory T cells abrogates their function in vivo. *J Immunol*. 2006; 177: 4376-4383.

Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+) CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 2000; 192: 295-302.

Regele H, Böhmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S, Watschinger B, Kerjaschki D, Exner M. Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol*. 2002; 13: 2371-2380.

Reischig T, Jindra P, Svecova M, Kormuda S, Opatrny K Jr, Treska V. The impact of cytomegalovirus disease and asymptomatic infection on acute renal allograft rejection. *J Clin Virol*. 2006; 36: 146-151.

Rezzani R, Bonomini F, Rodella LF. Histochemical and molecular overview of the thymus as site for T-cells development. *Prog Histochem Cytochem*. 2008; 43: 73-120.

Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA. Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. *J Am Soc Nephrol*. 2004; 15: 390-397.

Robertson H, Morley AR, Talbot D, Callanan K, Kirby JA. Renal allograft rejection: beta-chemokine involvement in the development of tubulitis. *Transplantation*. 2000; 69: 684-687.

Robey E, Fowlkes BJ. Selective events in T cell development. *Annu Rev Immunol*. 1994; 12: 675-705.

Rocha PN, Plumb TJ, Crowley SD, Coffman TM. Effector mechanisms in transplant rejection. *Immunol Rev*. 2003; 196: 51-64.

Roncador G, Brown PJ, Maestre L, Hue S, Martínez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, Powrie F, Banham AH. Analysis of FOXP3 protein expression in human CD4+ CD25+ regulatory T cells at the single-cell level. *Eur J Immunol*. 2005; 35: 1681-1691.

Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006; 212: 28-50.

Roussey-Kesler G, Giral M, Moreau A, Subra JF, Legendre C, Noël C, Pillebout E, Brouard S, Soulillou JP. Clinical operational tolerance after kidney transplantation. *Am J Transplant*. 2006; 6: 736-746.

Rudner XL, Happel KI, Young EA, Shellito JE. Interleukin-23 (IL-23)-IL-17 cytokine axis in murine *Pneumocystis carinii* infection. *Infect Immun*. 2007; 75: 3055-3061.

Rüegger A, Kuhn M, Lichti H, Loosli HR, Huguenin R, Quiquerez C, von Wartburg A. Cyclosporin A, a Peptide Metabolite from Trichoderma polysporum (Link ex Pers.) Rifai, with a remarkable immunosuppressive activity. *Helv Chim Acta*. 1976; 59 (4): 1075-1092.

Ruggenenti P, Perico N, Gotti E, Cravedi P, D'Agati V, Gagliardini E, Abbate M, Gaspari F, Cattaneo D, Noris M, Casiraghi F, Todeschini M, Cugini D, Conti S, Remuzzi G. Sirolimus versus cyclosporine therapy increases circulating regulatory T cells, but does not protect renal transplant patients given alemtuzumab induction from chronic allograft injury. *Transplantation*. 2007; 84: 956-964.

Rush D. Protocol transplant biopsies: an underutilized tool in kidney transplantation. *Clin J Am Soc Nephrol*. 2006; 1: 138-143.

Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol*. 2002; 20: 323-370.

Russell PS, Chase CM, Colvin RB. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation*. 1997; 64: 1531-1536.

**S**

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995; 155: 1151-1164.

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev*. 2001; 182: 18-32.

Sakaguchi S, Takahashi T, Nishizuka Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med*. 1982; 156: 1577-1586.

Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008; 133: 775-787.

Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004; 22: 531-562.

Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol*. 2005; 6: 345-352.

Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25+ T cells in human kidney transplant recipients. *J Am Soc Nephrol*. 2003; 14: 1643-1651.

Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med*. 1998; 187: 875-883.

Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science*. 1997; 277: 2005-2007.

Sayegh MH, Fine NA, Smith JL, Rennke HG, Milford EL, Tilney NL. Immunologic tolerance to renal allografts after bone marrow transplants from the same donors. *Ann Intern Med*. 1991; 114: 954-955.

Sayegh MH, Turka LA. The role of T-cell costimulatory activation pathways in transplant rejection. *N Engl J Med*. 1998; 338: 1813-1821.

Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem*. 2001; 276: 37672-37679.

Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, de St Groth BF. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006; 203: 1693-1700.

Segall M, Noreen H, Edwins L, Haake R, Shu XO, Kersey J. Lack of correlation of MLC reactivity with acute graft-versus-host disease and mortality in unrelated donor bone marrow transplantation. *Hum Immunol*. 1996; 49: 49-55.

Segerer S, Cui Y, Eitner F, Goodpaster T, Hudkins KL, Mack M, Cartron JP, Colin Y, Schlondorff D, Alpers CE. Expression of chemokines and chemokine receptors during human renal transplant rejection. *Am J Kidney Dis*. 2001; 37: 518-531.

Segundo DS, Ruiz JC, Izquierdo M, Fernández-Fresnedo G, Gómez-Alamillo C, Merino R, Benito MJ, Cacho E, Rodrigo E, Palomar R, López-Hoyos M, Arias M. Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+ CD25+ FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation*. 2006; 82: 550-557.

Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002; 2: 116-126.

Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. *Annu Rev Immunol*. 1993; 11:385-402.

Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells. *Immunol Rev.* 2006; 212: 60-73.

Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+) CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol*. 2002; 3: 135-142.

Shishido S, Asanuma H, Nakai H, Mori Y, Satoh H, Kamimaki I, Hataya H, Ikeda M, Honda M, Hasegawa A. The impact of repeated subclinical acute rejection on the progression of chronic allograft nephropathy. *J Am Soc Nephrol*. 2003; 14: 1046-1052.

Shresta S, Pham CT, Thomas DA, Graubert TA, Ley TJ. How do cytotoxic lymphocytes kill their targets? *Curr Opin Immunol*. 1998; 10: 581-587.

Simon MM, Hausmann M, Tran T, Ebnet K, Tschopp J, ThaHla R, Müllbacher A. *In vitro*- and *ex vivo*-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. *J Exp Med.* 1997; 186: 1781-1786.

Simpson E, Scott D, James E, Lombardi G, Cwynarski K, Dazzi F, Millrain M, Dyson PJ. Minor H antigens: genes and peptides. *Transpl Immunol*. 2002; 10: 115-123.

Simpson E. Minor transplantation antigens: mouse models for human host-versus-graft, graft-versus-host and graft-versus-leukemia reactions. *Transplantation*. 1998; 65: 611-616.

SivaSai KS, Smith MA, Poindexter NJ, Sundaresan SR, Trulock EP, Lynch JP, Cooper JD, Patterson GA, Mohanakumar T. Indirect recognition of donor HLA class I peptides in lung transplant recipients with bronchiolitis obliterans syndrome. *Transplantation*. 1999; 67: 1094-1098.

Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature*. 1993; 363: 156-159.

Smyk-Pearson SK, Bakke AC, Held PK, Wildin RS. Rescue of the autoimmune scurfy mouse by partial bone marrow transplantation or by injection with T-enriched splenocytes. *Clin Exp Immunol*. 2003; 133: 193-199.

Sojka DK, Huang YH, Fowell DJ. Mechanisms of regulatory T-cell suppression – a diverse arsenal for a moving target. *Immunology*. 2008; 124: 13-22.

Sojka DK, Hughson A, Sukiennicki TL, Fowell DJ. Early kinetic window of target T cell susceptibility to CD25+ regulatory T cell activity. *J Immunol*. 2005; 175: 7274-7280.

Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, Croker BP, Droz D, Dunnill MS, Halloran PF, *et al*. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int*. 1993; 44: 411-422.

Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, Cosio F, David DS, Drachenberg C, Einecke G, Fogo AB, Gibson IW, Glotz D, Iskandar SS, Kraus E, Lerut E, Mannon RB, Mihatsch M, Nankivell BJ, Nickeleit V, Papadimitriou JC, Randhawa P, Regele H, Renaudin K, Roberts I, Seron D, Smith RN, Valente M. Banff '07 classification of renal allograft pathology: updates and future directions. *Am J Transplant*. 2008; 8: 753-760.

Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, Drachenberg CB, Gibson IW, Grimm PC, Haas M, Lerut E, Liapis H, Mannon RB, Marcus PB, Mengel M, Mihatsch MJ, Nankivell BJ, Nickeleit V, Papadimitriou JC, Platt JL, Randhawa P, Roberts I, Salinas-Madriga L, Salomon DR, Seron D, Sheaff M, Weening JJ. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant*. 2007; 7: 518-526.

Spriggs MK. Interleukin-17 and its receptor. *J Clin Immunol*. 1997; 17: 366-369.

Starzl TE, Klintmalm GB, Weil R 3rd, Porter KA, Iwatsuki S, Schroter GP, Fernandez-Bueno C, MacHugh N. Cyclosporin A and steroid therapy in sixty-six cadaver kidney recipients. *Surg Gynecol Obstet*. 1981; 153: 486-494.

Stasikowska O, Wagrowska-Danilewicz M. Chemokines and chemokine receptors in glomerulonephritis and renal allograft rejection. *Med Sci Monit*. 2007; 13: RA31-36.

Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. *J Immunol*. 2007; 178: 320-329.

Suvas S, Rouse BT. Treg control of antimicrobial T cell responses. *Curr Opin Immunol*. 2006; 18: 344-348.

Szabo A, Heemann U. Ischemia reperfusion injury and chronic allograft rejection. *Transplant Proc*. 1998; 30: 4281-4284.

Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med*. 1997; 185: 817-824.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000; 100: 655-669.

**T**

Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+) CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol*. 2001; 31: 1122-1131.

Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S. Immunologic self-tolerance maintained by CD25+ CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*. 1998; 10: 1969-1980.

Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. Immunologic self-tolerance maintained by CD25(+) CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. 2000; 192: 303-310.

Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol.* 2008; 9: 239-244.

Tedesco-Silva H, Mourad G, Kahan BD, Boira JG, Weimar W, Mulgaonkar S, Nashan B, Madsen S, Charpentier B, Pellet P, Vanrenterghem Y. FTY720, a novel immunomodulator: efficacy and safety results from the first phase 2A study in de novo renal transplantation. *Transplantation*. 2005; 79: 1553-1560.

Terasaki PI, Ozawa M. Predicting kidney graft failure by HLA antibodies: a prospective trial. *Am J Transplant*. 2004; 4: 438-443.

Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. *Immunity*. 1997; 7: 445-450.

Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+ CD25+ T cell suppressor function. *J Immunol*. 2004; 172: 6519-6523.

Thornton AM, Shevach EM. $CD4^+$ $CD25^+$ immunoregulatory T cells suppress polyclonal T-cell activation *in vitro* by inhibiting interleukin-2 production. *J Exp Med.* 1998; 188: 287-296.

Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. *Kidney Int*. 2005; 68: 1866-1874.

Toso C, Edgar R, Pawlick R, Emamaullee J, Merani S, Dinyari P, Mueller TF, Shapiro AMJ, Anderson CC. Effect of different induction strategies on effector, regulatory and memory lymphocyte sub-populations in clinical islet transplantation. *Transplant Int*. 2008; in print.

Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. A blinded, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. *Transplantation*. 1996; 61: 1029-1037.

**U**

Uboldi de Capei M, Dametto E, Fasano ME, Messina M, Pratico L, Rendine S, Segoloni G, Curtoni ES. Cytokines and chronic rejection: a study in kidney transplant long-term survivors. *Transplantation*. 2004; 77: 548-552.

**V**

Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+ CD25hi T-regulatory cells. *Blood.* 2006; 108: 253-261.

VanBuskirk AM, Burlingham WJ, Jankowska-Gan E, Chin T, Kusaka S, Geissler F, Pelletier RP, Orosz CG. Human allograft acceptance is associated with immune regulation. *J Clin Invest*. 2000; 106: 145-155.

Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, Sayegh MH. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation*. 1997; 64: 795-800.

Verbsky JW. Therapeutic use of T regulatory cells. *Curr Opin Rheumatol*. 2007; 19: 252-258.

Veronese FV, Noronha IL, Manfro RC, Edelweiss MI, Goldberg J, Gonçalves LF. Prevalence and immunohistochemical findings of subclinical kidney allograft rejection and its association with graft outcome. *Clin Transplant*. 2004; 18: 357-364.

Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC, O'Garra A. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+ CD25+ regulatory T cells. *J Immunol*. 2004; 172: 5986-5993.

Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. 2008; 8: 523-532.

Vincenti F, de Andrés A, Becker T, Choukroun G, Cole E, González-Posada JM, Kumar MA, Moore R, Nadalin S, Nashan B, Rostaing L, Saito K, Yoshimura N. Interleukin-2 receptor antagonist induction in modern immunosuppression regimens for renal transplant recipients. *Transpl Int*. 2006; 19: 446-457.

Vincenti F, Friman S, Scheuermann E, Rostaing L, Jenssen T, Campistol JM, Uchida K, Pescovitz MD, Marchetti P, Tuncer M, Citterio F, Wiecek A, Chadban S, El-Shahawy M, Budde K, Goto N; DIRECT (Diabetes Incidence after Renal Transplantation: Neoral C Monitoring Versus Tacrolimus) Investigators. Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. *Am J Transplant*. 2007; 7: 1506-1514.

Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K, Charpentier B; Belatacept Study Group. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med*. 2005; 353: 770-781.

Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science*. 1996; 273: 104-106.

Viola A, Molon B, Contento RL. Chemokines: coded messages for T-cell missions. *Front Biosci*. 2008; 13: 6341-6353.

Von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol*. 2005; 6: 338-344.

Von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med*. 1995; 181: 1519-1526.

**W**

Waldmann H, Adams E, Fairchild P, Cobbold S. Regulation and privilege in transplantation tolerance. *J Clin Immunol*. 2008; 28: 716-725

Waldmann H, Hale G. CAMPATH: from concept to clinic. *Philos Trans R Soc Lond B Biol Sci*. 2005; 360: 1707-1711.

Waldmann TA. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem*. 1989; 58: 875-911.

Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+ CD25- T cells. *J Clin Invest*. 2003; 112: 1437-1443.

Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1994; 1: 405-413.

Wang HY, Wang RF. Regulatory T cells and cancer. *Curr Opin Immunol*. 2007; 19: 217-223.

Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol*. 2007; 37: 129-138.

Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005; 23: 23-68.

Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. 2006; 24: 677-688.

Weimar W, Rischen-Vos J, de Kuiper P, Gregoor PJ, IJzermans JN, van Besouw NM, Baan CC, van der Mast BJ. Tapering immunosuppression in recipients of living donor kidney transplants. *Nephrol Dial Transplant*. 2004; 19 Suppl 4: iv61-63.

Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, Nuñez G, Tang A, Sayegh M, Hancock WW, Strom TB, Turka LA. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*. 1999; 5: 1303-1307.

Whitelegg A, Barber LD. The structural basis of T-cell allorecognition. *Tissue Antigens*. 2004; 63: 101-108.

Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001; 27: 18-20.

Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*. 2007; 8: 277-284.

Williams NS, Engelhard VH. Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. *J Immunol*. 1996; 156: 153-159.

Woodward RS, Kutinova A, Schnitzler MA, Brennan DC. Renal graft survival and calcineurin inhibitor. *Transplantation*. 2005; 80: 629-633.

Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. 2006; 126: 375-387.

**X**

Xu Q, Lee J, Jankowska-Gan E, Schultz J, Roenneburg DA, Haynes LD, Kusaka S, Sollinger HW, Knechtle SJ, VanBuskirk AM, Torrealba JR, Burlingham WJ. Human CD4+ CD25low adaptive T regulatory cells suppress delayed-type hypersensitivity during transplant tolerance. *J Immunol*. 2007; 178: 3983-3995.

Xystrakis E, Urry Z, Hawrylowicz CM. Regulatory T cell therapy as individualized medicine for asthma and allergy. *Curr Opin Allergy Clin Immunol*. 2007; 7: 535-541.

**Y**

Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, Sakaguchi S. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity*. 2007; 27: 145-159.

Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*. 2008; 28: 29-39.

Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. 2006; 116: 1310-1316.

Yong Z, Chang L, Mei YX, Yi L. Role and mechanisms of CD4+ CD25+ regulatory T cells in the induction and maintenance of transplantation tolerance. *Transpl Immunol*. 2007; 17: 120-129.

**Z**

Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS. Inhibition of CD4+ CD25+ regulatory T-cell function by calcineurin dependent interleukin 2 production. *Blood*. 2006; 108: 390-399.

Zelenika D, Adams E, Mellor A, Simpson E, Chandler P, Stockinger B, Waldmann H, Cobbold SP. Rejection of H-Y disparate skin grafts by monospecific CD4+ Th1 and Th2 cells: no requirement for CD8+ T cells or B cells. *J Immunol*. 1998; 161: 1868-1874.

Zeller JC, Panoskaltsis-Mortari A, Murphy WJ, Ruscetti FW, Narula S, Roncarolo MG, Blazar BR. Induction of CD4+ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J Immunol*. 1999; 163: 3684-3691.

Zhang ZX, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med*. 2000; 6: 782-789.

Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4+ CD25+ T cells selectively kill B lymphocytes. *Blood*. 2006; 107: 3925-3932.

Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced $CD4^+$ $CD25^+$ cells educate $CD4^+$ $CD25^-$ cells to develop suppressive activity: the role of IL-2, TGFβ, and IL-10. *J Immunol.* 2004; 172: 5213-5221.

Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood*. 2008; 112: 1157-1569.

# 7. ABBREVIATIONS

**aa** .....amino acid
**ADCC**.....antibody-dependent cell-mediated cytotoxicity
**AICD** .....activation-induced cell death
**AP-1** .....activator protein 1
**APC**.....antigen-presenting cell
**AR** .....acute rejection
**ATGs**.....antithymocyte globulins
**aTreg**....."adaptive" regulatory T cells
**aza** .....azathioprine
**BCR**.....B cell receptor
**BSX** .....basiliximab, or basiliximab study group
**cAMP** .....cyclic adenosine monophosphate
**CAN** .....chronic allograft nephropathy
**CHR** .....chronic humoral rejection
**CMV**.....cytomegalovirus
**CNI(s)**.....calcineurin inhibitor(s)
**CR** .....chronic rejection
**CsA**.....cyclosporine A
**CTL**.....cytotoxic T lymphocyte
**CTLA-4**.....cytotoxic T lymphocyte-associated antigen 4
**CV** .....coefficient of variation
**DC(s)** .....dendritic cell(s)
**DGF**.....delayed-graft function
**DSA(s)**.....donor-specific alloantibody(ies)
**DTH** .....delayed-type hypersensitivity
**EBV**.....Epstein-Barr virus
**ELISA** .....enzyme-linked immunosorbent assay
**ELISPOT**.....enzyme-linked immunospot (assay)
**EMT** .....epithelial-mesenchymal transition
**FKBP12**.....FK506-binding protein 12
**GITR**.....glucocorticoid-induced tumor necrosis factor receptor
**GVHD** .....graft-versus-host disease
**HAR** .....hyperacute rejection
**HCV** .....hepatitis C virus
**HLA** .....human leukocyte antigen
**IFNγ** .....interferon gamma

**Ig** .....immunoglobulin
**IL**.....interleukin
**IPEX**.....immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
**IS** .....immunosuppression
**IVIg**.....Intravenous immune globulins
**LAG-3** .....lymphocyte activation gene-3
**LDA**.....limiting dilution assay
**MFI** .....mean fluorescence intensity
**MHC** .....major histocompatibility complex
**MLR**.....mixed leucocyte reaction (assay)
**MMF** .....mycophenolate mofetil
**MPA**.....mycophenolic acid
**mRNA** .....messenger ribonucleic acid
**mTOR** .....mammalian target of rapamycin
**NFAT** .....nuclear factor for activated T cells
**NF-κB**.....nuclear factor- κB
**NK**.....natural killer (cells)
**Nrp1** .....neuropilin-1
**nTreg**....."natural" regulatory T cells
**PBMC** .....peripheral blood mononuclear cell(s)
**PD-1** .....programmed cell death-1
**pred**.....prednisone
**PTLD** .....post-transplantation lymphoproliferative disease
**TAC**.....tacrolimus
**TCR**.....T cell receptor
**TGFβ**.....transforming growth factor β
**$T_H$** .....helper T lymphocyte
**THYMO**.....thymoglobulin, or thymoglobulin study group
**TNFα**.....tumor necrosis factor alpha
**TNFR** .....tumor necrosis factor receptor
**Treg**.....regulatory T cells

# 8. MATERIALS AND METHODS

Materials and methods for the retrospective as well as for the prospective parts of our study are included in the "Materials and Methods" section of the related publication, and will not be reiterated here.

Additional methods for the retrospective part (*"Homing properties of $CD4^+$ $CD25^{high}$ T cell sub-populations"*) are listed below.

**Flow cytometric assessment of chemokine receptors**

Cryopreserved peripheral blood mononuclear cells from kidney transplant recipients were thawed according to standard procedure. The monoclonal antibodies included CD3 PerCP-Cy5.5 (BD Biosciences), CD4 Alexa Fluor® 700 (BioLegend), CD25 FITC (BD Biosciences), CD45RO ECD (Beckman Coulter), CD127 APC-Alexa Fluor® 750 (eBioscience), CCR1 APC-Alexa Fluor® 647 (BD Biosciences), CCR3 PE (BD Biosciences), CxCR1 PE (BD Biosciences), CxCR2 APC (BD Biosciences), CxCR4 PE-Cy5 (BD Biosciences), CCR9 Alexa Fluor® 647 (BD Biosciences), CxCR5 PE (R&D Systems), CxCR3 APC (BD Biosciences), CCR4 PE-Cy7 (BD Biosciences), CCR5 PE-Cy5 (BD Biosciences), CCR6 PE (BD Biosciences), and CCR7 FITC (R&D Systems). PBMCs were cell surface stained according to the manufacturer's recommendations (30 minutes incubation at 4°C), washed and subsequently fixed.

PBMCs were collected on a LSRII SORP (BD Biosciences), and lymphocytes were gated according to their forward and side scatter characteristics. Flow cytometric analyses were performed using the FACSDiVa software (BD Biosciences).

# 9. APPENDICES

# 9.1. Appendix I

Adapted from: Solez K *et al*. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant*. 2008; 8: 753-760.

**Banff '97 diagnostic categories for renal allograft biopsies – Banff '07 update**

1. **Normal**
2. **Antibody-mediated changes** (may coincide with categories 3, 4 and 5 and 6)
   Due to documentation of circulating antidonor antibody, and C4d or allograft pathology
   *C4d deposition without morphologic evidence of active rejection*
   C4d+, presence of circulating antidonor antibodies, no signs of acute or chronic TCMR or ABMR (i.e. g0, cg0, ptc0, no ptc lamination). Cases with simultaneous borderline changes or ATN are considered as indeterminate
   *Acute antibody-mediated rejection*[1]
   C4d+, presence of circulating antidonor antibodies, morphologic evidence of acute tissue injury, such as (Type/Grade):
   I. ATN-like minimal inflammation
   II. Capillary and or glomerular inflammation (ptc/g >0) and/or thromboses
   III. Arterial—v3
   *Chronic active antibody-mediated rejection*[1]
   C4d+, presence of circulating antidonor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries
3. **Borderline changes:** 'Suspicious' for acute T-cell-mediated rejection (may coincide with categories 2 and 5 and 6)
   This category is used when no intimal arteritis is present, but there are foci of tubulitis (t1, t2 or t3) with minor interstitial infiltration (i0 or i1) or interstitial infiltration (i2, i3) with mild (t1) tubulitis
4. **T-cell-mediated rejection** (TCMR, may coincide with categories 2 and 5 and 6)
   *Acute T-cell-mediated rejection (Type/Grade:)*
   IA. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of moderate tubulitis (t2)
   IB. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of severe tubulitis (t3)
   IIA. Cases with mild-to-moderate intimal arteritis (v1)
   IIB. Cases with severe intimal arteritis comprising >25% of the luminal area (v2)
   III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)
   *Chronic active T-cell-mediated rejection*
   'chronic allograft arteriopathy' (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)
5. **Interstitial fibrosis and tubular atrophy**, no evidence of any specific etiology
   (may include nonspecific vascular and glomerular sclerosis, but severity graded by tubulointerstitial features)
   Grade:
   I. Mild interstitial fibrosis and tubular atrophy (<25% of cortical area)
   II. Moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area)
   III. Severe interstitial fibrosis and tubular atrophy/loss (>50% of cortical area)
6. **Other**: Changes not considered to be due to rejection - acute and/or chronic (may include isolated g, cg or cv lesions and coincide with categories 2, 3, 4 and 5)

[1] Suspicious for antibody-mediated rejection if C4d (in the presence of antibody) or alloantibody (C4d+) not demonstrated in the presence of morphologic evidence of tissue injury.

## 9.2. Appendix II

## *Appendix II: Demographic data of the patients of the retrospective study*

| | All kidney transplant recipients | Stable kidney transplant recipients on standard IS | Kidney transplant recipients with CHR | Stable kidney transplant recipients on no or minimal IS |
|---|---|---|---|---|
| | n=91 | n=60 | n=7 | n=24 |
| *General characteristics* | | | | |
| Recipient gender (males / females) | 59 / 32 | 42 / 18 | 2 / 5 | 15 / 9 |
| Mean recipient age at tx (years) | 44.3 (3 - 69) | 47.1 (12 - 69) | 28.6 (3 - 43) | 42.0 (19 - 63) |
| Donor organ source: | | | | |
| - deceased | 64 (70%) | 37 (62%) | 5 (71%) | 22 (92%) |
| - living-related | 17 (19%) | 15 (25%) | 2 (29%) | 0 |
| - living-unrelated | 10 (11%) | 8 (13%) | 0 | 2 (8%) |
| Retransplants | 6 (7%) | 1 (2%) | 0 | 5 (21%) |
| *Immunologic characteristics* | | | | |
| Gender mismatches between recipient and donor | 48 (53%) | 33 (55%) | 3 (43%) | 12 (50%) |
| HLA mismatches between recipient and donor: | | | | |
| - HLA-A | 1.20 | 1.19 | 1.24 | 1.42 |
| - HLA-B | 1.36 | 1.37 | 1.41 | 1.30 |
| - HLA-DR | 1.29 | 1.26 | 1.39 | 1.36 |
| ***Clinical characteristics*** | | | | |
| Serum creatinine at the time of the study (µmol/l) (mean ± SD) | 136.3 ± 62.1 | 137.3 ± 48.8 | 206.6 ± 139.1 | 111.1 ± 26.9 |
| Immunosuppression at the time of the study: | | | | |
| - CNI (CsA or TAC) | 61 (67%) | 54 (90%) | 7 (100%) | 0 |
| - sirolimus | 6 (7%) | 6 (10%) | 0 | 0 |
| - MMF | 61 (67%) | 46 (77%) | 3 (43%) | 12 (50%) |
| - azathioprine | 14 (15%) | 5 (8%) | 0 | 9 (38%) |
| - prednisone | 47 (52%) | 23 (38%) | 4 (57%) | 20 (83%) |

## Stable kidney transplant recipients (n=60)

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx no. | Tx type | Serum creat. | Immunosuppression |
|---|---|---|---|---|---|---|---|---|
| RN5 | 1940 | 1989 | M | Renal polykystosis | 1 | C | 166 | CsA |
| RN7 | 1951 | 1998 | M | Secondary amyloidosis | 1 | C | 107 | CsA |
| RN11 | 1936 | 1998 | F | Glomerulonephritis | 1 | C | 83 | MMF |
| RN12 | 1954 | 2004 | M | Diabetes | 1 | LUR | 132 | TAC + aza + pred |
| RN13 | 1957 | 1999 | M | Diabetes | 1 | C | 117 | CsA |
| RN14 | 1981 | 2005 | F | Reflux | 1 | LR | 87 | TAC + MMF |
| RN15 | 1958 | 1990 | F | Reflux | 1 | C | 142 | TAC + MMF + pred |
| RN16 | 1939 | 2000 | F | HTA | 1 | LR | 142 | CsA |
| RN17 | 1939 | 1994 | F | Renal polykystosis | 1 | C | 132 | CsA + MMF |
| RN18 | 1945 | 2005 | M | Renal polykystosis | 1 | LUR | 127 | TAC + pred |
| RN19 | 1975 | 1987 | M | Nephronophtisis | 1 | C | 163 | TAC + MMF + pred |
| RN21 | 1986 | 2005 | M | Renal malformation | 1 | LR | 122 | rapa + aza + pred |
| RN22 | 1958 | 2005 | M | Renal polykystosis | 1 | C | 179 | TAC + MMF+ pred |
| RN23 | 1976 | 2003 | M | Glomerulonephritis | 1 | C | 129 | TAC + MMF |
| RN24 | 1943 | 2004 | M | Renal polykystosis | 1 | C | 135 | rapa + MMF |
| RN25 T1 | 1955 | 1987 | M | FSH | 1 | C | 273 | TAC + MMF |
| RN25 T2 | | | | | | | 214 | rapa + MMF |
| RN29 | 1934 | 2003 | M | Glomerulonephritis | 1 | C | 173 | CsA |
| RN33 | 1941 | 2006 | M | Glomerulonephritis | 1 | C | 140 | TAC + MMF + pred |
| RN34 | 1976 | 2003 | M | Glomerulonephritis | 1 | C | 119 | TAC + MMF |
| RN35 | 1953 | 2004 | F | Reflux | 1 | C | 146 | TAC + MMF + pred |
| RN36 | 1965 | 2004 | F | Reflux | 1 | LUR | 115 | TAC + aza + pred |
| RN37 | 1947 | 2004 | F | Glomerulonephritis | 1 | C | 143 | TAC + MMF |
| RN38 | 1937 | 2006 | F | HTA | 1 | LR | 153 | TAC + MMF |
| RN39 | 1988 | 2004 | M | Malformation | 1 | C | 309 | CsA + pred |
| RN40 | 1966 | 2005 | M | Glomerulonephritis | 1 | LR | 169 | TAC + MMF + pred |
| RN41 | 1968 | 2004 | M | Glomerulonephritis | 1 | LR | 144 | TAC + MMF |
| RN42 | 1943 | 2005 | M | FSH | 1 | LR | 161 | TAC + MMF |
| RN43 | 1955 | 2006 | F | Reflux | 1 | LUR | 171 | TAC + MMF + pred |

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx no. | Tx type | Serum creat. | Immunosuppression |
|---|---|---|---|---|---|---|---|---|
| RN44 | 1967 | 2000 | M | Reflux | 3 | C | 152 | rapa + MMF + pred |
| RN45 | 1951 | 2005 | M | Renal polykystosis | 1 | C | 116 | TAC + MMF |
| RN46 | 1947 | 2004 | M | Renal polykystosis | 1 | C | 113 | CsA + MMF + pred |
| RN47 | 1956 | 2005 | F | Diabetes | 1 | LR | 79 | TAC + MMF + pred |
| RN49 | 1940 | 2003 | F | HTA | 1 | C | 113 | TAC + aza |
| RN50 | 1955 | 2005 | M | Chronic pyelonephritis | 1 | LR | 108 | TAC + MMF |
| RN51 | 1961 | 2003 | M | Renal polykystosis | 1 | LUR | 108 | TAC + MMF |
| RN52 | 1951 | 2005 | M | HTA | 1 | LUR | 115 | TAC + MMF + pred |
| RN53 | 1945 | 2005 | M | Glomerulonephritis | 1 | C | 92 | TAC + MMF |
| RN54 | 1946 | 2005 | M | Alport syndrome | 1 | LR | 144 | TAC + MMF |
| RN55 | 1965 | 2006 | F | Reflux | 1 | LR | 106 | TAC + MMF |
| RN56 | 1961 | 2005 | M | Und. | 1 | C | 134 | TAC + MMF |
| RN57 | 1956 | 2006 | M | Diabetes | 1 | C | 130 | TAC + MMF + pred |
| RN59 | 1954 | 2004 | F | Glomerulonephritis | 1 | LR | 88 | TAC + MMF |
| RN60 | 1950 | 2004 | M | Diabetes and HTA | 1 | C | 95 | TAC + MMF |
| RN61 | 1953 | 2003 | M | Renal polykystosis | 1 | C | 94 | TAC + MMF |
| RN62 | 1968 | 2003 | M | FSH | 1 | C | 111 | TAC + MMF |
| RN63 | 1952 | 2005 | M | Und. | 1 | C | 108 | TAC + MMF + pred |
| RN64 | 1970 | 2005 | M | Glomerulonephritis | 1 | C | 341 | CsA + MMF + pred |
| RN65 | 1977 | 2004 | F | Alport syndrome | 1 | C | 103 | TAC + MMF |
| RN66 | 1961 | 2004 | M | Glomerulonephritis | 1 | LUR | 167 | TAC + aza |
| RN67 | 1943 | 2005 | M | Diabetes and HTA | 1 | LR | 112 | TAC + MMF |
| RN68 | 1949 | 2005 | M | Renal polykystosis | 1 | C | 121 | TAC + MMF + pred |
| RN69 | 1950 | 2004 | M | Glomerulonephritis | 1 | C | 108 | TAC + MMF |
| RN70 | 1957 | 2006 | M | Renal polykystosis | 1 | C | 142 | TAC + MMF |
| RN71 | 1947 | 2004 | F | Lupus | 1 | C | 58 | TAC +MMF |
| RN72 | 1954 | 2005 | M | HTA | 1 | LR | 97 | TAC + MMF + pred |
| RN73 | 1952 | 2004 | M | Glomerulonephritis | 1 | LUR | 139 | TAC + MMF |
| RN79 | 1960 | 2004 | M | Chemotherapy toxicity | 1 | C | 124 | TAC + MMF + pred |
| LMC-22.09.49 | 1949 | 2005 | F | HTA | 1 | C | 176 | rapa + pred |
| DJ-18.07.64 | 1964 | 1996 | F | FSH | 1 | LR | 151 | rapa + MMF |

## Kidney transplant recipients with chronic humoral rejection (n=7)

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx no. | Tx type | Serum creat. | Immunosuppression |
|---|---|---|---|---|---|---|---|---|
| RN4 | 1978 | 1997 | F | Glomerulonephritis | 1 | LR | 186 | TAC + pred |
| RN8 | 1966 | 1993 | F | Und. | 1 | C | 533 | TAC |
| RN9 | 1956 | 1999 | M | Glomerulonephritis | 1 | C | 213 | TAC + MMF + pred |
| RN10 | 1949 | 1991 | M | Glomerulonephritis | 1 | C | 115 | CsA |
| RN27 | 1960 | 2002 | F | Und. | 1 | C | 125 | TAC |
| RN80 | 1969 | 1993 | F | Glomerulonephritis | 1 | LR | 180 | TAC + MMF + pred |
| RN90 | 1987 | 1990 | F | Reflux | 1 | C | 94 | TAC + MMF + pred |

## Kidney transplant recipients on no or minimal immunosuppression (n=24)

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx no. | Tx type | Serum creat. | Immunosuppression | Reason for low immunosuppression |
|---|---|---|---|---|---|---|---|---|---|
| RN2 | 1977 | Und. | F | Und. | 1 | C | Und. | no | Stopped IS on her own |
| RN3 | 1946 | 1999 | F | FSH | 1 | C | 87 | pred | PTLD |
| RN26 | 1960 | 1979 | M | Glomerulonephritis | 1 | C | 108 | aza + pred | Long-term IS |
| RN28 | 1954 | 1998 | M | Sarcoidosis | 1 | C | 143 | MMF + pred | CNI nephrotoxicity |
| RN58 | 1963 | 1982 | F | FSH | 1 | C | 98 | aza | Und. |
| RN74 | 1959 | 1993 | F | Glomerulonephritis | 2 | C | 131 | MMF | CNI nephrotoxicity |
| RN76 | 1947 | 2001 | M | Diabetes and HTA | 1 | C | 167 | MMF | Und. |
| RN77 | 1945 | 1995 | M | Renal polykystosis | 1 | LUR | 103 | MMF + pred | CNI nephrotoxicity |
| RN78 | 1954 | 1974 | F | Reflux | 1 | C | Und. | aza + pred | Long-term IS |
| RN82 | 1962 | 1981 | M | Alport syndrome | 1 | C | 100 | aza + pred | Long-term IS |
| RN83 | 1954 | 1979 | M | Glomerulonephritis | 1 | C | 168 | aza + pred | Long-term IS |
| RN84 | 1944 | 1990 | F | HTA | 1 | C | 65 | MMF + pred | TMA |
| RN85 | 1961 | 1998 | M | HTA | 1 | C | 103 | MMF + pred | CNI nephrotoxicity |
| RN86 | 1935 | 1982 | M | Glomerulonephritis | 1 | C | 106 | aza + pred | Long-term IS |
| RN87 | 1949 | 2003 | F | Renal polykystosis | 2 | C | 94 | MMF + pred | Psychotic troubles, ulcers |
| RN88 | 1935 | 1990 | M | Sarcoidosis | 1 | C | 106 | MMF + pred | CNI nephrotoxicity |
| RN89 | 1940 | 1985 | M | Glomerulonephritis | 2 | C | 65 | aza + pred | Long-term IS |

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx no. | Tx type | Serum creat. | Immunosuppression | Reason for low immunosuppression |
|---|---|---|---|---|---|---|---|---|---|
| RN91 | 1952 | 2005 | M | Diabetes and HTA | 1 | C | 115 | MMF + pred | CNI nephrotoxicity |
| RN92 | 1946 | 1985 | M | Glomerulonephritis | 2 | C | 96 | MMF + pred | CNI nephrotoxicity |
| RN93 | 1931 | 1970 | M | Glomerulonephritis | 1 | C | 150 | aza + pred | Long-term IS |
| RN94 | 1951 | 2003 | F | Renal polykystosis | 1 | LUR | 89 | MMF + pred | GI troubles |
| RN95 | 1926 | 1984 | M | Glomerulonephritis | 1 | C | 125 | MMF + pred | Long-term IS |
| RN96 | 1955 | 1985 | F | Glomerulonephritis | 2 | C | 105 | aza + pred | Neurological problems |
| BB-23.12.38 | 1938 | 2001 | M | Chemotherapy toxicity | 1 | C | 120 | pred | Visceral leishmaniasis |

## Liver transplant recipients on no or minimal immunosuppression (n=4)

| Code | Year of birth | Year of tx | Gender | Cause of liver failure | Tx no. | Tx type | Immunosuppression | Reason for low immunosuppression |
|---|---|---|---|---|---|---|---|---|
| G46 | 1987 | 1989 | M | CMV hepatitis | 1 | C | no | Long-term IS |
| CFM-22.12.49 | 1949 | 1988 | F | Primary biliary cirrhosis | 1 | C | pred | PTLD |
| BD-17.05.55 | 1955 | 1997 | M | Neuro-endocrine tumor | 1 | C | CsA[1] + pred | Malabsorption |
| G50 | 1992 | 1992 | M | α1-antitrypsin deficiency | 1 | C | low TAC | Long-term IS |

**Abbreviations:**

| | | | | | |
|---|---|---|---|---|---|
| aza | azathioprine | HTA | hypertension | PTLD | post-transplant lymphoproliferative disease |
| C | cadaveric | IS | immunosuppression | | |
| CNI | calcineurin inhibitor | LR | living-related | rapa | rapamycin (sirolimus) |
| creat | creatinine | LUR | living-unrelated | TAC | tacrolimus |
| CsA | ciclosporin A | M | male | TMA | thrombotic microangiopathy |
| F | female | MMF | mycophenolate mofetil | tx | transplantation |
| FSH | focal and segmental hyalinosis | pred | prednisone | Und. | undetermined |
| GI | gastro-intestinal | | | [1] | undetectable level |

## 9.3. Appendix III

# *Appendix III: Demographic data of the patients of the prospective study*

| | Kidney transplant recipients | |
|---|---|---|
| | **THYMO** | **BSX** |
| | **n=19** | **n=16** |
| ***General characteristics*** | | |
| Recipient gender (males / females) | 13 / 6 | 9 / 7 |
| Mean recipient age at tx (years) | 42.6 (19 - 81) | 49.7 (27 - 78) |
| Donor organ source: | | |
| - cadaveric | 0 | 3 (19%) |
| - living-related | 13 (68%) | 6 (38%) |
| - living-unrelated | 6 (32%) | 7 (44%) |
| ***Immunologic characteristics*** | | |
| Gender mismatches between recipient and donor | 11 (58%) | 8 (50%) |
| HLA mismatches between recipient and donor: | | |
| - HLA-A | 0.84 | 1.29 |
| - HLA-B | 1.00 | 1.36 |
| - HLA-DR | 0.89 | 1.15 |
| ***Clinical characteristics*** | | |
| Serum creatinine at the time of the study (μmol/l) (mean ± SD): | | |
| - before tx | 654.1 ± 297.6 | 544.9 ± 205.7 |
| - at 6 months after tx | 124.6 ± 34.3 | 130.6 ± 40.1 |
| Acute rejection episode at 6 months after tx | 2 (10.5%) | 2 (12.5%) |
| Patient survival at 6 months after tx | 19 (100%) | 16 (100%) |
| Graft survival at 6 months after tx | 19 (100%) | 16 (100%) |

## Kidney transplant recipients with THYMOGLOBULIN induction therapy (n=19)

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx type | Rejection | Serum creat. at 6 months | Immunosuppression at 6 months |
|---|---|---|---|---|---|---|---|---|
| GER-R02 | 1967 | 2005 | M | Renal polykystosis | LR | no | 122 | TAC + MMF |
| GER-R03 | 1971 | 2005 | F | Renal polykystosis | LUR | no | 103 | CsA[3] + MMF |
| GER-R04 | 1982 | 2006 | F | Bartter syndrome | LR | no | 128 | TAC + MMF |
| GER-R05 | 1925 | 2006 | M | Renal polykystosis | LUR | no | 97 | TAC + MMF |
| GER-R06 | 1935 | 2006 | M | IgA nephropathy | LR | no | 131 | TAC + MMF |
| GER-R07 | 1971 | 2006 | F | HTA | LR | no | 144 | [4] MMF + pred |
| GER-R08 | 1954 | 2006 | M | Renal polykystosis | LR | no | 139 | TAC + MMF |
| GER-R09 | 1976 | 2006 | F | Reflux + renal aplasia | LR | no | 123 | TAC + MMF |
| GER-R10 | 1978 | 2006 | M | Reflux | LR | no | 139 | TAC + MMF |
| GER-R11 | 1972 | 2006 | M | Glomerulonephritis | LUR | yes[1] | 134 | TAC + MMF + pred[5] |
| GER-R12 | 1958 | 2007 | M | Glomerulonephritis | LUR | no | 135 | TAC + MMF |
| GER-R13 | 1949 | 2007 | M | Glomerulonephritis | LUR | yes[2] | 117 | TAC + MMF + pred[6] |
| GER-R14 | 1964 | 2007 | M | Glomerulonephritis | LR | no | 235 | TAC + pred[7] |
| GER-R15 | 1970 | 2007 | F | HTA post-eclampsia | LR | no | 75 | TAC + MMF |
| GER-R16 | 1963 | 2007 | M | Und. | LR | no | 118 | TAC + MMF |
| GER-R17 | 1988 | 2007 | M | Und. | LR | no | 104 | TAC + MMF |
| GER-R18 | 1982 | 2008 | F | Und. | LR | no | 74 | TAC + MMF |
| GER-R19 | 1946 | 2008 | M | Und. | LR | no | 97 | TAC + MMF |
| GER-R20 | 1981 | 2008 | M | Renal polykystosis | LUR | no | 150 | TAC + MMF |

[1] Acute cellular rejection, severe, tubulo-interstitial, C4d negative (at day 14)
[2] Acute cellular rejection, severe, diffuse, C4d negative (at day 40)
[3] TAC switched to CsA at 4 months post-tx due to alopecia (related to TAC therapy)
[4] TAC stopped and prednisone reintroduced at 4 months post-tx due to thrombotic microangiopathy
[5] Prednisone reintroduction due to acute cellular rejection at day 14 post-tx
[6] Prednisone reintroduction due to acute cellular rejection at day 40 post-tx
[7] MMF stopped and prednisone reintroduced at 3 months post-tx due to CMV primoinfection/reactivations and chronic sinusitis

## Kidney transplant recipients with BASILIXIMAB induction therapy (n=16)

| Code | Year of birth | Year of tx | Gender | Cause of renal failure | Tx type | Rejection | Serum creat. at 6 months | Immunosuppression at 6 months |
|---|---|---|---|---|---|---|---|---|
| LAR-R02 | 1938 | 2007 | M | Glomerulonephritis | LUR | yes[1] | 154 | TAC + MMF + pred |
| LAR-R03 | 1951 | 2007 | F | Renal polykystosis | C | no | 97 | TAC + MMF + pred |
| LAR-R05 | 1973 | 2007 | F | Glomerulonephritis | LR | no | 111 | TAC + MMF + pred |
| LAR-R07 | 1929 | 2007 | M | HTA | C | no | 111 | TAC + MMF + pred |
| LAR-R08 | 1963 | 2007 | F | Renal polykystosis | LR | no | 151 | TAC + aza[3] + pred |
| LAR-R09 | 1946 | 2007 | M | Diabetes | LR | no | 129 | TAC + MMF + pred |
| LAR-R10 | 1980 | 2007 | M | Nephronophtisis | C | no | 162 | TAC + MMF + pred |
| LAR-R11 | 1954 | 2007 | F | Diabetes | LUR | no | 81 | TAC + MMF + pred |
| LAR-R12 | 1957 | 2007 | F | Glomerulonephritis | LR | yes[2] | 105 | [4] TAC + pred |
| LAR-R13 | 1971 | 2007 | F | Diabetes | LR | no | 120 | TAC + MMF + pred |
| LAR-R14 | 1952 | 2007 | M | Diabetes + HTA | LUR | no | 144 | TAC + MMF + pred |
| LAR-R15 | 1967 | 2007 | M | Reflux | LR | no | 141 | TAC + MMF + pred |
| LAR-R16 | 1947 | 2007 | M | Glomerulonephritis | LUR | no | 150 | [5] CsA + pred |
| LAR-R17 | 1955 | 2007 | M | HTA | LUR | no | 88 | TAC + MMF + pred |
| LAR-R18 | 1973 | 2007 | F | Lupus | LUR | no | 93 | TAC + MMF + pred |
| LAR-R19 | 1943 | 2008 | M | Hyperoxaluria | LUR | no | 252 | CsA + pred |

[1] Acute mild cellular rejection (at day 38)
[2] Acute moderate humoral rejection (at day 7)
[3] MMF switched to aza at 6 months post-tx due to gastro-intestinal intolerance
[4] TAC switched to CsA and MMF stopped at 4.5 months post-tx due to BK virus nephropathy
[5] TAC switched to CsA and MMF stopped at 2 months post-tx due to BK virus nephropathy

**Abbreviations:**

| | | | | | |
|---|---|---|---|---|---|
| C | cadaveric | IS | immunosuppression | pred | prednisone |
| creat. | creatinine | LR | living-related | TAC | tacrolimus |
| CsA | cyclosporine A | LUR | living-unrelated | Tx | transplantation |
| F | female | M | male | Und. | undetermined |
| HTA | hypertension | MMF | mycophenolate mofetil | | |

## 9.4. Appendix IV

In complement to the above-mentioned studies performed essentially in renal transplant recipients, collaborators of our laboratory were also involved in a similar project that studied liver transplant recipients.

In their study, they assessed the frequency of the activated T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ $CD127^{high}$ cells, as in our study on kidney transplant recipients) and of the regulatory T cell sub-population (defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ $FoxP3^+$ $CD127^{low}$ cells) in stable patients, more than one year after orthotopic liver transplantation. They also analyzed the influence of chronic infection by the hepatitis C virus (HCV) on the frequency of these T cell sub-populations, as well as the effect of PEG-interferon and ribavirin antiviral treatment.

In accordance with our results, they found that the frequency of the activated T cell sub-population was increased in liver transplant recipients, as compared to healthy individuals, whilst the frequency of the regulatory T cell sub-population was decreased. Interestingly, in patients with HCV infection, the increase of the activated T cell sub-population was lower than in HCV non-infected liver transplant recipients, and regulatory T cells were less decreased. Moreover, successful anti-HCV treatment induced a significant increase in the percentage of the activated T cell sub-population in responders, whilst after unsuccessful treatment this percentage remained unmodified in non-responders.

These results show that the distribution of $CD4^+$ $CD25^{high}$ T cell subsets was also modified after orthotopic liver transplantation, confirming our results obtained after kidney transplantation. In addition, HCV infection seems to exert an immunomodulatory effect on T cell subsets that have been demonstrated to play a crucial role in the outcome of transplantation; again, this observation could modify the therapeutic approach of liver transplant recipients, depending on their HCV status.

My contribution to this study was to perform some of the experiments and to collect some of the clinical data concerning the liver transplant recipients.

The results of this study were recently submitted as a manuscript to the *American Journal of Transplantation*.

## HCV INFECTION AFTER LIVER TRANSPLANTATION IS ASSOCIATED WITH LOWER LEVELS OF ALLOREACTIVE $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T CELLS

D Ciuffreda[1,2], L Codarri[1], L Buhler[3], L Vallotton[1], E Giostra[3], G Mentha[3], P Morel[3], G Pantaleo[1], M Pascual[2].

1 Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland
2 Center of Transplantation, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Centre Universitaire Romand de Transplantation, Switzerland
3 Division of Visceral and Transplantation Surgery, University Hospital Geneva (HUG), Geneva, Centre Universitaire Romand de Transplantation, Switzerland

Correspondence to:
Manuel Pascual, Center of Transplantation (CHUV), Rue de Bugnon
1011 Lausanne,Switzerland
Telephone: +41 21 314 24 04
Fax +41 21 314 23 76
Email: Manuel.Pascual@chuv.ch

Running title: HCV as immunomodulant in liver transplant recipients

**ACKNOWLEDGMENTS**
We are grateful to all the patients who accepted to participate to the study.

## ABSTRACT

Expression of IL-7Rα discriminates between activated $CD25^{+}CD45RO^{+}CD4^{+}$ T cells (IL-7Rα high and FoxP3 negative) and regulatory T cells (IL-7Rα low and FoxP3 positive). The $IL\text{-}7R\alpha^{high}CD25^{+}CD45RO^{+}CD4^{+}FoxP3^{-}$ T-cell population has been shown to be expanded in blood and tissue in patients after kidney transplantation and to contain alloreactive T-cells.

In the present study, we have analysed the distribution of the $IL\text{-}7R\alpha^{high}CD25^{+}CD45RO^{+}CD4^{+}FoxP3^{-}$ T-cells in blood of 53 patients after liver transplantation. The $IL\text{-}7R\alpha^{high}CD25^{+}CD45RO^{+}CD4^{+}FoxP3^{-}$ T-cell population was significantly expanded ($P<0.0001$) in stable transplant recipients as compared to healthy donors. However, the magnitude of the expansion was significantly higher ($P<0.0001$) in liver transplant recipients with no HCV infection as compared to those with pre-existing HCV infection. Of interest, effective suppression of HCV viremia after antiviral therapy was associated with an increase of the $IL\text{-}7R\alpha^{high}CD25^{+}CD45RO^{+}CD4^{+}FoxP3^{-}$ T cell population to levels comparable to HCV non-infected liver transplant recipients.

The present results indicate that: a) the $IL\text{-}7R\alpha^{high}CD25^{+}CD45RO^{+}CD4^{+}FoxP3^{-}$ T-cell population is expanded after liver transplantation, b) it is a valuable immunologic marker to monitor activated and potential alloreactive CD4 T-cells in liver transplantation and c) pre-existing HCV infection negatively influences the expansion of this population in liver transplant recipients.

## INTRODUCTION

End stage liver disease related to hepatitis C virus infection (HCV) is currently the leading indication for orthotopic liver transplantation (OLT) (1). Recurrence of HCV infection is almost universal after liver transplantation (2) leading to cirrhosis in approximately 20% of patients within 5 years or in 50% of patients within 10 years after transplantation (3). Immunosuppressive therapy is likely responsible for a more severe and accelerated course of HCV hepatitis post-transplantation as compared to the non-transplanted setting (3, 4). In particular high doses of steroids, multi-drug combinations, powerful induction treatments and treatment of acute rejection can lead to worse outcomes (5).Whether HCV-infected liver transplant recipients have a different risk for liver allograft rejection, compared to non-HCV infected recipients remains unclear. Rosen et al. prognosed that HCV re-infection of the liver allograft can precipitate an anti-donor response as suggested by the identification of HCV-specific cytotoxic T lymphocytes (CTLs) restricted by donor alleles (6). Other some studies have however shown no difference in the incidence of acute cellular rejection in HCV-infected liver recipients (7). Recently, it has been shown that patients with chronic HCV infection have a down-regulated expression of the IL-7Rα on CD4+ and CD8+ T lymphocytes when compared to individuals who have cleared HCV infection (8). IL-7 receptor is expressed differently in different stages of T-cell differentiation. It is expressed on cells that have not differentiated into effectors (9, 10). Furthermore, recent data have indicated that the IL-7R expression is lower on CD8+ T cells specific in viral chronic infections (11). The previous reported evidence that downregulation of IL-7R is associated with HCV viral persistence provides new insights which stimulate the study of HCV as a virus potentially influencing activated and/or regulatory T cells.

IL-7Rα (CD127) is a surface marker able to discriminate between two distinct CD4+ T cells populations: CD4+ T regulatory cells (Tregs) and CD4+ activated T cells. Tregs express low levels of IL-7Rα, whereas activated CD4+ T cells have been shown to express high levels (12,13). Recent data indicate that a subset of CD4+CD25+ T cells (Foxp3 negative) expressing CD45RO and IL-7R$\alpha^{high}$ is significantly increased in solid organ transplant recipients as compared to healthy subjects, particularly in patients with documented chronic allograft humoral rejection. This T cell population (CD4+CD25+CD45RO+IL-7R$\alpha^{high}$) appears to be donor-specific and it increases in the circulation within a few weeks after transplantation (14).

Analysis of these CD4+ T cell subsets has not been reported in HCV-infected liver transplant recipients. We hypothesized that chronic HCV infection may downregulate expression of IL-7Rα after liver transplantation, thus possibly decreasing the frequency of CD4+CD25+CD45RO+IL-7R$\alpha^{high}$ T cells. In this perspective, we analyzed CD4+ T cell subsets in the blood of stable liver transplant recipients, either infected or non-infected by HCV.

## PATIENTS AND METHODS

### Patients

A total of 53 liver transplant recipients, 29 patients with pre-existing HCV-infection (end-stage liver disease, ESLD, was HCV-related) and 24 with no HCV-infection (their etiologies for ESLD were alcoholic cirrhosis (n=16), autoimmune hepatitis (n=2), sclerosing cholangitis (n=2), primary biliary cirrhosis (n=1), haemangioma (n=1), HBV related ESLD (n=1) and alpha1 antitripsine deficiency (n=1)) were studied at the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne and at the University Hospital Geneva (HUG)

As control groups, 38 healthy subjects and 53 non-transplanted HCV-chronically infected patients were studied.

All patients gave informed written consent to participate to the study which had been approved by the local Institutional Review Boards (IRB).

All patients had received liver transplants from deceased donors and they were studied at more than one year after transplantation.

### FACS analysis

Peripheral blood mononuclear cells were isolated using standard Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) gradients centrifugation. The antibodies used for flow cytometric analyses included PerCP, PerCP-Cy5.5 or PE conjugated mouse anti-human CD4 (Becton Dickinson, Franklin, NJ), APC-conjugated mouse anti-human CD25 (BD Pharmingen, San Diego, CA), FITC (BD Pharmingen, San Diego, CA) or ECD (Immunotech, Beckman-Coulter, Marseille, France) conjugated mouse anti-human CD45RO, and PE (Immunotech, Beckman-Coulter, Marseille, France) or APC (R&D Systems, Minneapolis, MN) conjugated mouse anti-human IL-7Rα. For intracellular FoxP3 analysis, cell preparations were fixed and permeabilized with fixation/permeabilization buffers (eBioscience, San Diego, CA) after staining of cell surface markers, and stained with FITC-conjugated rat anti-human FoxP3 (eBioscience, San Diego, CA). All flow cytometric analyses were performed on a FACSCalibur and LSRII (Becton Dickinson Systems, Franklin, NJ).

### Statistical analysis

Statistical significance was calculated by two tailed t student test. P values $<0.05$ were considered significant.

## RESULTS

### Baseline patient characteristics and post-transplant course

The 29 HCV-infected liver recipients (22 males and 7 females) had a median age at time of the study of 54 years (range 41-70 years) and a medium time after transplantation of 5.5 years (range 1-14 years). The 24 non-HCV-infected liver recipients (17 males and 7 females) had a median age at time of the study of 52 years (range 20-70 years) and a medium time after transplantation of 6.6 years (range 1-13 years). Immunosuppressive therapy consisted of cyclosporine or tacrolimus (FK) alone (n=15 in HCV-infected, n=8 in non-HCV- infected) combined with prednisone (n=4 in HCV-infected, n=3 in non-HCV-infected), or with mycophenolate mofetil (MMF) (n=8 in HCV-infected, n=8 in non-HCV-infected) and combined with both (n=2 in HCV-infected, n=5 in non-HCV-infected).

The diagnosis of acute rejection (AR, according to the biopsy) within the first year post transplant was made in 8 out 29 HCV-infected patients and in 12 out 24 non HCV-infected patients (p=0.057).
The 38 healthy subjects (17 males and 21 females) had a median age at time of the study of 50 years (20-74) and the 53 non-transplanted HCV-chronically infected patients (32 males, 21 females) had a median age at time of the study of 40 years (28-59).

During the study, 10 HCV-infected liver transplant recipients were treated with a lower dose combined antiviral therapy of pegylated interferon alpha 2a (PEG-IFN-alpha2a, Pegasys, Hoffmann La- Roche; Switzerland) 135 mg/week, together with ribavirin (RBV, Copegus, Hoffmann La- Roche; Switzerland) 200-400 mg/day, as previously described (15). The antiviral therapy was planned for 12 months depending on genotype (i.e. 6 months in patients with genotype 2 and 3) and tolerability. Reasons for treatment discontinuation were: bacterial pneumonia, persistent bronchitis, cerebral hemorrhagic stroke and retinal haemorrhage. Six out 10 patients achieved a sustained viral response with serum HCV-RNA levels not detectable at 6 months after treatment interruption. During antiviral therapy patients were studied for CD4 T cell subpopulations profile at 4 weeks, 3, 6 and 12 months of therapy and 6 months after treatment discontinuation. Sustained viral response to antiviral therapy was defined by suppression of HCV viremia below the limit of detection of PCR HCV RNA assay at 6 months after treatment interruption.

**Analysis of $CD4^+CD25^+$ T cells**

The distribution of $CD4^+CD25^+$ T cells was analysed in blood mononuclear cells of 53 liver transplant recipients (24 HCV negative and 29 HCV-chronically infected) and of 90 non-transplanted subjects (52 HCV-chronically infected patients and 38 healthy donors).

In non-transplanted HCV-infected subjects the percentage of $CD4^+CD25^+$ T cells was not significantly different from healthy subjects (2.5+/-0.3% versus 2.2+/-0.1%, $p>0.05$) (**Fig. 1A**).

However, the percentage of $CD4^+CD25^+$ T cells was significantly lower ($P<0.05$) in the total cohort (n=53) of liver transplant recipients (**Fig 1A**).

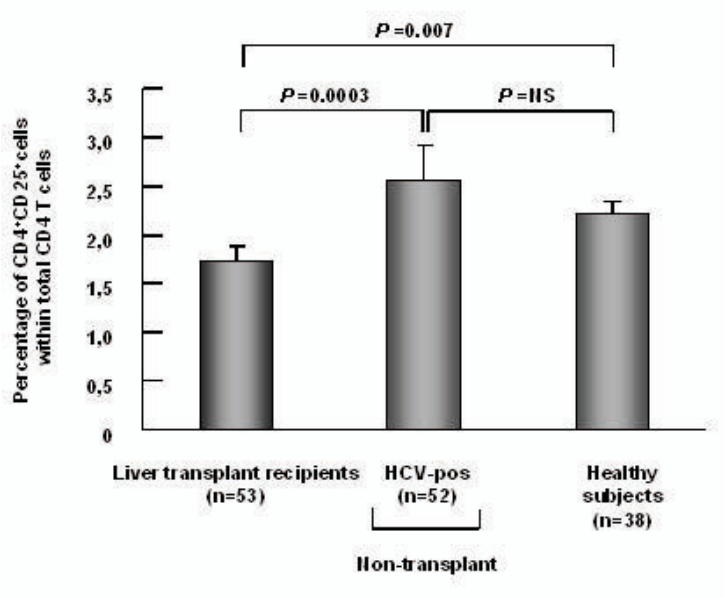



**Figure 1A**

**Expression of $CD4^+CD25^+$ T cells.** The distribution of $CD4^+CD25^+$ T cells was analysed in blood mononuclear cells of 53 liver transplant recipients, 52 non-transplanted HCV-infected subjects and 38 healthy subjects. In non-transplanted HCV-infected subjects, the percentage of $CD4^+CD25^+$ T cells was not significantly different from healthy subjects. The percentage of $CD4^+CD25^+$ T cells was significantly lower in the total cohort of transplant recipients compared both to non-transplanted HCV-infected subjects and to healthy subjects.

We then analysed the proportion of $CD4^+CD25^+$ T cells in the liver transplant recipients stratified on the basis of pre-existing HCV infection. $CD4^+CD25^+$ T cells were significantly lower ($p<0.0005$) in HCV-negative liver transplant recipients (1.5+/-0.1%) compared to healthy subjects (2.2+/-0.1%), while the percentage of $CD4^+CD25^+$ T cells was not significantly different ($p>0.05$) in HCV-infected liver transplant recipients (1.9+/-0.2%) and healthy subjects (2.2+/-0.1%).

In HCV-infected liver transplant recipients the percentage of $CD4^+CD25^+$T cells (1.9+/-0.2%) was significantly lower ($p<0.05$) compared to non-transplanted HCV-infected (2.5+/-0.3%) (**Fig 1B**).

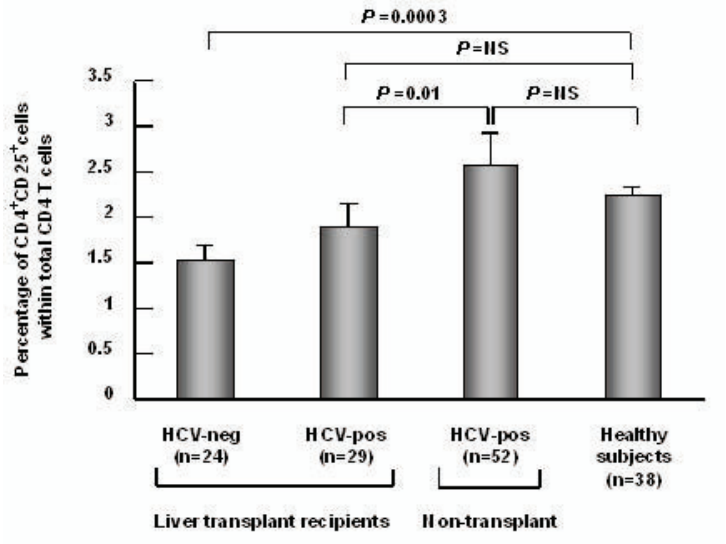



**Figure 1B**

**Expression of $CD4^+CD25^+$ T cells in liver transplant recipients stratified on the basis of the presence of HCV infection.** The distribution of $CD4^+CD25^+$ T cells was not statistically different between HCV-infected transplant recipients and HCV-negative transplant recipients. $CD4^+CD25^+$ T cells were significantly lower in HCV-negative liver transplant recipients compared to healthy subjects but not significantly different in HCV-infected transplant recipients and healthy sujects. In HCV-infected liver transplant recipients the percentage of $CD4^+CD25^+$ T cells was significantly lower compared to non-transplanted HCV-infected.

## $CD4^+CD25^+CD45RO^+FoxP3^+IL\text{-}7R\alpha^{low}$ T cells, i.e. T regulatory cells (Tregs) in liver transplant subjects

Since $CD4^+CD25^+$ T cell population is heterogeneous we analysed allospecific and T regulatory cells based on the expression of IL-7Rα and FoxP3.

In healthy subjects, a majority of $CD4^+CD25^+$ T cells (79.3+/-2.3%) were $FoxP3^+$ and IL-$7R\alpha^{low}$ i.e., Tregs. In HCV-infected non-transplanted patients the proportion of Tregs was not significantly different than in healthy donors (**Fig 2**). In HCV-negative liver transplant recipients, the population of Tregs was significantly decreased (59.3+/-2.9%) compared to healthy subjects and non-transplanted HCV-infected patients ($P<0.0005$) (**Fig 2**). In HCV-infected liver transplant recipients, the population of Tregs was significantly lower compared to healthy subjects ($p<0.05$) and non-transplanted HCV-infected patients ($p<0.005$) while was significantly increased compared to HCV-negative liver transplant recipients ($p=0.009$) (**Fig 2**).

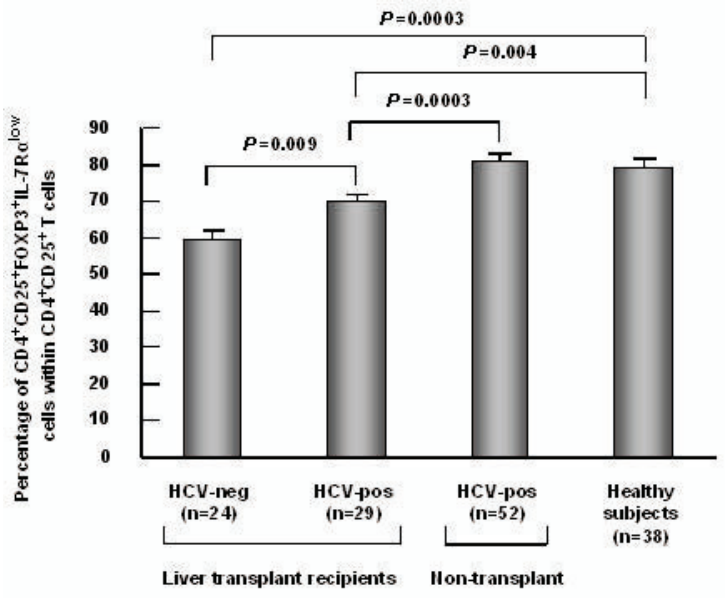



**Figure 2**

**Expression of CD25, CD45RO, FoxP3 and IL-7Rα$^{low}$ on CD4 T cells, i.e. T regulatory cells, in liver transplant recipients, non-transplanted HCV-infected subjects and healthy subjects.**

In healthy subjects, majority of CD4$^+$CD25$^+$ T cells were FoxP3$^+$ and IL-7Rα$^{low}$ i.e. T regulatory cells. In HCV-infected non-transplanted patients the proportion of T regs was not significantly different than in healthy subjects. In HCV-negative liver transplant recipients the population of T regs was significantly decreased compared to healthy subjects and non-transplanted HCV-infected patients. In HCV-infected transplant recipients, the population of Tregs was significantly lower compared to healthy subjects and non-transplanted HCV-infected patients, while it was significantly increased compared to HCV-negative liver transplant recipients.

## Expansion of activated CD4$^+$CD25$^+$CD45RO$^+$FoxP3$^-$IL-7Rα$^{high}$ T cells in liver transplant recipients

Expression of CD45RO and IL-7Rα$^{high}$ on CD4$^+$CD25$^+$FoxP3$^-$ T-cells defines the population of activated alloreactive CD4 T-cells as previously described (14). This CD4 T-cell population ranged about 6.1+/-0.7% in healthy subjects (**Fig 3A**). In non-transplanted HCV-infected patients, the population of activated T cells was not different from healthy subjects (6.6+/-0.9%, p=0.26) (**Fig 3A**). However, this CD4 T-cell population was significantly increased in transplant recipients, both in HCV-negative or HCV-infected recipients (17.6+/-1.4% and 10.5+/-0.9%, respectively, p<0.001), as compared to healthy subjects or non-transplanted HCV-infected patients (**Fig 3A**). Of interest, this activated CD4 T-cell population was significantly lower in HCV-infected compared to HCV negative recipients (P<0.0001) (**Fig 3A**).

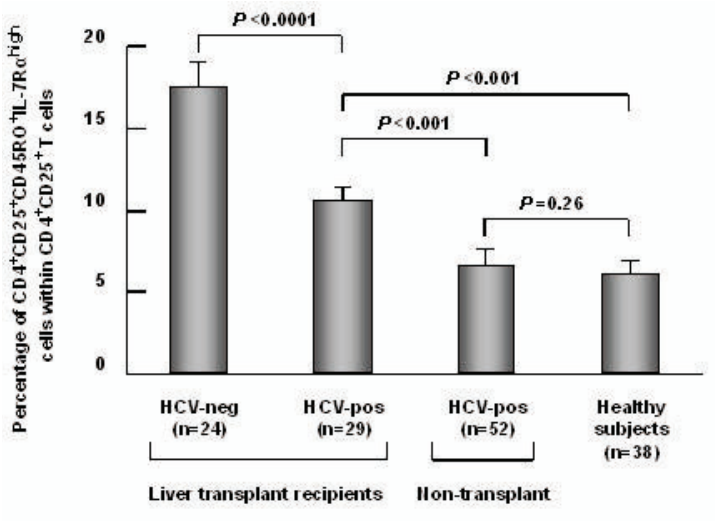



**Figure 3A**

**Expression of CD25, CD45RO, and IL-7Rα$^{high}$ on CD4 T cells, i.e., activated T cells, in liver transplant recipients, non-transplanted HCV-infected subjects and healthy subjects.**

In non-transplanted HCV-infected patients, the percentage of activated CD4 T cells, defined by the expression of CD25, CD45RO, and IL-7Rα$^{high}$, was not different compared to healthy subjects; in contrast, this population was significantly increased in transplant recipients both HCV-negative and HCV-infected recipients compared to healthy subjects and non-transplanted HCV-infected patients. The population of activated CD4 T cells was significantly lower in HCV-infected transplant recipients, compared to HCV-negative recipients.

A representative flow cytometry profile of the CD4$^+$CD25$^+$CD45RO$^+$FoxP3$^-$IL-7Rα$^{high}$ activated T-cell population in HCV-negative and HCV-infected liver transplant recipient is shown in **Figure 3B**.

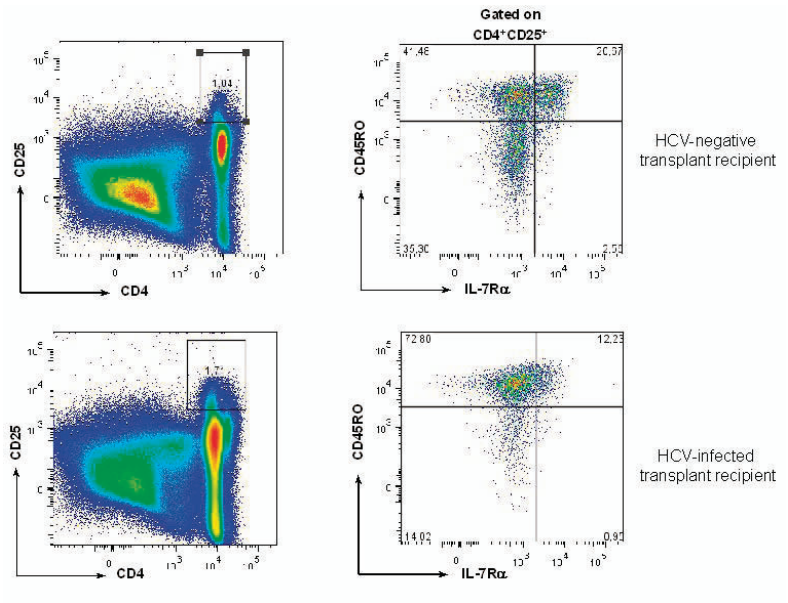



**Figure 3B.**

Representative flow cytometry profile of CD4$^+$CD25$^+$CD45RO$^+$IL-7Rα$^{high}$ activated T cell population in HCV-negative and HCV-infected transplant recipients.

**Increase of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T-cell population in HCV-infected transplant recipients after antiviral therapy**

To further analyze the impact of HCV infection on the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cell population, we analysed potential changes in this population in HCV-infected transplant recipients before and after antiviral therapy with PEG-IFN and ribavirin. In patients who responded to antiviral therapy, as defined by sustained viral suppression of HCV viremia below the limit of detection of the PCR HCV RNA assay, the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T-cell population increased from 10.73+/-2.63% (prior therapy) to 21.7+/-6.3% (after suppression of HCV viremia) ($p<0.05$), to levels similar to those measured in HCV negative liver transplant recipients. In HCV-infected non-responder to antiviral therapy recipients, $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T-cells remained unchanged prior and after therapy (11.8+/-3.3% and 11.3+/-3.3%, respectively) (**Fig. 4A and 4B**).

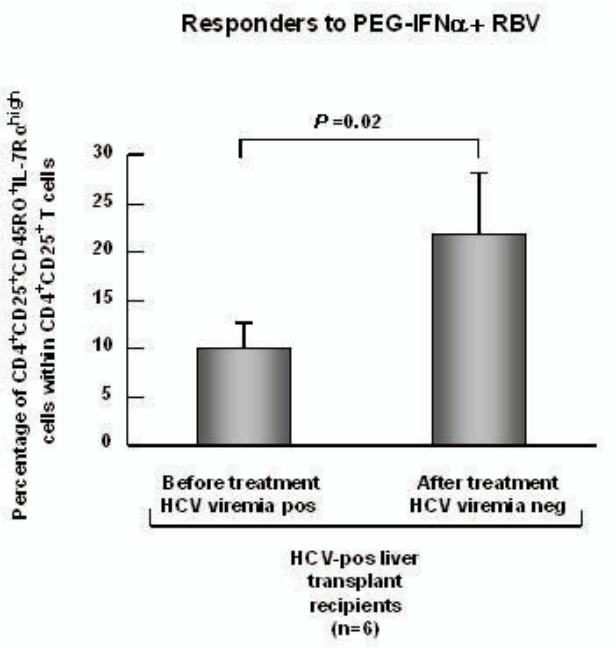



**Figure 4A.**

We analysed the changes of $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cell population in six HCV-infected transplant recipients who responded to treatment by PEG-IFN and ribavirin and showed viral clearance. The figure shows the changes before and after therapy when HCV viremia was negative. After viral clearance, the $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ T cell population significantly increased.

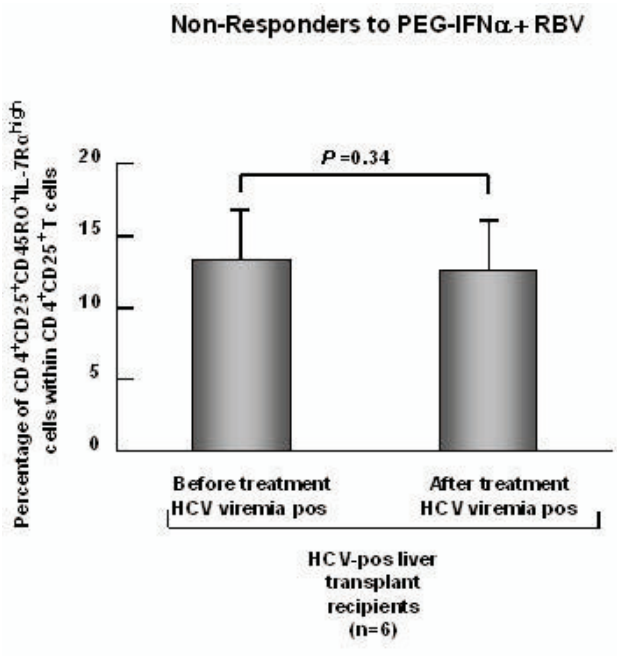



**Figure 4B.**
In six HCV-infected transplant recipients who did not respond to antiviral therapy and showed persistent HCV-viremia, the $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{high}$ T cell population did not change over time, neither during nor after antiviral therapy.

## DISCUSSION

In this study, we analyzed the expression of a CD4+ alloreactive T cell population, phenotypically defined by expression of CD25, CD45RO and high levels of IL-7Rα, in stable liver transplant recipients more than one year after transplantation. Both HCV-infected and HCV negative liver transplant recipients were studied and compared to non-transplanted HCV-infected patients and to healthy subjects.

First, we analysed overall $CD4^{+}CD25^{high}$ T cells and the results indicated a trend towards higher level of these T cells in non-transplanted HCV-infected patients, but the difference did not achieve statistical significance compared to healthy subjects. In contrast, we found a significant decrease of these cells in HCV negative transplant recipients compared to healthy subjects. This decrease in transplant recipients is most likely due to the immunosuppressive therapy. We observed no difference between HCV-infected and negative liver transplant recipients.
To better define the type of cells included in the $CD4^{+}CD25^{+}$ T cell population, we analysed the expression of IL-7Rα (absence/low expression) and FoxP3 (presence) as markers of regulatory T cells. In healthy subjects, the majority of $CD4^{+}CD25^{+}$ T cells were IL-7Rα low (i.e., regulatory T cells) as previously described (12,13). In contrast, these cells were

decreased in liver transplant recipients, but there was a significant difference between HCV-infected and non-infected transplant recipients, with higher levels of T regs in HCV-infected transplant recipients. This result in HCV-infected liver transplant recipients was in accordance with the observed increase of T regs in non-transplanted HCV-infected patients, as compared to healthy individuals (16,17). The analysis of IL-7Rα expression allows identification of regulatory T cells (i.e., IL7-Rα low and FoxP3 positive) versus activated CD4 T cells (i.e., IL-7Rα high and FoxP3 negative). We measured an important increase of activated $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{+}$ T cells in liver transplant recipients compared to healthy subjects. Interestingly, this increase of activated T cells in transplant recipient group was significantly lower in HCV-infected recipients when compared to HCV negative recipients. As such the presence of HCV infection in liver transplant recipients resulted in a significant increase of regulatory T cells and a decrease of alloreactive T cells, when compared to HCV-non infected liver transplant recipients.

These two findings indicated a potentially important effect of HCV on the immune system after transplantation by increasing the suppressive role of T regs and or decreasing alloreactive T cells. To further study the impact of HCV infection after liver transplantation we analysed the levels of $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{high}$ T cells before and after antiviral therapy both in responders (i.e., who became HCV-RNA negative) and in non-responders (i.e., HCV-RNA persistently positive despite antiviral therapy). All recipients were treated according to a protocol previously described with PEG-IFN and ribavirin (15).

In responders, we observed a significant increase of $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{high}$ T cells when compared to non-responders, in whom levels of $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{high}$ T cells remained unmodified. The observed changes in levels of $CD4^{+}CD25^{+}CD45RO^{+}IL\text{-}7R\alpha^{high}$ T cells were associated only with viral clearance and not to decrease of HCV-RNA levels. We observed an increase of the T cell population only after the virus was cleared and not after starting antiviral therapy.

Overall, these results further suggest that HCV infection has some immune modulation properties. This immune modulation may have importance in the transplant setting and it is possible that stable HCV-infected transplant recipients greater than one year post-transplantation, may be at lower risk for graft rejection compared to HCV negative recipients. This may have implications for the long term administration of IS therapy.

Recipients with low levels of activated T cells might represent a group of patients who need lower levels of immunosuppressive therapy. If confirmed, our results may also be relevant for the design of “tolerogenic” or minimisation protocols, as HCV-infected may actually be ideal candidates for such strategies. Furthermore lower levels of immunosuppressive therapy for

HCV-infected transplant recipients could be positive for a less severe and a better outcome of HCV hepatitis post-transplantation. Finally, the precise mechanism by which HCV might interfere with the allospecific immune response is not understood and it remains to be further investigated. Future studies investigating the immune status of liver transplant recipients should take into account the presence or not of chronic HCV infection, as this may alter the interpretation or the conclusion of these studies.

## REFERENCES


(1) Berenguer M, Lopez-Labrador FX, Wright TL. Hepatitis C and liver transplantation. J Hepatol. 2001; 35: 666-678.

(2) Wright T, Donegan E, Hsu H, Ferrel L, Lake JR, Kim M et al. Recurrent and acquired hepatitis C viral infection in liver transplant recipients. Gastroenterology. 1992; 103: 317-322

(3) Berenguer M, Prieto M, Rayon JM, Mora J, Pastor M; Ortiz V et al. Natural history of clinically compensated hepatitis C virus-related graft cirrhosis after liver transplantation. Hepatology. 2000; 32: 852-858.

(4) Berenguer M, Ferrell L, Watson J, Prieto M, Kim M, Rayon M, et al. HCV-related cirrhosis progression following liver transplantation: increase in recent years. J Hepatol. 2000; 32: 673-684.

(5) Charlton M, Seaberg E. Impact of immunosuppression and acute rejection on recurrence of hepatitis C: results of the National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. Liver Transpl Surg. 1999; 5: S107-S114.

(6) Rosen HR, Hinrichs DJ, Leistikow RL, Callender G, Wertheimer AM, Nishimura MI, Lewinson DM. Cutting edge: identification of HCV –specific CD8 T cells restricted by donor HLA alleles following liver transplantation. J Immunology. 2004;173: 5355-5359.

(7) Casavilla FA, Rakela J, Kapur S, Irish W, McMichael J, Demetris AJ et al. Clinical outcome of patients infected with hepatitis C virus infection on survival after primary liver transplantation under tacrolimus. Liver Transpl Surg. 1998; 4: 448-454.

(8) Golden-Mason L, Burton JR, Castelblanco N, Klarquist J, Benloch S, Wang C, Rosen HR. Loss of IL-7 receptor alpha chain (CD127) expression in acute HCV infection associated with viral persistence. Hepatology. 2006; 44: 1098-1109.

(9) Prlic M, Lefrançois L, Jameson SC. Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. J Exp Med. 2002; 195: F49-F52.

(10) Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol. 2002; 2: 251-262.

(11) Van Leeuwen EM, de Bree GJ, Remmerswaal EB, Yonq SL, Tesselaar K, ten Berqe IJ, van Lier RA. IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human $CD8^+$ T cells. Blood. 2005; 106: 2091-2098.

(12) Seddiki N, Santaner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression on interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006; 203: 1693-1700.

(13) Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, et al. CD127 expression inversely correlates with Foxp3 and suppressive function of human $CD4^+$ Treg cells. J Exp Med. 2006; 203: 1701-1711.

(14) Codarri L, Vallotton L, Ciuffreda D, Venetz JP, Garcia M, Hadaya K et al. Expansion and tissue infiltration of an allospecific $CD4^+CD25^+CD45RO^+IL\text{-}7R\alpha^{high}$ cell population in solid organ transplant recipients. J Exp Med. 2007; 204: 1533-1541.

(15) Fontana M, Ciuffreda D, Moradpour D, Pascual M. Combined antiviral therapy for recurrent hepatitis C virus after liver transplantation.Transplantation. 2007; 83: 525-526.

(16) Cabrera R, Tu Z, Xu Y, Firpi RB, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for $CD4^+CD25^+$ regulatory T lymphocytes in hepatitis C virus infection. Hepatology. 2004; 40: 1062-1071.

(17) Sugimoto K, Ikeda, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated *ex vivo* in persistent HCV infection. Hepatology. 2003; 38: 1437-1448.