
# PHENOTYPIC AND TRANSCRIPTIONAL BIOMARKERS IN ORGAN TRANSPLANTATION

Thesis presented by

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to obtain the degree of Doctor

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Als meus pares
Als meus germans
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# TABLE OF CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| ALG | Anti-lymphocyte globulin |
| ATG | Anti-thymocyte globulin |
| ATP | Adenosine-triphosphate |
| AZA | Azathioprine |
| BMT | Bone marrow transplantation |
| CDR | Complementary determining region |
| CMV | Cytomegalovirus |
| CNI | Calcineurin inhibitor |
| CsA | Cyclosporin A |
| CTL | Cytotoxic T lymphocyte |
| DTH | Delayed-type hypersensibility |
| ELSIPOT | Enzyme-linked immunosorbent spots |
| FK506 | Tacrolimus |
| GVHD | Graft-versus-host-disease |
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antibody |
| HMBP | Hydroxymethylbutyl pyrophosphate |
| HSV | Herpes simplex virus |
| IFN | Interferon |
| Ig | Immunoglobulin |
| ILT3 | Immunoglobulin-like transcript 3 |
| IPP | Isopentenyl pyrophosphate |

| | |
|---|---|
| IS | Immunosuppressive treatment |
| KIM-1 | Kidney injury molecule-1 |
| KIR | Killer-cell immunoglobulin-like receptor |
| KT | Kidney transplantation |
| LT | Liver transplantation |
| mAb | Monoclonal antibody |
| MHC | Major histocompatibility complex |
| MICA | MHC class-I chain-related A |
| MPA | Mycophofenolic acid |
| mTOR | Mammalian target of rapamycin |
| NAG | N-acetyl-beta-D-glucosaminidase |
| NGAL | Neutrophil gelatinase-associated lipocalin |
| OKT3 | Muromonab-CD3 |
| PBC | Primary billiar cirrhosis |
| PHA | Phytohaemagglutinin |
| STAT | Signal transducers and activators of transcription |
| TCR | T-cell receptor |
| TGF | Transforming growth factor |
| TNF | Tumour necrosis factor |

# I. INTRODUCTION

# 1. Organ transplantation and immunosuppression

Organ transplantation constitutes the treatment of choice to prolong life by replacement of damaged or non-functional organs. Tissue engraftment was a distant challenge in the seventies, but currently is a routinary procedure in the medical practice that has contributed to extend survival and quality of life within the general population.

The essence of the surgical technique has not changed dramatically, however efforts have been tried and tested to optimize the outcome. Advances in the understanding of the overall transplant process, including ischemia-reperfusion injury, organ preservation techniques, and immunological mechanisms underlying rejection and graft function, together with a more individualized immunosuppressive therapy have been combined to progressively increase the success of the human allotransplantation.

### 1.1 History of clinical transplantation

In December 1954 in Boston, a kidney was transplanted from one healthy twin to his identical brother. Joseph Murray was leading the clinical team that performed this first successful transplantation. Some 50 years before, Emerich Ullmann performed the first experimental transplantation of a kidney between dogs in Vienna in 1902. Since then, efforts were done to improve techniques and knowledge related to organ transplantation [1]. The work performed by scientists during the 1940s until the 1960s, yielded new concepts in “transplantation science” like rejection as an immunologic event, chimerism-associated central tolerance, induction peripheral tolerance and the importance of immunosuppressive agents to ensure graft survival. Then, transplantation turned to be seen from an immunologic point of view.

Although it was entirely empiric, the practical framework required for the maturation of clinical transplantation was essentially between the 1960s and 1980s decade, when the first successful allotransplantations in humans of the liver (Denver, 1967) [2], heart (Cape Town, 1967) [3], heart/lung (Stanford, 1981) [4], pancreas and intestine (Minnesota, 1967) [5], multiple abdominal viscera (Pittsburgh, 1988) [6], and bone marrow (Paris,1963) were achieved in humans. Once the surgical and preservation techniques were developed (still used with minor modifications), the field of organ transplantation stalled and entered a phase euphemistically termed “consolidation.” The underlying reason was the failure to find improved means to exploit the principles for controlling rejection, meaning an effective and safe immunosuppressive treatment which would ensure long-term graft survival with stable organ function [7].

### 1.1.1 Leading causes for liver transplantation

Liver transplantation (LT) represents the first choice treatment for patients with fulminant acute hepatitis and for patients with chronic liver disease and advanced functional failure.

In Europe, cirrhosis of adult patients accounts for the majority of transplants performed (58%), with alcoholism (18%) and HCV infection (15%) being the two most common underlying conditions. Other transplant indications include cholestatic liver diseases (PBC and primary sclerosing cholangitis), metabolic diseases (Wilson’s disease, familial amyloidotic polyneuropathy, $\alpha$-1 antitrypsin deficiency), and chronic hepatitis (HBV infection, autoimmune). Transplantations are also performed for hepatocellular carcinoma (13%). Furthermore, 9% of patients are transplanted for acute hepatic failure, the main causes of which are acute viral hepatitis and drug toxicity. Pediatric LTs account

for 10% of all liver transplants performed so far by the European transplant programs, being cholestatic liver disease the predominant indication [8]

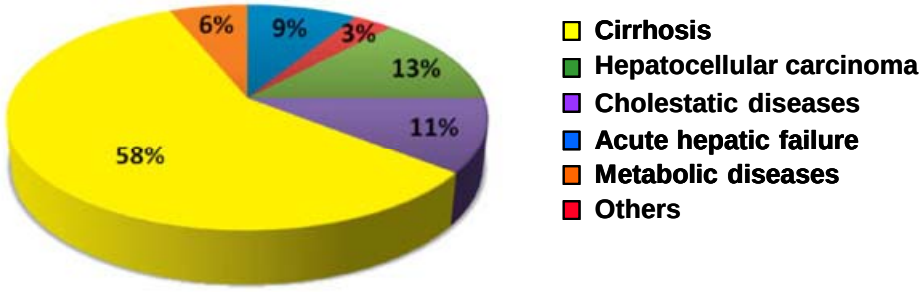


**Figure 1**: Primary disease leading to liver Transplantation in Europe (01.1998-06.2007) (Modified after Adam R, Seminars in liver disease, 2009)

The only therapeutic option for irreversible failure of the graft is liver retransplantation, which will has worse outcomes than primary LTs. Indications for retransplantation are loss of primary graft non-function, acute resistant rejection, liver arterial thrombosis or primary disease recurrence [9].

### 1.1.2 Leading causes for kidney transplantation

Renal transplantation is the standard therapy for patients with end-stage renal disease. In the absence of a compatible living donor, potential renal transplant recipients have to be on dialysis while they wait to receive an organ from a deceased donor. Living donor grafts, in comparison to deceased donor grafts, have improved health care outcomes with 5-year survival rates of 80% and 68%, respectively [10].

The most frequent indication for renal transplantation is glomerulonephritis, followed by diabetic nephropathy and cystic kidney disease. Other transplant indications are systemic immunological disease, vascular disease, interstitial nephritis and hereditary or congenital kidney disease.

Leading causes for graft loss are recipient death with functioning graft and chronic allograft nephropathy (in this case patients would need retransplantation) [11].

### 1.2 Immunosuppressive therapy

The success of organ transplantation is in very large part attributable to advances in immunosuppressive treatment (IS). Today, transplantation clinicians have an armamentarium of immunosuppressive agents at their disposal, all of which are used in various combinations both for induction and maintenance of immunosuppression. Therefore, loss of organs due to acute, irreversible rejection is now uncommon and one-year graft-survival rates of 80 to 90 % are the norm for all types of organ transplantation. Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic or blocking lymphocyte response pathways. On the other hand, immunodefiency state provokes undesired consequences (cancer and infections). Moreover each immunosuppressant has its own non-immune toxic effects [1, 12]. Appearance of new immunosuppressive agents brought uncountable potential combined therapies and the emergence of new protocols directed to find the safest combined regimens (meaning low doses, few interactions and minimal side effects).

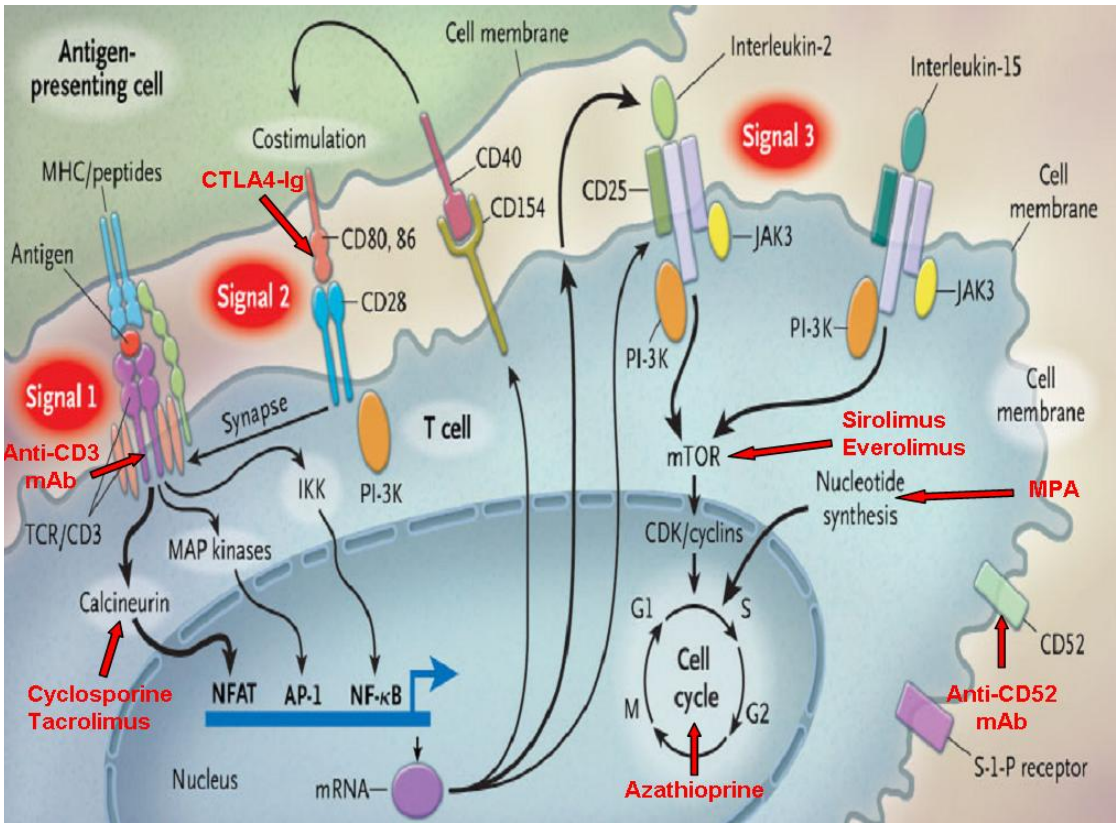


**Figure 2**: Immunosuppressive drugs and sites of action according to the Three-Signal Model (Signal 1: Antigen on dendritic cells triggers T cell with cognate receptor (TCR); signal 2: CD80 and CD86 provide co-stimulation engaging CD28; signal 3: activation of 'target of rapamycin' pathway precipitates T cell proliferation). Modified after Halloran P, The New England Journal of Medicine, 2004

Detailed explanation regarding T cell activation is provided in section 2.3. of the Introduction

### 1.2.1 Historical perspective

The first transplants between non-identical individuals suffered from early acute rejection and graft failure. Total body irradiation prior to transplantation was used in the late 1950s to ablate the recipient's immune system and overcome rejection, but the results were invariably fatal. The breakthrough in chemical immunosuppression for transplantation came with the observation that 6-mercaptopurine could induce immunological unresponsiveness in rabbits to a foreign protein. Around the same time, R. Calne tested the ability of several novel chemotherapeutic agents to prolong kidney allograft survival in dogs. One

of the compounds, BW57-322 (azathioprine), stood out in terms of efficacy and tolerability. But until the early 1960s effective chemical immunosuppression did not become a reality, when corticosteroids were combined with azathioprine by Starzl. Despite improvements, by the late 1970s kidney allograft survival barely exceeded 50% at 1 year.

Transplantation history changed with the discovery of cyclosporin A (CsA), originally classified as an anti-fugal, in 1976. Clinical trials studying its anti-lymphocyte properties showed to not only facilitate kidney transplantation, but also transplantation of pancreas and liver and later heart and lungs.

The 1970s were also notable for the development of monoclonal antibodies (mAbs). The first mAb clinically relevant was OKT3, a mouse anti-human CD3 mAb, which was initially used to treat acute rejection.

Two agents with interesting results in rodent models were identified in the late 1980s, namely tacrolimus (FK506) and sirolimus.

In the 1990s the pace of new drug development increased with the introduction of mycophenolate mofetil, together with two anti-CD25 mAbs, daclizumab and basiliximab.

### 1.2.2. Corticosteroids

Universally used as first-line treatment for acute allograft rejection, the two main corticosteroids are prednisolone and prednisone (used mainly in Europe and the USA, respectively). Corticosteroids have a variety of anti-inflammatory and immunomodulatory effects. Concretely, they mediate a reduced production of cytokines, including IL-1, IL-2, IL-6, IFN-$\gamma$ and TNF-$\alpha$. They also impair monocyte/ macrophage function and decrease the number of circulating CD4+ T cells [12].

### 1.2.3 Azathioprine

Metabolism of azathioprine (AZA) results in several compounds that are incorporated into replicating DNA and thereby halt replication. These metabolites also block the *de novo* pathway of purine synthesis, conferring specificity of action on lymphocytes. It has also been suggested that AZA interferes with CD28 co-stimulation of alloreactive T lymphocytes [12].

### 1.2.4 Mycophenolic acid

Mycophenolic acid (MPA) has got two parent compounds, mycophenolate mofetil and mycophenolate sodium. The target of MPA is inosine monophosphate dehydrogenase, the rate-limiting enzyme in the de novo synthesis of guanosine nucleotides; hence such a blockade results in relatively selective interference with lymphocyte proliferation, preferentially the activated lymphocyte population [12].

Since MPA and AZA block DNA replication, proliferating populations, like B and T cells, are impeded in the adult body.

### 1.2.5 Calcineurin inhibitors: Cyclosporin A and Tacrolimus

Calcium-calcineurin is a calmodulin-dependent protein phosphatase. When activated promotes a transcriptional activation by NFAT, a T cell specific transcription factor that regulates IL-2 gene expression in human T cells, resulting in a blockade of T cell activation and proliferation [13].

CsA engages cyclophilin and tacrolimus enganges FK506-binding protein 12 (FKBP12). In both cases the immunosuppressants form a complex with the mentioned immmunophilins that inhibits calcineurin activity. Tacrolimus potency as immunosuppressant is superior to CsA, and dosage of the latter directly correlates with its rate of inhibition and the severity of its side effects.

### 1.2.6 The mTOR inhibitors: sirolimus and everolimus

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase involved in both innate and adaptive immune responses. mTOR regulates cell growth and proliferation, motility and survival, protein synthesis and transcription. Blockade of mTOR impairs dendritic cell maturation and function and inhibits T cell proliferation [14]. Sirolimus and everolimus are derivatives from rapamycin with potent immunosuppressive activity. mTOR inhibitors neutralize the signalling required for the progression beyond the $G_1$ to S phase in the cell cycle, blocking cellular proliferation [15]. These agents may also have antineoplasic [16] and arterial protective effects [17].

### 1.2.7 Polyclonal and Monoclonal Antibodies

Polyclonal antibodies (ALG and ATG) are prepared by inoculating rabbits or horses with human lymphocytes or thymocytes. After their IV administration, this leads to a rapid and profound lymphopenia. In addition, they may cross-link the TCR, causing partial T cell activation and blockade of T cell proliferation. The presence of xenogeneic proteins causes a febrile episode in 80% of the recipients [12].

mAbs were developed in order to improve immunosuppressive specificity. The idea was to use a hybridoma cell line which would yield an infinite supply of antigen-specific antibodies against a particular antigen. The above mentioned OKT3, which specifically reacts with the T cell receptor, was the pioneer. The new generation of suitable mAbs was characterized by a lack of immunogenicity, long half-life with prolonged biologic effects and minimal acute toxicity. Examples of these compounds are anti-IL2R (CD25) mAbs; including basiliximab and daclizumab, the lymphocyte depleting anti-CD52 mAb

(Campath-1) and alemtuzumab (humanized version). Ultimately, the major trust in clinical development is to block the co-stimulatory pathway on T cells; an analogue of CTLA4-Ig (belatacept) could be a promising agent [18]. Nowadays therapeutic antibodies are used in "inducing strategies" to reduce the incidence of rejection or to promote tolerance [19].

### 1.2.8 Side effects of immunosuppressive drugs

Accommodation of immunosuppressive regimens, meaning development of new drugs and combined therapy, allowed transplantation to be implanted as the optimal treatment for many patients with end-stage organ failure. However, patients pay a prize for being under short/long-term IS maintenance. Since all currently available agents cause widespread non-specific immunosuppression, they increase the risk of infection and certain types of malignancy (skin cancer and post-transplant-lymphoproliferative disease). In addition, each kind of immunosuppressive drug has its own agent-specific side-effects, which could be summarized in metabolic side effects, such as hypertension, dyslipidemia, hyperglicemia, ulcers, nauseas, vomiting and liver and kidney toxicity [20, 21]. Immunosuppressive agents also interact with other medications and affect their metabolism, action and concentration on blood.

| | Cyclosporine | Tacrolimus | Sirolimus | Azathioprine | Mycophenolate | Corticosteroids |
|---|---|---|---|---|---|---|
| Immunosuppressive potency | +++ | +++± | ++± | + | ++ | + |
| Nephrotoxicity | ++ | ++ | – | – | – | – |
| Neurotoxicity | + | ++ | – | – | – | – |
| Hirsutism/hypertrichosis | ++ | – | – | – | – | ++ |
| Skin rash | – | – | + | – | – | – |
| Diabetogenic | + | ++ | – | – | – | ++ |
| Diarrhoea | – | – | + | – | ++ | – |
| Hepatotoxicity | ± | ± | + | + | – | – |
| Marrow suppression | – | – | + | + | + | – |

Key: –: equals no effect; +: mild (or low incidence) toxicity/potency; ++++: extreme toxicity or potency.

**Table 1:** Immunosuppressive drugs: potency and side effects. (Taylor A.L; Clinical Reviews in Oncoly/Hematology, 2005)

### 1.3 New perspectives to improve graft and patient survival

Since overall survival rates had been ameliorated, the focus has shifted towards patient quality of life after transplantation, as well as immunosuppression management and recurrence of the primary organ disease as major contributors to morbidity and mortality in long-term survivors.

#### 1.3.1 Predicting graft function

Robust and accurate tests to predict graft function, together with tailor made therapies according to the needs of each individual patient are major goals in the management of transplant recipients.

Currently, the presence of anti-HLA donor antibodies and antibodies against MICA detected prior to allotransplantation are associated with increased frequency of renal allograft loss [22]. Nowadays, several laboratories are developing non-invasive tools to identify molecular subgroups to predict chronic allograft nephropathies, including investigations at gene expression level (transcriptomics), protein translation (proteomics) and even the metabolite network (metabolomics). An independent set of prospective samples, coming from accessible body fluids, should be used to validate candidate biomarkers. In case of acute kidney graft injury several biomarkers have been described, like NGAL and cystatin C in the plasma, or KIM-1, NGAL, NAG and others in the urine [23].

#### 1.3.2 New immunosuppressive agents

From a pharmacological point of view, efforts are done to improve the specificity of immunoregulation. New approaches in drug development include the targeting of different immunological pathways interfering with cell-surface molecules, or the inhibition of signaling mechanisms. Small molecules, biologics

(agents developed from protein living cells employing recombinant DNA technology) and mAbs (like belatacept) are under study for clinical usage [23].

### 1.3.3 Cell therapy regulatory T cells

Regulatory T cells (Tregs) are known to recognize alloantigens and regulate allospecific immune responses. Hence, they are an attractive target to promote graft acceptance through the suppression of allospecific effector cells. It has been proposed that Tregs could be generated and clonally expanded *in vitro* from precursor T cells for posterior re-injection into the organ recipient [24]. Furthermore, their function could be boosted *in vivo* through drugs, since conventional immunosuppressants have various direct and indirect effects on Tregs (Table Y) [25]. Several clinical studies in kidney transplantation already analyzed the effect of different IS. Concretely, it has been reported that Tregs are present upon clearance of daclizumab (administered at the time of transplantation) [26]; alemtuzumab positively affected the ratio of Tregs to effector T cells [27]; and belatacept regimen did not yield in decreased CD4+CD25$^{high}$ cells neither Foxp3 mRNA expression, despite interfering with the costimulation pathway (interaction of CD80/86 and CD28) during T cells activation [28].

| Immunosuppressive drug | Effect on Tregs |
|---|---|
| mTOR inhibitors | + |
| CNIs | - |
| Anti-IL2R mAbs | +/- ? |
| T-cell depletion | + ? |

**Table 2**: Effect of different immunosuppressive drugs on Tregs (Modified after Wekerle T; Transplantation Proceedings, 2008)

### 1.3.4 Induction of tolerance: a major challenge in human transplantation

A theoretical solution to avoid the side effects of chronic IS and also chronic rejection would be the induction of transplantation tolerance. This state is defined as the indefinite acceptance of the transplanted graft, in the absence of IS, in an immunocompetent host capable of accepting a second graft from the same donor but able to reject a third-party graft. Immunological tolerance was first reported by Billingham *et al.* in new born mice [29]. These pioneers in the tolerance field were already aware that graft survival prolongation was "great but not necessarily indefinite" and that "tolerance is not of an all-or-nothing character". On the other hand, the demonstration of immunological tolerance as is commonly done in experimental animal transplantation is unsuitable for clinical application, since donor specificity is impossible to establish *in vivo* and normal graft histology can not always be assessed because late biopsies are not available. Hence, in the clinical setting, the term operational tolerance (TOL) was chosen to designate a state of graft acceptance whereby a patient could enjoy stable graft function without the need for IS [30].

In human transplantation allograft tolerance has been intentionally induced via hematopoietic macrochimerism, using bone marrow or hematopoietic stem cell transplantation combined with simultaneous or delayed KT [31]. This strategy, however, only appears to be effective in selected non-sensitized recipients, and is associated with substantial side effects which for the time being preclude their widespread clinical application. On the other hand, operational tolerance can occasionally occur spontaneously in patients receiving conventional immunosuppressive drugs. This phenomenon was first reported in the case of

non-compliant liver recipients [32] and has also been observed in the context of KT, although it is much less prevalent than in LT [33].

In fact, the liver is considered to harbour unique tolerogenic properties compared to the rest of organs, resulting in a decreased tendency to rejection. Worldwide experience demonstrates that operational tolerance can be achieved in approximately one-quarter of selected liver recipients, and there are at list 8 clinical trials assessing the feasibility of intentionally weaning IS from stable liver recipients [34-41]. A well planned and slow weaning of IS under strict clinical control in selected patients (long-term survivors who experience side effects from IS) appears to be the work scheme to rely on in the present clinical context. While this strategy is considered to be reasonably safe, its application is limited by the lack of a diagnostic test capable of identifying tolerant recipients before IS is discontinued. Thus, there is a real need to develop a robust, clinically applicable, diagnostic algorithm of allograft tolerance which could help clinicians to select ideal groups of patients to start immunosuppressive weaning protocols. In this sense, it has been reported that microarray analyses of PBMCs from both TOL liver and kidney recipients, revealed gene signatures able to predict tolerance in independent cohorts of TOL recipients. These signatures were even capable of distinguishing TOL recipients from other recipients under IS and from healthy individuals [42, 43].

# 2. The immune system: innate and adaptive immune responses

The immune system comprises a network of cells, tissues and organs that protect the body by identifying and attacking potentially harmful foreign molecules.

This system bears two distinct arms, the innate and the adaptive immune response. The innate immune response includes all defence mechanisms that are encoded in the germ line genes of the host. The activation of this response is fast but rather unspecific. On the contrary, the adaptive immune response manifests exquisite specificity for its targets and develops immunological memory capable of eliciting an accelerated and heightened secondary response [44]. Although both responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact and fully effective immune response.

### 2.1 Transplantation: elements of the alloimmune response

Allotransplantation between two genetically disparate (histoincompatible) individuals of the same species leads to an immune response directed against the donor tissues, which determines the acceptance or rejection of the graft. This alloresponse is mainly due to the capacity of T cell receptors to cross-react with high affinity with foreign MHC molecules (i.e. MHC molecules not previously encountered in the thymus during T cell development). [45].

The alloimmune response orchestrates a cascade of events involving both the innate and the adaptive arms of the immune system. Ultimately, if left un-treated, recognition of the allogeneic tissue results in a complex effector response rejecting the allograft. This combined cellular and molecular response can take place according to different patterns:

- Hyperacute rejection describes very early graft loss which usually occurs within the first two days after transplantation. In this situation, preformed antibodies specific for donor antigens are expressed on graft vascular endothelial cells and therefore, present in the recipient's serum. Such antibodies fall into two main categories: low affinity immunoglobulin M (IgM) antibodies, which are specific for ABO blood group antigens, and high affinity IgG antibodies directed against HLA antigens. The binding of these antibodies to their targets triggers activation of clotting, and complement cascades leading to intravascular thrombosis, ischemia and subsequent necrosis [45].

- Acute rejection (AR) is the form of rejection established between five days and three months after transplantation, in the presence of IS. Histologically, AR shows a diffuse interstitial cellular infiltration composed of both CD4 and CD8 T cells, with a significant presence of activated or memory phenotype. Whereas, when vascular rejection occurs, macrophages are the predominant cells found in the intimal arteritis lesions of the biopsies [45]. AR is the most common type of rejection reported after liver transplantation.

- Chronic rejection (CR), describes an active but slow injury leading to graft loss, which is caused by a host-anti-graft immune response mediated by several pathogenic factors. The incidence of CR in liver transplantation is low (around 2%), although it is considered that liver CR is preceded by at least one episode of AR. On the other hand, CR in renal transplant is frequently seen (at least 20%). The array of changes found in kidney biopsies of grafts showing progressive dysfunction is known as chronic allograft nephropathy. This clinic-pathological state that causes progressive kidney allograft injury probably develops due to immunologic factors, such as T cell mediated immune

responses and presence of anti-HLA donor antibodies. Non-immunologic variables may also influence, including chronic CNI toxicity, donor disease, ischemia, infection, hypertension, hyperlipidemia and recurrence of primary disease [46-48].

Another aspect that requires special consideration is graft-versus-host-disease (GVHD).

It occurs when donor T cells respond to genetically defined proteins on host cells (HLA proteins mainly). Yet GVHD constitutes the major complication of allogeneic hematopoietic stem cell transplantation, and it remains lethal for those patients not responding to steroid therapy. It can develop in various clinical settings, when tissues containing T cells are transferred to a recipient who is unable to eliminate those cells since he is immunosuppressed (*e.g.*, solid organ transplants including liver, intestine, lung and kidney) [49].

Based on GVHD onset after surgery, two different subtypes have been defined:

- Acute GVHD, appearing prior to 100 days, is directly related to the degree of mismatch between HLAs. Donor T cells and host APCs play an essential role in the immunopathogenesis of GVHD. The principal targeted organs are skin, gastrointestinal tract and liver [45, 49].
- Chronic GVHD, occurring more than 100 days after engraftment, is a major cause of morbidity and mortality in long-term survivors of allogeneic hematopoietic stem cell transplantation. Advanced age of recipients and history of acute GVHD are considered to be the main risk factors, although pathologic mechanisms are still poorly understood [49, 50].

## 2.2 Elements of the innate response

The innate immune system consists of natural or nonspecific mechanisms for the protection against foreign antigens. These defences do not require prior exposure to pathogens for their activation, as they perform their sentry function by using non-rearranged receptors, named pattern recognition receptors (PRRs) [51].

### 2.2.1 Toll-Like Receptors

Toll-like receptors (TLRs) belong to a family of PRRs, whose primary function is to signal that microbes have breached the body's barrier defences. They recognize common structural features of microbes, so called pathogen-associated molecular patterns (PAMPs), including LPS, peptidoglycan and lipoteichoic acid. In addition, during transplantation, ischemic and surgical trauma releases endogenous molecules capable of binding to TLRs and thereby activating innate responses [51]. TLRs are mainly found on macrophages and dendritic cells, but also on neutrophils, eosinophils, epithelial cells and keratinocytes. Activation of most TLRs induces cellular responses associated with acute and chronic inflammation, through the expression of genes encoding cytokines and other inflammatory mediators [44].

### 2.2.2 The complement system

The complement system consists of a complex set of plasma proteins that react with one another in a series of enzymatic reactions in a cascading manner. Its activation leads to either opsonize pathogens or recruit inflammatory cells or directly kill pathogens. Factors responsible for their activation are: the formation of insoluble antigen-antibody complexes, platelet aggregation, release of endotoxins, presence of viruses or bacteria in the circulation, and the release of

proteases from injured tissues. Studies in animal models in solid organ transplantation have identified new roles for complement, including the mediation of reperfusion damage and cellular rejection [52].

### 2.2.3. Antigen Presenting Cells

Antigen presenting cells (APC), including dendritic cells (DC), macrophages and B cells, are activated after the encounter of microbes or environmental insults. This event is central for the priming of alloreactive T cell responses. Experiments depleting and restoring graft “passenger leukocytes” implicated DCs as a key player in alloantigen presentation [53]. Upon functional maturation, DCs convey antigens from peripheral tissues and become potent stimulators of T cells, through the induction of pro-inflammatory cytokines or increasing the expression of Tcells co-stimulatory molecules [51].

### 2.2.4 Natural Killer cells

Natural Killer (NK) cells are cytotoxic lymphocytes that contribute to surveillance against transformed cells, certain viruses, and other intracellular pathogens. Human NKs are distinguished based on their CD56 receptor expression density. In peripheral blood, the majorities (90%) are $CD56^{dim}$ and they are regarded as the classical NK subset, mediating cytotoxic effector functions and antibody-dependent cellular cytotoxicity. The remaining 10%, expressing $CD56^{high}$, produce high amounts of pro-inflammatory cytokines (such as IFN-$\gamma$ and TNF-$\alpha$) indicating a primary role in the immunoregulatory function. NK cells possess a variety of inhibitory and activating receptors that engage MHC class-I like molecules [54]. Their exact role in solid organ rejection remains to be clarified, although it has been shown that they act as facilitators by amplifying early graft inflammation and supporting the activity of alloreactive T cells [55].

### 2.2.5 Natural Killer T lymphocytes

Natural killer T (NKT) cells represent a small population of T lymphocytes reactive to glycolipids which are presented in the context of MHC class-I like molecules. Upon activation, NKT cells can mediate rapid and sustained production of an excess of cytokines capable of impacting both innate and acquired immune responses. In the transplantation field, they may be activated by the milieu of cytokines generated by nonspecific inflammation [56]. NKT cells could promote graft damage by mediating interferon IFN-$\gamma$ release that subsequently would lead to the recruitment of neutrophils, resulting in islet loss [57].

### 2.2.6 $\gamma\delta$ TCR T lymphocytes

Whereas most mature T cells express the $\alpha\beta$ TCR heterodimer, a small proportion expresses an alternative $\gamma\delta$ TCR heterodimer. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells seem to directly recognize antigens in tissues and respond to a variety of stress-induced MHC-like self-antigens, by using an extremely limited $\gamma\delta$ TCR diversity [58]. In fact, the variable region repertoire of $\gamma\delta$ TCR is highly restricted, especially on peripheral blood [59]. $\gamma\delta$ T cells have been implicated in innate responses concerning infectious diseases, regulation of immune responses (including cell recruitment and activation), and also tissue repair [60].

$\gamma\delta$ T cells comprise <10% of total peripheral blood T cells. The most common subpopulation, expresses the V$\gamma$9V$\delta$2 TCR which specifically react against 'phosphoantigens'. These pyrophosphomonoesters represent metabolites of isoprenoid biosynthetic pathways from either foreign origin (HMBP) or self-derived (IPP). Non-V$\delta$2 subsets, primarily expressing V$\delta$1 regions, comprise

around 10% of the γδ T cell pool. MHC class-I-like molecules, including CD1, as well as MICA and MICB have been implicated as ligands for Vδ1 T cells [61].

Vδ1 and Vδ2 T cells appear to differ functionally from one another. It has been reported that following non-specific stimulation, Vδ2 T cells tend to express more pro-inflammatory genes, including TNF-α, IFN-γ, IL-17, and IL-21, whereas the Vδ1 T cells express higher levels of regulatory cytokine genes (IL-10 and IL-11) [62]. On the other hand, they share many innate receptors, like NK receptors and several TLRs [61]. γδ subpopulations are able to recognize and destroy tumour cells. Furthermore, Vδ2 T cells predominate in mycobacterial infections, while Vδ1 T cells are preferentially expanded in infections such as HIV, CMV and malaria [60].

### 2.2.7 Innate immunoregulatory cells

Several studies suggest that different cells of the innate immune system may have dual roles in solid organ transplantation. They are able to interact with the allograft and exert their effector function, but under specific conditions, they can act as immunoregulators or tolerance inductors.

- DCs exist in an “immature” state expressing little or no co-stimulatory molecules in the absence of danger signals, then cognate engagement of Ag-specific T cells results in anergy or apoptosis. Given this, the potential use of immature or “tolerogenic” DCs as therapy to promote peripheral tolerance upon organ transplantation has been an area of active research [51].

- NK cells can also facilitate tolerance induction. In this way, Yu *et al.* [63] demonstrated that NK cells were critical for promoting tolerance by reducing the survival and dissemination of graft-derived donor APCs in transplant recipients [51].

- Evidence suggests that in the transplant setting, NKT may dampen rejection rather than exacerbate it. It has been shown that NKT may produce tolerogenic cytokines (*e.g.*, IL-10) [64], and they could serve to aid the generation of Tregs [64].

- It is under study the role of Vδ1 T lymphocytes in the protection against microorganisms which can cause latent infection [65]. In addition, augmented gene expression of γδ T cells and increased relative amounts of the Vδ1 subset have been reported in operationally tolerant liver recipients [42, 66].

### 2.3 Elements of the adaptive response

The adaptive immune system occupies a preferential place in the field of transplantation immunology, since it was demonstrated that T cells are both necessary and sufficient for the recognition of allogenic tissues and the accomplishment of a complete effector response.

#### 2.3.1. αβ TCR T lymphocytes and allorecognition

The allorecognition in the context of the adaptive immune response is orchestrated by αβ T lymphocytes that recognize MHC molecules, among others, expressed on the surface of the transferred cells. They engage a specific receptor complex, the T cell receptor (TCR), which is encoded by gene elements that somatically rearrange to assemble antigen-binding molecules with exquisite specificity for individual unique microbial and environmental structures. The generation of the TCR is a complex process that creates an impressive repertoire in the order of $>10^{14}$ through combinatorial joining of V, D, and J gene segments. The recombination process is triggered by IL-7 and involves many enzymes [67].

Alloantigens can be divided into major histocompatibility complexes (MHC), designated as HLA in human, and minor histocompatibility antigens (mHAg). The former, is responsible for eliciting the strongest immune responses to allogeneic tissues.

#### 2.3.1.1 Class-I MHC molecules

There are three major HLA class-I molecules, designated HLA-A, HLA-B, and HLA-C, which are constitutively expressed on most nucleated cells. The display of antigenic peptides bound within the HLA molecules on the surface of cells is described as antigen presentation. Generally, the peptides bound in the grooves of the HLA class-I molecules are derived from proteins synthesized within the cell that bears the class-I molecules. They are, consequently, described as endogenous antigens.

Critical for their biologic function, HLA molecules manifest a high structural polymorphism, which underlies the extreme difficulty finding perfect matched organs that will not induce a strong anti-MHC alloresponse.

Given that most individuals in the human population are heterozygous for HLA and that every single class-I protein can bind many different antigenic peptides, each individual is able to form a bond with a very diverse collection of peptides. On a population level, the diversity of peptide-binding motifs is enormous [44].

#### 2.3.1.2 Class-II MHC molecules

There are three major class-II proteins designated HLA-DR, HLA-DQ, and HLA-DP, which are constitutively expressed only by macrophages, DCs, B lymphocytes and thymic epithelial cells. The peptides that are presented by class-II HLA molecules are generally derived from exogenous proteins that were taken up by APCs by means of phagocytosis. Subsequently they are

degraded into peptides within a lysosomal or endosomal compartment before being transported to the cell surface via specialized class-II loading compartment. As in the case of class-I HLA, structural polymorphisms are central to their biological function [44].

#### 2.3.1.3 The MIC system

The MIC system consists of two polymorphic families of MHC class-I related genes, termed MICA and MICB. These molecules function as restricting elements for intestinal $\gamma\delta$ T cells and they behave like cell stress molecules. MICA is expressed in endothelial cells, keratinocytes and monocytes. It is likely that the polymorphic MICA molecule may be a target for specific antibodies and T cells in solid organ grafts or in GVHD [45]. Anti-MICA antibodies induce a prothrombotic state, and associated with HLA, they could induce graft failure in KT [68].

#### 2.3.1.4 Minor histocompatibility antigens

In principle, any protein that has polymorphisms within a species can act as mHAg. Peptide fragments from these proteins are presented to T cells in an MHC class-I or class-II restricted manner. The number of possible mHAgs in allotransplants is very large. The *in vivo* correlate of an immune response to a mHAg is transplant rejection or GVHD.

Several mHAgs have been described in transplantation descending from different cellular origins:

- Encoded by sex chromosomes: a set of proteins encoded on the male-specific Y chromosome that are known collectively as H-Y antigens [45]..

- Encoded by autosomes: non-Y-linked mHAgs have also been described upon recognition by T cells from patients with GVHD after BMT between HLA identical individuals [45].

- Encoded by mitochondrial DNA: studies of a maternally transmitted transplantation antigen described that such peptides could act as histocompatibility antigens, although no worsening of graft condition was reported when its effect was studied in a Japanese cohort [45, 69].

### 2.3.2 Mechanisms of antigen recognition

Allorecognition can proceed via several mechanisms:

- Direct allorecognition, whereby T cells recognize determinants on the intact donor MHC molecules displayed on the surface of transplanted cells.
- Indirect allorecognition in which donor MHC molecules are processed and presented as peptides by self-MHC molecules.
- Semi-direct or linked allorecognition, where trafficking recipient DCs acquire intact donor MHC:peptide complexes from cells of the graft enabling them to stimulate antigen-specific immune responses.

The presence of passenger APCs in donor tissues at the time of transplantation dictates that the direct anti-donor alloresponse is vigorous in the early period post-engraftment and diminishes with the death and removal of these APCs over time. The indirect alloresponse, on the contrary, requiring antigen capture and processing, is less rapid but continues indefinitely as graft-derived antigens are continuously acquired and processed [45].

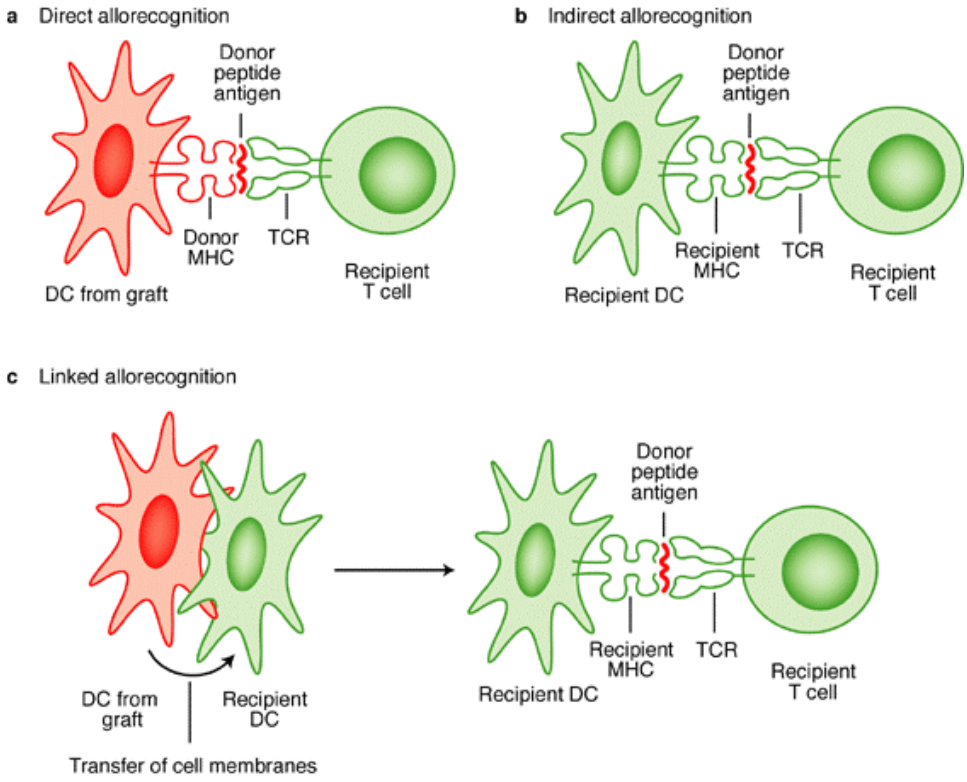


**Figure 3**: Pathways of allorecognition in transplantation (Coates P.T, Expert Reviews in Molecular Medicine, 2002)

### 2.3.3 T lymphocyte differentiation and activation

T cells emerge from pluripotent stem cells in the bone marrow and require successive differentiation stages, terminating at the thymus. During this process, T cells are educated to discriminate 'self' and 'nonself' antigens through the expression of antigen-specific receptors known as TCR. Approximately 90% of peripheral blood T cells have a TCR comprised of $\alpha\beta$ subunits. Whilst random combinatorial generation of TCRs occurs, T cells require signaling through their antigen receptor to survive and proliferate. Only 5% of T cell precursors turn out expressing the appropriate TCR.

In the course of early differentiation, immature T cells express both CD4 and CD8 co-receptors. In the next phase, CD4 T cells are selected through

interaction with class-II MHC molecules, whereas CD8 T cells are selected based upon their interaction with class-I MHC molecules.

Antigen encounter is needed to achieve successful T cell maturation. The sustained physical contact between antigenic APC and the specific TCR is known as 'immunological synapse'. Interaction of the TCR/CD3 complex with the peptide presented by an HLA molecule provides only a partial signal for cell activation. Full activation requires the additional participation of a co-stimulatory pathway, via CD28 on the T cell and B7 molecules (also called CD80 and CD86) on the APC. CD28 activates anti-apoptotic molecules and is also up-regulating additional co-stimulatory molecules like CD40L, which increases cytokine secretion, memory T cell generation and B cell activation [67, 70].

The family of co-stimulatory molecules further includes the inhibitory receptor, CTLA-4, that binds to B7 molecules (CD80 and CD86) with higher affinity than CD28. This interaction provides down-modulating signals that help terminating immune responses and maintain self-tolerance [71].

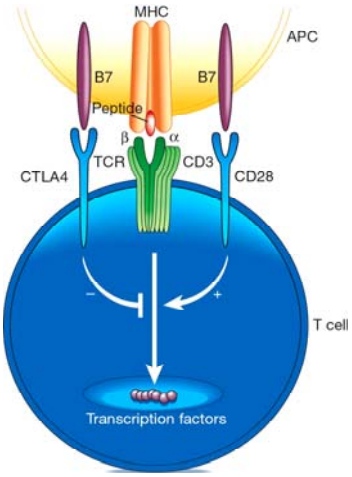


**Figure 4**. T cell regulation by CD28 and CTLA-4. (Appelbaum F.R, Nature, 2001)

### 2.3.4 T lymphocyte effector phase

Activated T cells migrate from the lymph node to pathogen/antigen location to exert their effector functions.

#### 2.3.4.1 CD4 T lymphocytes

CD4 T cells are classically designated “helper cells”, including different populations. These T helper (Th) cell subsets may be defined by their expression of a combination of specific cytokines, transcription factors, and the signaling pathways through which their differentiation is mediated. At least four Th cell types have been consistently identified and characterized to date, namely Th1, Th2, Tregs and more recently, Th17.

- Th1 differentiation is promoted by IL-12 signalling mediated via STAT-4. In addition, IL-27 signaling via STAT-1 plays an important role. When these pathways are activated Th2 differentiation is blocked.

  The defining features of Th1 are the expression of IFN-γ, IL-2 and TNF-β. Th1 cells provide host immunity to intracellular pathogens, and enhance macrophage activity due to IFN-γ production [67, 72].
- Th2 polarization is induced by IL-4 signaling via STAT-6, provoking the activation of the GATA-3 transcriptional factor, which stimulates Th2 cytokine production and inhibits Th1 differentiation.

  Th2 responses are stimulated by cytokine release of IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. Th2 support humoral and allergic responses. Moreover, they are thought to blunt the severity of allograft rejection by inhibiting Th1-mediated activity. However, Th2 cytokines *per se* are not indicators of graft survival and allograft rejection [73, 74].
- Th17 cells are defined by the expression of IL-17, the retinoic acid-related orphan receptor (ROR)-γT, and differentiation through the STAT3 pathway.

  The factors that initiate Th17 development are not completely clear. Several studies indicated that both TGF-β and IL-6 are required for Th17

differentiation. Additionally, IL-1β has been identified as promoting Th17, both alone and in conjunction with IL-23.

Th17 cells are known to be pro-inflammatory mediators through a variety of mechanisms. In this sense, it has been shown that IL-23 drives Th17 responses, like the induction of experimental autoimmune encephalitis and collagen-induced arthritis [75]. In the field of transplantation, it is has been reported that IL-17 protein is elevated in different tissues during acute rejection [72].

Classically, Th1 responses were considered responsible for a wide range of autoimmune and inflammatory diseases, as well as acute organ rejection and GVHD [67]. However, description of Th17 resulted in a novel hypothesis, proposing that skewing of responses towards Th1 or Th17 may cause the development and progression of autoimmune diseases and transplant rejection. Otherwise, blockade of critical cytokines may favour a shift of polarization towards Th2 and Treg phenotypes [75].

A number of cell types with immunoregulatory capacity and broad-spectrum phenotype have been described in the literature. The most important T cell population able of suppressing immune responses is named Treg.

- Tregs are characterized as CD4+CD25+ T cells, and the forkhead transcription factor 3 (Foxp3) was identified as determinant for their development. Although Foxp3 is used as phenotypic biomarker, it is not ideal, as it is an activation marker of human T cells. Recent evidences have also implicated the IL-7 receptor (CD127) as a suitable marker, since its down-regulation is highly associated with Foxp3+ Tregs [76].

Tregs have been divided into natural and adaptive subsets. The former arise in the thymus during the early T cell developmental stage. Adaptive Tregs are derived in the periphery either from naïve CD4+CD25- T cells, promoted due to surrounding conditions like low antigen doses and/or weaker stimuli, or by expansion of natural Tregs after recognizing alloantigens [76]. In this way, allogeneic Tregs developing from indirect recognition have been shown to limit chronic rejection in animal studies [79].

Recently it has been highlighted that natural Tregs harbour the ability to become Th17 cells in the presence of IL-6 [77].

Although the exact mechanism by which Tregs exert their effect or function is unknown, it is believed that their suppressive function is cell-contact-dependent. *In vitro* and *in vivo* data showed an important role for TGF-β, IL-2, IL-10 and IL-35 as mediators of Tregs activity. Other regulatory mechanisms include the expression of CTLA-4 on DCs, granzyme B, which acts as effector molecule for Tregs, and INF-γ that limits Th17 while enhances Treg production [76, 78].

#### 2.3.4.2 CD8 T lymphocytes

CD8 T cells show a major cytotoxic activity against cells infected with intracellular microbes and against tumour cells, but also contain a regulatory subset that downregulate immune responses (suppressor cells).

- Cytotxic T lymphocytes (CTLs), displaying a CD8+CD28+ phenotype, exert their function via two distinct effector mechanisms; depending either on perforin or on Fas ligand. Perforin is a membrane pore-forming molecule, which enables the release of granular enzymes into the cytosol of the target cell, and thereby induces rapid apoptosis of the targeted cell through

caspase-dependent and caspase-independent manners [70]. Fas ligand pathway leads to apoptosis via caspase reaction casacade.

Some authors divide CTLs into two populations; Tc1 and Tc2, where Tc1 secrete IFN-γ, and Tc2 secrete IL-4 and IL-5. Naïve CD8 T cells have a strong tendency to differentiate into Tc1 cells. IFN-γ and IL-12 encourage this process. Both subsets kill their targets equally well; however Tc2 cells might further provide help to B cells [67].

- Suppressor T cells (Ts) are MHC class-I restricted CD8 T cells lacking CD28, which act via direct cell-contact, and exercise a tolerogenic effect on APCs [80]. Tolerogenic APCs express inhibitory receptors that initiate a cascade of events which results in the generation of CD4 MHC class-II restricted Treg, while blocking the complete full Th activation [81]. One of these receptors, ILT3, was shown to signal not only intracellularly, biasing the APC into a tolerogenic pathway, but also extracellularly, eliciting the differentiation of CD8 Ts [82]. Related to their origin, it is postulated that chronic antigenic exposure or tolerogenic conditions, would lead CTLs to loose their cytolytic potential to acquire the ability to induce inhibitory receptors in APCs. This would drive the differentiation of antigen specific Ts [80].

  Their potential in transplantation lies in the fact that they could suppress the response to the graft in an antigen-specific manner, while the patient would remain immunologically competent. In this clinical context, the organ endothelium fills the role of APCs, expressing HLA proteins. This could indicate that Ts are primary effectors of tolerance in organ transplantation,

inducing a tolerogenic phenotype and inhibiting CD4 Th alloreactivity [80, 83].

### 2.3.5 B lymphocytes

B cells are central players of the humoral immune response. They are defined by their production of antibodies [immunoglobulin (Ig)] and thereby provide a protective immune defence against bacteria, viruses and harmful protein antigens.

B cells differentiate from hematopoietic stem cells in the bone marrow. It is here that their antigen receptors (surface Ig) are compounded. During this process the B cell receptor (BCR) develops, achieving unique antigen specificity. The whole procedure ends with the assembly of the antigen binding component of the BCR. B cells also have a co-receptor complex consisting of CD19, CD81 and CD21. Binding of specific ligands leads to transduction pathways inducing cell proliferation and maturation [70].

Naïve B cells express IgM and IgD. B cell maturation is mediated by Th derived cytokines, which induce isotype switching while maintaining antigenic specificity. At the same time, activation of somatic mutation and clonal expansion of B cells with high antigen affinity occur. These processes are associated with the development of B cell memory. Memory responses are characterized by production of IgG, IgA and IgE, which are critical for the success of vaccination against pathogens, and to perpetuate the pathology of autoimmune and allergic syndromes [70].

B cells also contribute to the immune response by important antibody-independent mechanisms such as presentation of antigen. They internalize, process and present antigens in the context of MHC class-II. Furthermore, they

elicit the production of pro-inflammatory cytokines and chemokines (*e.g.*, IL-6, IL-12, TNF-$\alpha$ and INF-$\gamma$). Additionally, B cells are able to behave as tolerogenic APCs, and produce immunomodulatory cytokines like IL-10 and TGF-$\beta$ [50].

Nowadays it is under study if the use of B cell depletion through anti-CD20 mAb (rituximab) could serve as a prophylactic treatment for GVHD in allogenic stem cell transplantation [50].

# 3. Immune monitoring strategies in clinical organ transplantation

The development of reliable and non-invasive assays to explore the current state of the alloimmune response in transplant recipients is of interest for several reasons. These assays would provide data to identify rejection prior to its clinical manifestation without resorting to invasive test procedures (protocol biopsies). They could also allow for the implementation of a personalized immunosuppressive therapy. Additionally, in some cases, the identification of an immunological tolerance profile would enable for a partial or complete cessation of immunosuppressants. This would result in reducing treatment costs and avoiding long-term side effects of IS. Moreover, immune monitoring techniques could improve the global understanding of the mechanisms underlying the generation of tolerance and rejection, and this could aid in the design of tolerance-inducing clinical transplantation trials.

Assays developed for the immunological monitoring of the alloimmune response can be broadly divided into antigen-specific and antigen non-specific.

In clinical research, no exclusive assays have been already developed for monitoring organ graft function, and the immune response may be altered between pre- and post-transplantation time points. Therefore, a combination of monitoring assays needs to be performed over time.

### 3.1 Antigen specific immune monitoring assays

These assays are developed to quantify lymphocytes recognizing donor antigen through both direct and indirect MHC-allorecognition.

#### 3.1.1 Cell proliferation assays

Cell proliferation assays include a set of quantitative *in vitro* determinations of the T lymphocytes present in a culture before and after the addition of stimuli.

- Limiting dilution assays (LDA) consist of multiple replicates of serial dilutions of responder cells (recipient's PBMC) in wells containing a non-limiting stimulus (donor stimulator cells). This technique provides a precise quantification of immunity to a given agent and allows the estimation of frequencies of antigen-specific cells participating in a given immune response. Proliferation, CTLs cytotoxicity and cytokine secretion can be measured [84].

- Mixed lymphocyte reactions (MLR) are based on the assessment of new DNA synthesis by measuring the incorporation of tritiated thymidine. This assay allows an estimate of primary response to direct allorecognition in an entire cell population, but does not provide information on the proliferation of individual cells [85]. In the context of transplantation, the predictive value of MLR has been used for increasing the safety in IS minimization procedures through the detection of donor-specific CTL precursor frequency in either liver or kidney transplantation [86, 87]. However the predictive value of MLR is very little and it is a labour-intensive assay.

- Measurement of cell division by CFSE labelling is based on the property of carboxyfluorescein succinimidyl ester (CFSE), an intracellular fluorescent label, which disperses equally between daughter cells upon cell division in a MLR. This results in sequential halving of fluorescence intensity with each successive generation accessible by flow cytometry [88]. This method allows studying different phenotypically defined cell subsets simultaneously and it has been already proposed to distinguish rejection in living donor LT; co-expression of CD8 and CD25 on responder T cells reflected their cytotoxic activity towards donor cells, and provided evidence of low incidence of acute rejection compared to histological diagnose [89].

- Tetramer staining depends on four MHC-peptide complexes covalently linked to a fluorochrome. These MHCs can be loaded with a peptide antigen with known MHC restriction, and they are able to bind the TCR of T cells which identify the complex as antigen specific, allowing them to detect these T cells *ex vivo* by flow cytometry. Tetramers are mainly used in the clinic for immune monitoring of autoimmune and infectious diseases, like HIV or HBV. In solid organ transplantation, this technique would require unique tetramers for each donor/recipient combination and for direct and indirect immune responses [90].

### 3.1.2 ELISPOT assay

ELISPOT quantifies the frequency of memory T cells that respond to donor antigens by producing a selected cytokine, like IFN-γ, granzyme B, IL-2, IL-4, IL-5 and IL-10 *in vitro*. This assay is able to detect an alloresponse to donor antigens presented either via the direct or the indirect pathways [84]. INF-γ producing cells detected by ELISPOT have been widely reported in renal transplant as a valuable graft function predictor. It has been reported its efficacy either pre-transplant, to identify pre-sensitized patients, or early post-transplant to detect risk for immune-mediated graft deterioration [91]. Additionally, measurements of INF-γ employing ELISPOT showed an independent correlation between early cellular alloreactivity and long-term renal graft function [92, 93].

### 3.1.3 Delayed-type-hypersensivity assay

The *trans-vivo* delayed-type-hypersensivity (DTH) assay has the ability to identify donor specific unresponsiveness with linked recognition. For DTH, recipient PBMCs and donor antigen are transferred to the footpads of naïve mice. A control such as saline solution or third-party cells needs to be

performed. The measurement of the DTH-like swelling response allows for calculating and indexing alloreactivity. This assay enables the measurement of T cell reactions primed either through direct or indirect pathways [84]. DTH has the potential to distinguish between deletional tolerance (the absence of alloreactive cells and, thus, the absence of a response) and tolerance maintained by regulation (an absent response that returns when a regulatory cytokine is neutralized) [94]. Its application as a clinical monitoring tool is limited by the need to sacrifice mice.

### 3.1.4 Detection of donor-specific antibodies

Cross-matching is a routinely performed method to detect recipient antibodies responding to specific donor antigens. Concretely, *de novo* development of anti-HLA antibodies has been associated with increased acute and chronic rejection and decreased graft survival in kidney, heart, lung, liver, and corneal transplants [95]. Procedures like the complement-dependent cytotoxicity assay, ELISA, flow cytometry and recently, Luminex bead-based screening are well established techniques to identify HLA class-I and class-II antibodies in recipient blood or sera [96, 97].

### 3.1.5 Detection of alloespecific cytokines detection using flow cytometry

This method allows the individual characterization of a large number of cells. Multicolor staining permits to assess the co-expression of different cytokines in combination with surface markers, considering single cells. The introduction of secretion inhibitors to accumulate cytokines intracellularly substantially improved the signal/noise ratio, although this procedure can limit the viability of treated cells. This method has been used to investigate intracellular cytokine patterns in renal transplant recipients with and without chronic allograft

nephropathy compared to healthy controls. Specifically, the cytokine production of CD3 T cells (*e.g.*, IL-2, IL-4, IL-5 and IFN-γ) was increased in transplanted patients compared to healthy individuals [98]. IL-17 production has also been assessed through this assay, leading to report that Th17 polarization was induced by IL-1β, enhanced by IL-6 but suppressed by TGF-β and IL-12 in naïve CD4 T cells [99].

### 3.2 Antigen non-specific immune monitoring assays

Antigen non-specific assays, for the most part, determine the phenotype of surface markers or functional state of cells with the goal of identifying a pattern that is associated with a particular clinical status. Supported on technology advance, more sophisticated methods have been suggested, like high-throughput approaches which identify differentially expressed genes or proteins given the healthy and pathologic states.

#### 3.2.1 Soluble CD30 measurement

The CD30 molecule is a glycoprotein member of the tumor necrosis factor/nerve growth factor receptor superfamily. It is preferentially expressed on T cells that secrete Th2 cytokines. A soluble form of CD30 (sCD30) is released into the bloodstream after activation of CD30 T cells. Elevated serum sCD30 has been shown to correlate with increased disease activity related to predominant Th2 responses [100]. Several studies revealed good correlation between high sCD30 level and renal acute rejection [101]. This marker also predicted poor graft survival in KT [102].

### 3.2.2 Measurement of polyclonal non-antigen specific stimuli

ImmunKnow, the Cylex Immune Cell Function Assay, is the commercial name for an assay that quantifies the maximal degree to which recipient T cells can be activated by a non-donor specific, polyclonal stimulus. It consists of stimulating whole blood with PHA for 12 hours. The extent of early CD4 T cell activation is reflected by the synthesis and accumulation of intracellular ATP. This assay was designed to reflect the global state of immunosuppression and thereby facilitate decisions related to dosing immunosuppressive drugs [103].

### 3.2.3 Immune phenotyping

The flow cytometric analysis of PBMC surface markers bound by fluorochrome labelled monoclonal antibodies allows the quantification and characterization of numerous cell subsets. It is considered a fast, easy and reproducible method. In the transplantation field, phenotyping has been widely used to detect regulatory as well as effector T cells. An increased number of CD4+CD25$^{high}$ Treg has been reported in several trials studying operational liver tolerant recipients [66, 104]. Other populations detected through this method have been proposed to be suppressive T cells or useful predictors for tolerant patients; like CD3+CD4-CD8- DN T cells, CD8+CD28- Ts [105], the ratio of V$\delta$1/V$\delta$2 T cells [66] or the frequency of plasmacytoid and monocytoid DC precursors [106].

### 3.2.4 TCR repertoire

Analysis of the T cell receptor landscape is a method for defining changes in the TCR repertoire. These alterations are measured as the proportion of T cells using different variable TCR chains and simultaneously assessing the CDR3 length distributions of each gene product. For this assay, variable chain genes are specifically amplified from complementary DNA (cDNA) and subsequently

subjected to labelling runoff reactions. Products are analysed on DNA-sequence readers and plotted into Landscape-groups displaying frequency and size distribution of redundant TCR chains. An overrepresented TCR could indicate the expansion of alloreactive T cells, possibly responsible for mediating rejection. It may also reveal the expansion of protective regulatory T cells [107, 108]. However the lack of donor specificity may provoke that non-related antigens, like viral infections, induce an erroneous interpretation of the results.

### 3.2.5 Gene expression analysis

In the field of transplantation, therapeutic decisions are often based on histopathological results of graft biopsies. As correlation of histologic features with graft function is not always sufficient, additional sources for diagnosing and immune monitoring have been developed. Gene expression analysis rose as a powerful tool for the discovery of molecular fingerprints that underlie human disease.

This technique was developed for highly efficient and multiplexed sequencing of human genomes, and provided the option to analyse whole transcriptomes, meaning tens of thousands of mRNAs expressed in a given tissue at a specific time.

#### 3.2.5.1 Real-time PCR

Real-time PCR is a sensitive technique, which allows the rapid quantification of minute amounts of target mRNAs by determining the number of amplicons generated after each PCR cycle. Expression levels of the molecule of interest are monitored through the fluorescence of dyes or probes introduced into the reaction, which is proportional to the amount of product formed. The main

limitation of real-time PCR is the restricted number of genes that can be examined.

Using this technique, markers in graft biopsies have been described to help predicting acute rejection as well as ongoing chronic allograft dysfunction [109]. In a different clinical context, real-time PCR revealed an increased Foxp3 but reduced Granzyme B transcription in a group of kidney and bone marrow recipients who completely discontinuated IS [110].

#### 3.2.5.2 Microarray analysis

A microarray is a high-density array of cDNA or oligonucleotide probes immobilized on a solid support. Relative gene expression in a sample is determined by using a dual-colour fluorescent dye system, visualizing hybridization of probe sequences and reference samples. The power of this technology lies in the fact that it provides means to survey thousands of genes simultaneously [111]. It is a useful tool for discovering gene expression differences but presents challenges with respect to data analysis.

This technique is considered a promising tool as predictor for the identification of gene sets capable to prognosticate a defined syndrome or pathology. In transplantation, DNA microarrays have provided insights into the immune response leading to acute transplant rejection [112] and also generated gene expression profiles to distinguish stable from chronically injured allografts [113]. In addition, it has been reported that this tool constitutes a valid strategy to characterize TOL kidney and TOL liver recipients [42, 114, 115]. Despite of this, all data published until now need to be validated in appropriately designed clinical trials.

## 4. Genomics: a versatile tool for monitoring transplant recipients

Living the Era of the Human Genome brings the possibility to identify thousands of biomarkers in a relatively high-throughput fashion. The nature of end stage organ failure, the deficiency in donor organ availability and the toxicity resulting from long-term IS require standardised biomarkers as diagnostic tools. These biological markers would help diagnose disease in its early stages, predicting prognosis, suggesting treatment options and subserving in the implementation of therapies [116].

Transplant genomics refers to the analysis of genomes and functional genomics (meaning complete understanding of genome features) through global approaches for a clear comprehension of the functions of genes and proteins involved in the field of transplantation [117].

The matter of functional 'omics' includes a set of new technologies: microarray, proteomics, metabolomics and antibody-array technologies, which are capable of generating large amounts of information. The study of data arising from these techniques may develop novel source of biomarkers, and may also elucidate the biological mechanisms responsible for clinical processes such as acute and chronic rejection, drug toxicity or tolerance.

### 4.1 Transplant biomarkers

A biomarker is defined as a parameter objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention [118].

New genomic technologies have changed the accepted definition of a biomarker by expanding the concept to include a set of molecular measurements and complex patterns of interacting networks. In contrast to

functional biomarkers, there is another category of surrogate or disease-associated biomarkers [116]. They are intended to substitute a clinical endpoint (a characteristic used to assess the effect, benefit or risk, of a therapeutic intervention in a clinical trial) [118]. An example of surrogate biomarker is the carcinoembryonic antigen, found in the blood of patients with colon cancer [119].

The first studies searching for transplant biomarkers were performed in kidney recipients. Parameters like proteinuria, lymphocyturia and urinary proteins were described as human renal allograft predictors, although none of them was validated for clinical application [120-122].

At the present time, there is an increasing research for non-invasive transplant biomarkers, more specific and sensitive, to replace the biopsy as the gold standard for diagnosing graft function.

An ideal transplant biomarker should be able to predict outcomes prior to and after transplantation, prognosticate the onset and severity of specific events (*e.g.*, acute rejection) and also forecast injury responses due to immunosuppressive treatment.

These new promising technologies include gene and protein expression and metabolite profiling. They are still evolving although many studies have been performed including basic science and clinical trials. The resulting data require biological interpretation, through software applications, and novel identified biomarkers must be validated for their use as reliably clinical predictors.

## 4.2 DNA microarrays

Gene expression profiling is, by far, the most implanted 'omic' tool to monitor the function and immune status of the transplant. Microarrays have been increasingly introduced in various organ transplant studies including different pathological contexts. They have been used to either identify biomarkers or to predict rejection and tolerance, or to improve knowledge related to allograft dysfunction in studies on liver [115], heart [123], pancreas [124], kidney [43, 125, 126] and lung [127].

Despite of this, the scientific community believes that the potential of this technology is marginally exploited as there are still distinct and decisive issues to be discerned. The complexity of data analysis, assay reproducibility among different centres or laboratories, in addition to better global comprehension related to pathways that determine allograft progression remain to be clarified before microarrays can be used in clinics.

### 4.2.1 Data analysis

The large amounts of mRNA transcripts generated using DNA microarray technology could be useful generating transplant biomarkers via two different strategies:

- 'Class comparison' studies, where the goal is to determine whether the average expression pattern in one group (class) of specimens differs from that in another, and what genes appear to be responsible for the differences [128].

The selection of these genes involves two steps. The first step is to rank the genes. For this purpose, significance analysis of microarrays (*SAM*) can be used [129]. *SAM* includes the *t*-statistic, and assigns a score to each gene based on the change in gene expression related to standard deviation of

repeated measurements. The next step implies selecting a cutoff level within the ranking genes. This procedure is dependent on the false discovery rate (FDR) of the considered genes (a FDR of 10-20% is desirable [130]).

- 'Class prediction' studies, which imply the formulation of a rule among genes, gene signature, distinguish classes (*e.g.*, rejection versus normal). These studies consider an ensemble of genes and their interactions, and their performance employs a rigorous approach involving an independent test sample from the training set employed to formulate the rule [131]. In this type of studies it is important to avoid overfitting, which generally occurs when a model is excessively complex, as it has too many degrees of freedom, in relation to the amount of available data. Another critical point is creating classification rules. Effective classifier techniques are based on nearest centroids (meaning the mean of the expression level for a given gene list in a specimen class) and prediction around medioids (PAMs) [132]. The formulated classification rule has to be evaluated in a separate independent test sample.

The generation of gene expression profiles could provide researchers with data to decipher functional networks or mechanistic bounds within defined regulatory pathways. For this identification, gene set analysis (GSA) is a utile strategy for gene expression data investigation based on pathway knowledge. GSA focuses on sets of related genes. The employed methods are able to detect biologically relevant signals through the analysis of all of the available gene expression data. Moreover, GSA incorporates prior knowledge of biological pathways and from new developed gene sets [133]. Statistics for such analysis are focusing on either sample or gene randomization.

- Gene Set Enrichment Analysis (GSEA) is a representative sample randomizing method test that evaluates microarray data at gene sets level. Its goal is to determine whether members of a gene set tend to occur toward the top (or bottom) of a given list, in order to correlate specific groups of genes with a phenotypic class distinction [134]. GSEA is especially useful when gene expression changes in a given microarray data set is minimal or moderate.
- Parametric analysis of gene set enrichment (PAGE) is a gene randomization method testing the significance of gene sets based on permutations of gene labels or parametric distributions over genes [133]. PAGE applies to large expression datasets with multiple samples per experimental condition, and identifies significantly changed biological themes irrespective of data analysis methods or microarray platforms [135].

### 4.2.2 Limitations of the assays

While designing microarray studies it is fundamental to avoid any systematic bias.

- Disproportion between number of candidate genes (predictors) and the restricted number of available cases. This fact could provoke that several sets of genes could accurately classify randomized samples in a 'class prediction' study. Validation of the predictive model with an independent set of data is required. Internal validation strategies have been developed to address the biased estimate of accuracy.

A) One experimental approach is the 'split-sample validation', that divides the data into a training set for model development, and a test set for evaluation of the predicting accuracy of the model. A restriction of this procedure is the

inefficient use of the data, which may be too small in the generated groups [136].

B) Cross-validation methods are a different alternative. Such methods are based on repeated partitioning of the sample into a relatively large portion that is used for classifier development and a small portion that is used for classifier evaluation and then averaging the results over the multiple partitions. One representative model is the 'leave-one-out-cross-validation' (LOOCV), which generally performs very well in small sample sizes [137]. This method consists of using all but one of the training set data points and the process is repeated as many times as there are biologically independent samples.

- Intra-platform variability may cause experimental 'noise'. This interference appears when assays are performed in different laboratories, on different batches or by different researchers. Standardized protocols are required to achieve a good level of reproducibility [138].

- An incomplete view of the functional significance of differentially expressed genes, as information regarding protein expression levels does not necessarily correlate with mRNA expression levels. A better understanding of the studied condition could be inspired by the conjunction and cross-referencing of complementary information (*e.g.*, blood and tissue gene expression, protein post-translational modification, localization and interactions) [139].

### 4.2.3 Future perspectives

Ongoing and future multicenter studies in addition to meta-analyses of actual data, would enable validation of new sets of transplant biomarkers. A combination of genomics and the arising proteomics [140], antibiomics [117] and metabolomics assays [141] could yield powerful screening tools for

monitoring the alloimmune response in the transplant setting. Integrative analysis (antibiomics and genomics) and protein array analysis have been already reported by Li *et al.* [142]. Specifically, they provided an immunogenic and anatomic roadmap of the most likely non-HLA antigens that can generate serological responses after renal transplantation.

# II. EXPERIMENTAL PROJECTS

# 1. γδ T cells in transplantation

## 1.1 MAIN GOALS

The research of clinically-applicable biomarkers of operational tolerance involves an exhaustive study of molecules, cell populations and biological pathways that could induce tolerogenic conditions within an allograft.

In this study we focused on the contribution of peripheral blood γδ T cell subsets to the effector and/or regulatory arm of the alloimmune response in the context of organ transplantation.

Therefore we addressed the following specific aims:

1) Detailed investigation of the phenotype, functional properties and repertoire of Vδ1 and Vδ2 T cell populations in a cohort of liver recipients to improve the understanding of their role in clinical allograft transplantation.

2) Whether the type of transplanted organ, the regimen of immunosuppression and the presence of persistent viral infections could influence the distribution and properties of γδ T cell subsets.

3) Evaluation of the clinical value of γδ T cell subsets quantification as biomarkers to identify operationally tolerant liver transplant recipients.

## MATERIAL AND METHODS

### 1.2.1 Patients

Patients included in this study were from Hospital Clinic Barcelona (Spain) and from University of Rome “Tor Vergata” (Italy). The local Ethics Committees of both centers approved all aspects of the study and patients gave their informed consent.

Peripheral blood samples were collected using heparinized tubes from the following groups of patients (Table 1):

- TOL: 29 operationally tolerant liver transplant recipients intentionally weaned from immunosuppressive therapy under medical supervision. Inclusion criteria employed in selecting patients for immunosuppression weaning in the participating institutions were as follows: i) more than three years after transplantation; ii) single-drug IS; iii) absence of acute rejection episodes in the previous 12 months; iv) absence of signs of acute/chronic rejection in liver histology; and v) absence of autoimmune liver disease before or after transplantation. Blood was collected more than one year after successful immunosuppressive drug discontinuation.

- STA-Liver: 201 liver transplant recipients receiving single low-dose immunosuppressant and showing stable graft function. They also fulfilled the aforementioned clinical criteria for drug weaning.

- STA-Kidney: 50 kidney transplant recipients on maintenance IS (double or triple therapy based on either CsA, FK or sirolimus) with normal creatinine serum level and absence of proteinuria,

- ESLD: 50 patients with chronic end-stage liver disease listed for transplantation.

- CONT: 34 healthy volunteers with normal blood formula and no infections or other concomitant pathology.

Patient groups were age-matched.

**Table 1: Demographic and clinical data of patient groups included in the study**

| Clinical diagnosis | Number | Age [A] | Gender | Time from transplantation (years) [A] | Treatment [B] | Center [C] |
|---|---|---|---|---|---|---|
| Operationally tolerant (TOL) | 29 | 61(29-75) | 67% Male | 13 (6-19) | | B, R |
| Stable liver recipients (STA-Liver) | 201 | 57 (24-78) | 69% Male | 8 (3-20) | 47% FK 37% CsA 11.5% MMF 4% Rapa 0.5% AZA | B, R |
| Stable kidney recipients (STA-Kidney) | 50 | 61 (32-81) | 48% Male | 10 (4-18) | 48% Rapa based 48% CsA based 4% FK based | B |
| End stage liver disease (ESLD) | 50 | 55 (26-79) | 76% Male | | | B |
| Healthy controls (CONT) | 34 | 56 (42-72) | 40% Male | | | B |

[A] Median (range)
[B] AZA, azathioprine; CsA, cyclosporine A; FK, tacrolimus; MMF, mycophenolate mophetil; Rapa, rapamycin
[C] B, Hospital Clinic Barcelona, Spain; R, University Tor Vergata Rome, Italy

### 1.2.2 Peripheral blood immunophenotyping

#### Surface cell staining

100 μl of whole blood were incubated with the appropriate amount of antibody (following recommendations specified on the technical data sheet) during 15 minutes at room temperature (RT) in the dark.

Erythrocytes were lysed adding 2 ml of 1X BD FACS lysing solution (BD Biosciences, San Jose, CA, USA), vortexed and incubated 15 minutes at RT in the dark.

Cells were washed with PBS (AccuGENE, Lonza, Verviers, Belgium) and the pellet was resuspended in 500 μl of Fixation Buffer: PBS 2.5% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and 1% FCS (Biosera, East Sussex, UK).

Fluorescent cell surface monoclonal antibodies directed against the following targets were tested: CD3, CD4, CD8, CD25, CD28, CD56, CD16, CD19, CD45RA, CD62L, CCR7, HLA-DR, γδ TCR, αβ TCR, NKG2D and PD1 (from

BD Biosciences); NKG2A, NKG2C and GITR (from R&D Systems; Minneapolis, MN, USA), Vδ1 TCR (from ThermoScientific; Waltham, MA, USA); and Vδ2 TCR (from Immunotech; Marseille, France).

Donor cell chimerism was analyzed employing fluorescent monoclonal antibodies specific for HLA-A1 (One Lambda, Inc. CA, USA) and HLA-A2 (BD Biosciencies).

**Intracellular staining**

For intracellular staining, cells were pretreated as mentioned above performing cell surface staining and erythrocytes lyses.

Cell fixation and permeabilization was then performed by adding 500 µl of Cytofix/Cytoperm (BD Biosciences). Cells were vortexed and incubated at RT in the dark for 20 min. Permeabilized cells were then washed with 2 ml BD Perm/Wash™ buffer, spinned (5 min- 500 ×g- 4ºC) and supernatant decanted.

The pellet was resuspended in residual washing buffer containing an optimal concentration of fluorochrome-conjugated antibody specific for intracellular target protein. Incubation lasted 30 min at 4ºC in the dark. Cells were washed with Perm/Wash™ buffer (5 min- 500 xg- 4 ºC). The pellet was finally resuspended in Fixation Buffer. FACS acquisition was performed immediately.

The following monoclonal antibodies were tested: Foxp3 (eBioscience, San Diego, CA, USA), CTLA4 and perforin (both from BD Biosciences). Background fluorescence was assessed with appropriate IgG isotypes for each of the tested antibodies.

**Cytokine staining**

This procedure involves activation of PBMCs prior to intracellular cytokine detection.

PBMCs were isolated from whole blood using Ficoll density gradient (Histopaque 1077, Sigma-Aldrich).

-- To perform IL-10 and IFNγ staining (both from BD Biosciences), $1x10^6$ PBMCs were resuspended in 500 µl RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% male human AB serum, 1% pencillin-streptomycin and 10 mM L-glutamine (all from Biosera), and cultured in 24-well flat bottom plates (Greiner Bio-one, Frickenhausen, Germany) during four hours together with PMA (50 ng/ml, Sigma-Aldrich), ionomycin (1 µg/ml, Sigma-Aldrich) and brefeldin A (10 µg/ml, BD Biosciences). Cells were then harvested, washed and surface stained prior to intracellular staining using the Caltag Fixation/Permeabilization kit (Invitrogen, Carlsbad, CA, USA). Non-stimulated cells were used as control.

-- For IL-17A staining (BD Biosciences), PBMCs were first pre-stimulated for 1 week in the presence of IL-2 (100 UI/ml, Sigma-Aldrich). On day 4 the culture was re-stimulated. The staining procedure included cell surface and intracellular staining similar to the above mentioned cytokines.

All data acquisition was performed using a BDFacs Canto II flow cytometer (BD Biosciences), data analysis was conducted using FlowJo Software (Tree Star, Inc., Ashland, OR, USA) and GraphPad Prism (GraphPad Software, LaJolla, CA, USA) was employed to perform statistics.

### 1.2.3 Sequencing Vδ1 TCR CDR3

#### RNA extraction and cDNA preparation

PBMCs were separated from whole blood using Ficoll density gradient, and resuspended in TRIzol Reagent (1 mL TRIzol/$10^6$ PBMCs; Invitrogen). RNA isolation was conducted according to the manufacturer description. The following protocol was performed using RNAse-free tubes (from Ambion Inc., Austin, TX, USA) and filtered tips (Eppendorf AG, Hamburg, Germany) and pipettes for RNA-use only.

1. 200 μl of Chloroform (Sigma-Aldrich) were added to each tube, vortexed vigorously (10sec), incubated 2 min at RT and centrifuged (15 min- 12000 xg- 4ºC).
2. Upper phase was decanted using a 200μl pipette into a fresh tube.
3. 500 μl of Isopropanol (Sigma-Aldrich) were added and vortexed. Tubes were incubated 10 min at RT and centrifuged (10 min-15700 xg- 4ºC).
4. Supernatant was decanted and 800 μl of cold 70% EtOH (Sigma-Aldrich) were added.
5. Tubes were centrifuged (5 min- 7600 xg- 4ºC). Supernatant was decanted and air dried for 5-10 min.
6. Extracted RNA was resuspended in 20 μl of RNAse-free water (Ambion).

Genomic DNA was digested using the TURBO DNA–free kit (Ambion). RNA quantity and quality were determined through Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

1μg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA, USA) and following the manufacturer instructions. The procedure was performed using a Mastercycler (Eppendorf) programming the next conditions:

-- 10 min 25ºC
-- 120 min 37ºC
-- 5 sec 85ºC

### Vδ1 TCR chain CDR3 amplification

cDNA was used to amplify the Vδ1 TCR chain CDR3 employing primers specific for the variable Vδ1-region (designated VD1) and the constant Cδ-region (CD1) as described by Fujishima *et al.* [65].

Polymerase chain reaction (PCR) was performed for 40 cycles in a 20 µl reaction mixture containing Platinum *Taq* (Invitrogen) DNAPolymerase. The oligonucleotide sequences of the VD1 and CD1 primers were as follows:

- VD1: GTGGTCGCTATTCTGTCAACT
- CD1: AACAGCATTCGTAGCCCAAGCAC

PCR reagents:

| | |
|---|---|
| $H_2O$ | 11,5 µl |
| Taq | 0,3 µl |
| dNTPs | 1,0 µl |
| 10x Buffer | 2,5 µl |
| cDNA | 2,0 µl |
| $MgCl_2$ | 0,7 µl |
| Primer VD1 | 1,0 µl |
| Primer CD1 | 1,0 µl |
| | 20,0 µl |

PCR conditions: denaturation at 94°C for 1 min;
annealing at 55°C for 1 min;
extension at 72°C for 1.5 min.

PCR amplicons were gel extracted [1% Agarose in TAE Buffer (Gibco, Invitrogen) + SYBR®Green (5µl/100ml, Invitrogen)] applying a constant voltage of 120 V during 30 min to the electrophoresis chamber. DNA bands were visualised in a transilluminator (VilberLourmat, Cedex, France), cut out with a scalpel and extracted from the gel using the QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany).

**Cloning and transformation into *E.coli***

Eluted DNA was cloned into a TA cloning plasmid [either pGEM-T vector (Promega, Madison, Wisconsin, USA) or PCR 2.1 TOPO cloning vector (Invitrogen)] incubating the reagents during 4 hours at 14ºC.

| | |
|---|---|
| PGEM-T | 1 μl |
| Buffer ligation | 5 μl |
| T4 Ligase | 1 μl |
| DNA eluted | 3 μl |
| | 10 μl |

The plasmid (4 μl) was transformed into chemically competent *E.coli* (TOP 10, Invitrogen). Therefore, bacteria were thawed on ice and incubated for 20 min on ice.

Transformation of the bacteria was achieved via heat shock for 45 sec at 42ºC. An antibiotic-free incubation period was performed in 200 μl of S.O.C. medium (Invitrogen) during 1h at 37ºC.

Following this, 100 μl of S.O.C. culture were plated on LB-Agar plates containing 50 μg/ml Ampicillin (Sigma-Aldrich), 40 μl of X-Gal and 50 μl of IPTG (both from Sigma-Aldrich) and grown overnight at 37ºC.

**Plasmid Minipreparation**

According to blue/white screening 30 colonies were picked and individually inoculated in tubes containing 2 ml of LB medium. Bacteria grew overnight shaking (250 rpm) at 37ºC.

Bacteria were centrifuged at 4000 rpm for 10 min and supernatant was decanted.

Plasmid-DNA was prepared using a QIAprep Spin Miniprep kit and Vacuum manifold (Qiagen), following the instructions from the manufacturer.

**BigDye terminator PCR and sequencing**

The sequencing runoff reaction was performed using the BigDye Terminator Cycle Sequencing Kit (Version 3.1, Applied Biosystems).

PCR reagents:

| | |
|---|---|
| DNA sample | 1 µl |
| Big Dye Mix | 1 µl |
| Buffer (4ºC) | 1 µl |
| Primer (M13) | 1 µl |
| $H_2O$ | 16 µl |
| | 20 µl |

PCR conditions:

| | | |
|---|---|---|
| 1 min | 96ºC | |
| 10 sec | 96ºC | 25x Cycles |
| 15 sec | 55ºC | |
| 4 min | 60ºC | |

Sequence analysis was conducted using an ABI Prism 3730 automated DNA sequencer (Applied Biosystems).

## 1.3 RESULTS

Parts from the results presented here have been published in the following article: *Characterization of γδ T cell subsets in organ transplantation* (Puig-Pey *et al.*, 2010, May 5).

### 1.3.1 Immunophenotypic results

Two recent reports described that the distribution of Vδ1 and Vδ2 T cells was altered in the peripheral blood of tolerant liver recipients compared to non-tolerant and healthy individuals [66, 143]. We addressed whether these differences were detectable in a large cohort of liver and kidney recipients (29 TOL, 201 STA-Liver and 50 STA-Kidney), as well as in 50 patients listed for liver transplantation (ESLD) and 34 healthy volunteers (CONT).

All transplant recipients exhibited increased γδ T cell numbers as compared with healthy individuals (Figure 1A), and this was mainly attributable to an expansion of the Vδ1 T cell subset (Figure 1B). Vδ2 T cells were reduced in both liver and kidney recipients in comparison with healthy individuals (Figure 1C). As a result, Vδ1 T cells constituted the most abundant γδ T cell subset in peripheral blood of transplant recipients regardless of the type of organ being transplanted and of whether IS was administered or not. As observed in Figure 1D, a shift in the Vδ1/Vδ2 ratio was detected due to the mentioned alterations in γδ T cell subsets in comparison to non-transplanted groups (ESLD and CONT). The top Vδ1/Vδ2 ratio value was found in TOL liver recopients.

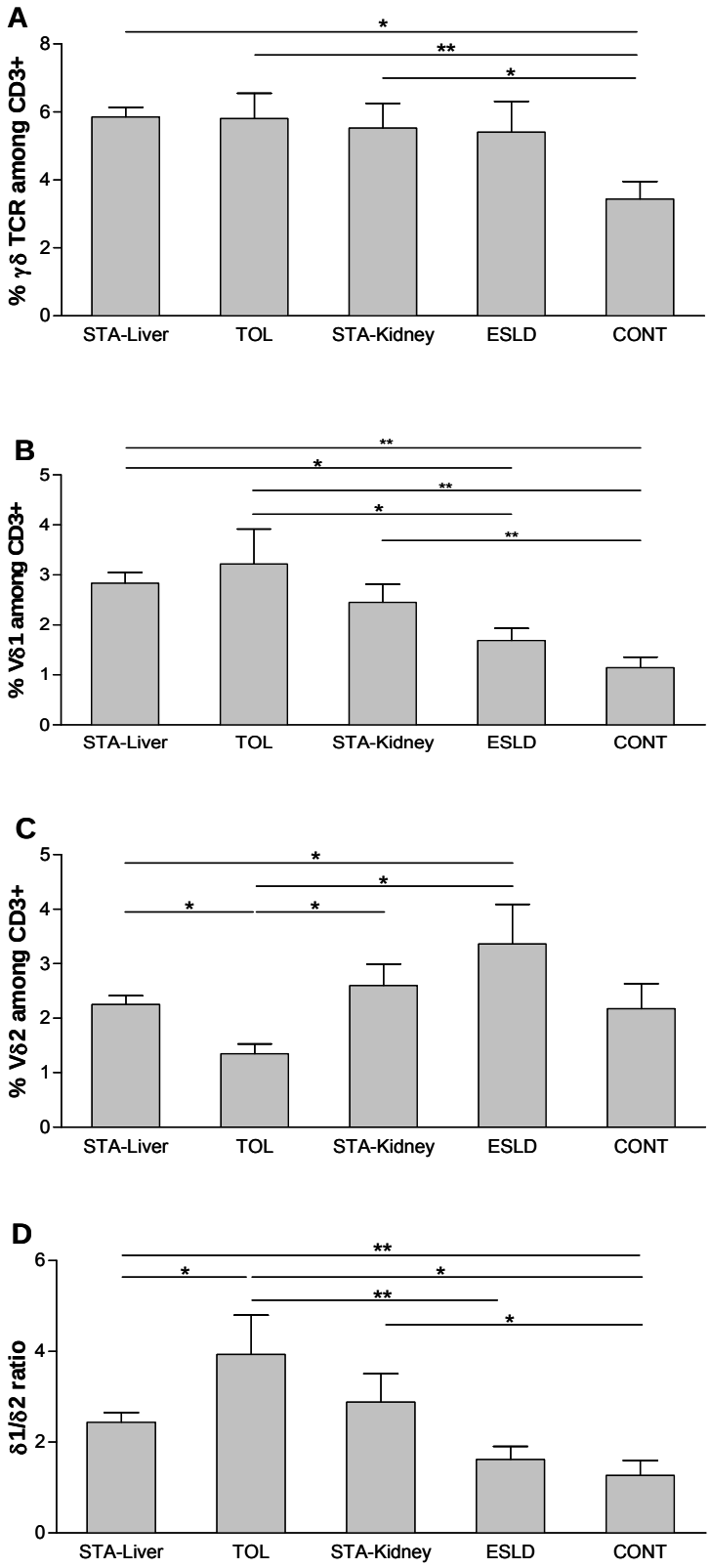


**Figure 1. Quantitative differences of γδ T cell subsets between TOL, STA-Liver, STA-Kidney, ESLD and CONT patient groups.** Proportion of γδ (A), Vδ1 (B) Vδ2 (C) T cells among peripheral blood CD3+ mononuclear cells. (D) Calculated Vδ1/Vδ2 T cell ratio of peripheral blood mononuclear cells. Bar plots represent mean (±SEM) values. Kruskall-Wallis test was

employed in γδ TCR analysis. ANOVA and Least Significant Difference (LSD) were used as post-hoc test for the residual parameters. (*)= *P*-value<0.05; (**)= *P*-value<0.01; (***)=*P*-value<0.001.

Similar differences were reported when absolute numbers of γδ T cells and their main subsets were analyzed using total lymphocyte count.

We investigated if the relative frequencies observed in γδ T cells blood phenotype were stable over time. Therefore, we immunophenotyped and analyzed two blood samples collected 14 months apart (ranging between 9 to 18 months) from a cohort of 30 STA-Liver recipients. We assessed the correlation of the measured values over time employing the *intraclass correlation coefficient* (ICC) [144]. The ICC is a measure that can be used to quantify the reproducibility of a variable, and it is a degree of the homogeneity within groups of replicate measurements relative to their total variation. The maximum value of the ICC is 1 (indicating similarity among samples), and the minimum is theoretically 0.

Comparison of the total γδ, Vδ1 and Vδ2 T cell frequencies and the subsequent ratio revealed not significant differences among the values over the established time points (Figure 2).

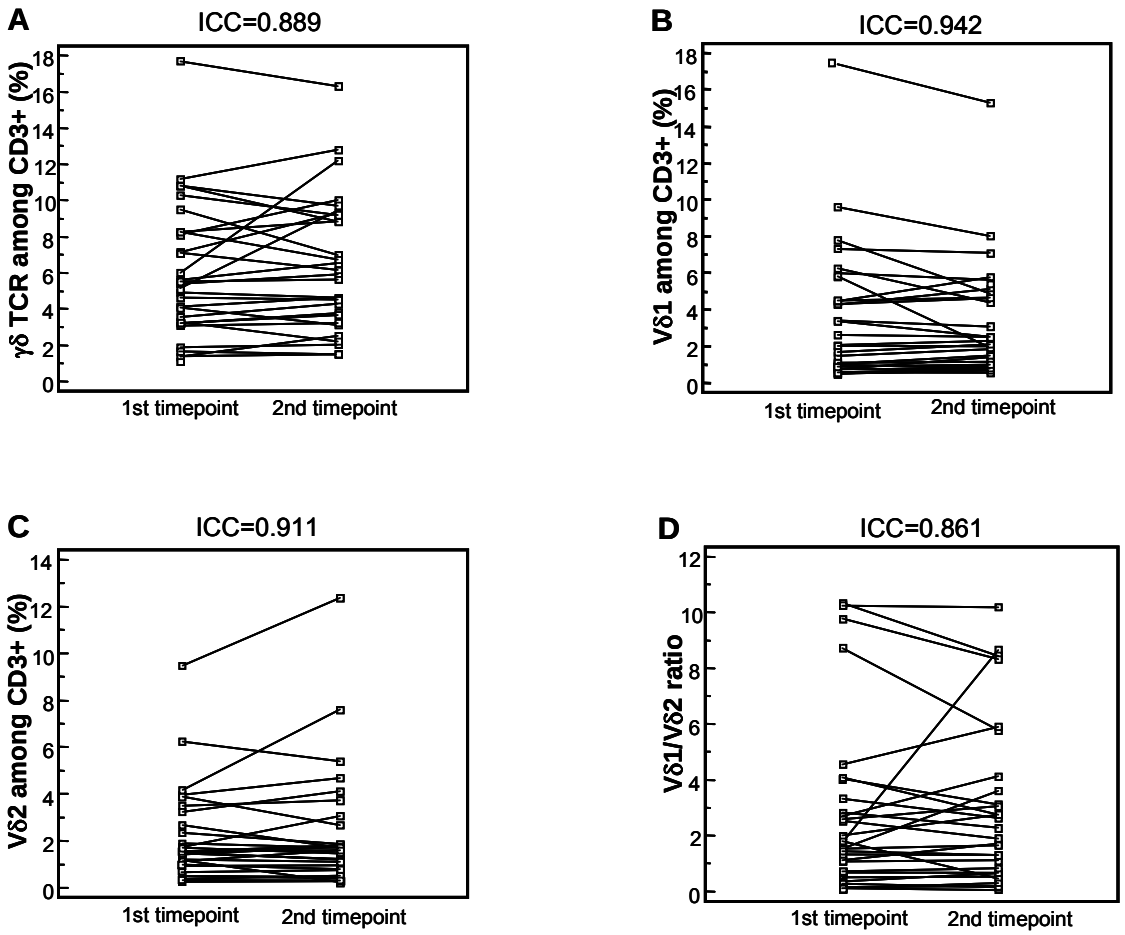


**Figure 2. The frequencies of γδ T cell subsets are stable over time in STA-Liver recipients.** Comparison of the relative number of γδ (A), Vδ1 (B), Vδ2 (C) T cells and Vδ1/Vδ2 ratio (D) in 2 sequential peripheral blood specimens obtained 14 months apart from 30 STA-Liver recipients. Calculated ICC value for each parameter is specified.

More drastic changes were observed in Vδ1/Vδ2 ratio measurements. Due to the mathematics of this parameter, the changes detected in the single subpopulations were amplified when the ratio was calculated.

In summary, as all ICC values were greater than 0.75, indicating an excellent agreement, we considered the size and subset distribution of the γδ T cell compartment in STA-Liver recipients as invariable over a substantial period of time.

We performed an exhaustive phenotypic and functional analysis to understand the role of Vδ1 and Vδ2 T cells in transplantation. A subset of 19 liver recipients (nine TOL and ten STA-Liver) and a group of eight age-matched non-transplanted healthy individuals (CONT) were examined using flow cytometry. Therefore, combinations of cell surface and intracellular markers together with *ex vivo* cytokine production were assessed (Table 3). Due to the fact that CONT harbor under-represented numbers of γδ T cell subsets, their phenotypic profile did not yield results suitable for analysis.

We performed the same calculus on the basis of total lymphocyte count (TLC), and we observed similar results compared to relative quantification. The mean absolute cell count of each subpopulation is shown in Table 3.

**Table 3. Immunophenotypic profile and *ex-vivo* cytokine production of Vδ1 and Vδ2 T cell subsets from 19 liver recipients.**

| | **Vδ1 T cells [%(SEM)]** | **Vδ2 T cells [%(SEM)]** | |
|---|---|---|---|
| | | | *P* value |
| **CD4** | 2.11 (0.47) | 0.86 (0.26) | 0.0276 |
| **CD8** | 21.85 (5.04) | 3.44 (1.16) | 0.0014 |
| **CD45RA-CCR7- ($T_{EM}$)** | 11.68 (1.73) | 92.18 (0.95) | <0.0001 |
| **CD45RA+CCR7- ($T_{EMRA}$)** | 83.10 (2.46) | 5.80 (0.77) | <0.0001 |
| **NKG2A** | 23.69 (3.79) | 57.94 (3.98) | <0.0001 |
| **NKG2C** | 21.71 (3.69) | 4.73 (0.86) | 0.0001 |
| NKG2D | 86.04 (2.61) | 92.54 (2.87) | ns |
| **HLA-DR** | 8.17 (1.61) | 3.88 (1.16) | 0.0375 |
| **CD28** | 12.74 (2.71) | 75.71 (4.51) | <0.0001 |
| **KLFR1** | 63.59 (5.63) | 23.46 (6.34) | <0.0001 |
| GITR | 4.76 (1.17) | 4.13 (1.00) | ns |
| PD-1 | 11.43 (2.24) | 5.61 (2.51) | ns |
| Foxp3 | 0.83 (0.44) | 1.08 (0.76) | ns |
| **CTLA-4** | 8.98 (1.16) | 4.16 (0.72) | 0.001 |
| **Perforin** | 11.26 (2.85) | 4.09 (1.10) | 0.0221 |
| **INFγ** | 24.44 (2.85) | 45.91 (4.89) | 0.0006 |
| IL10 | 8.62 (1.36) | 8.64 (2.55) | ns |
| **IL17-A** | 6.30 (0.90) | 2.11 (0.32) | 0.0002 |
| Mean absolute cell count ($10^9$/L) | 57.52 (16.90) | 36.64 (7.23) | |

ns: non significant

We employed the same surface markers and cytokines to establish the phenotypic differences of Vδ1 and Vδ2 T cells among TOL and STA-Liver recipients. Both groups expressed a similar expression profile among the

analyzed markers. Comparable results were achieved after analysis was conducted on the basis of relative frequency (Table 4) and total lymphocyte number (mean TLC for each group of recipients appears in Table 4).

**Table 4. Differences in γδ T cell subset phenotype and cytokine secretion between TOL and STA-Liver patients.**

| | Vδ1 T cells [%(SEM)] | | | Vδ2 T cells [%(SEM)] | | |
|---|---|---|---|---|---|---|
| | TOL (n=9) | STA-Liver (n=10) | *P* value | TOL (n=9) | STA-Liver (n=10) | *P* value |
| CD4 | 2,11 (0,83) | 2,11 (0,58) | ns | 0,78 (0,34) | 0,93 (0,38) | ns |
| CD8 | 29,29 (11,02) | 16,44 (3,19) | ns | 2,67 (1,63) | 4,06 (1,67) | ns |
| CD45RA-CCR7- ($T_{EM}$) | 11,73 (3,16) | 11,64 (2,04) | ns | 93,83 (1,09) | 90,99 (1,37) | ns |
| CD45RA+CCR7- ($T_{EMRA}$) | 82,26 (4,44) | 83,71 (2,91) | ns | 4,05 (0,96) | 7,04 (1,04) | ns |
| NKG2A | 19,22 (5,22) | 26,53 (5,24) | ns | 58,59 (7,28) | 57,49 (4,81) | ns |
| NKG2C | 22,80 (7,03) | 21,02 (4,34) | ns | 4,72 (1,62) | 4,73 (1,02) | ns |
| NKG2D | 84,99 (4,35) | 86,80 (3,38) | ns | 88,40 (6,27) | 95,85 (0,91) | ns |
| HLA-DR | 9,95 (2,31) | 6,74 (2,23) | ns | 1,82 (0,43) | 5,53 (1,94) | ns |
| **CD28** | 9,87 (4,14) | 14,83 (3,62) | ns | 86,65 (2,80) | 66,96 (6,71) | 0,0248 |
| KLFR1 | 70,79 (9,53) | 58,55 (6,20) | ns | 25,15 (12,93) | 22,13 (5,99) | ns |
| GITR | 5,98 (1,86) | 3,39 (1,30) | ns | 4,98 (1,69) | 3,27 (1,10) | ns |
| PD-1 | 12,68 (3,33) | 10,18 (3,13) | ns | 9,27 (4,79) | 1,96 (0,64) | ns |
| Foxp3 | 1.15 (0.68) | 0.29 (0.29) | ns | 1.73 (1.16) | 0.01 (0.00) | ns |
| CTLA-4 | 9,72 (1,05) | 8,31 (2,02) | ns | 4,76 (1,19) | 3,66 (0,88) | ns |
| **Perforin** | 5,30 (1,89) | 17,21 (4,69) | 0,0315 | 2,30 (1,07) | 5,69 (1,77) | ns |
| INFγ | 22,71 (3,34) | 25,98 (4,63) | ns | 47,46 (7,25) | 44,53 (7,00) | ns |
| IL-10 | 10,27 (2,41) | 7,34 (1,53) | ns | 11,31 (4,36) | 6,27 (2,86) | ns |
| IL17-A | 7,05 (1,30) | 5,44 (1,26) | ns | 2,13 (0,29) | 2,10 (0,64) | ns |
| Mean absolute cell count ($10^9$/L) | 69.11 (36.64) | 48.82 (12.67) | | 34.94 (7.49) | 37.91 (11.64) | |

ns: non significant

We further investigated the applicability of Vδ1 and Vδ2 subsets as a biomarker to discriminate TOL from liver recipients requiring IS, based on previously reported data [66, 143].

We plotted receiver operating characteristic (ROC) curves for Vδ1, Vδ2 and Vδ1/Vδ2 ratio from 29 TOL and 201 STA-Liver to determine the diagnostic utility of these parameters. ROC curve is a graphical plot of the sensitivity, or true positives, against (1 − specificity), or false positives, for a binary classifier system as its discrimination threshold is varied. A test with perfect discrimination would have a ROC plot passing through the upper left corner

(100% sensitivity, 100% specificity) [145], and the resulting area under the curve (AUC) would be 1.

Our results were not positive, as the three studied parameters showed an AUC<0.7, indicating than none of the measurements was capable of discriminating TOL from STA-Liver with accuracy (Figure 3).

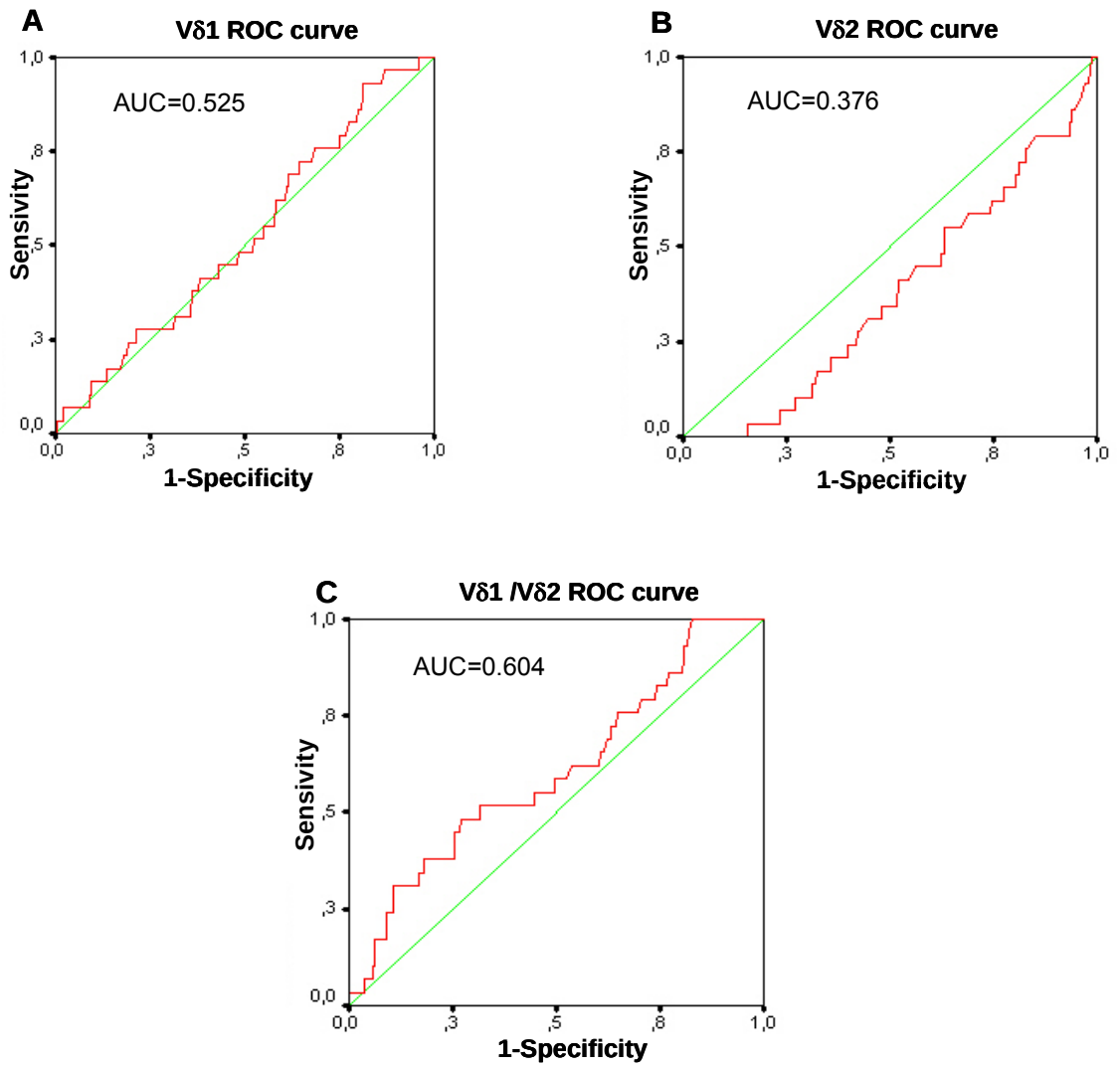


**Figure 3. γδ T cell subsets are not able to distinguish between TOL and STA-Liver using ROC curve as diagnostic test.** AUC of Vδ1 (A) and Vδ2 (B) T cells and Vδ1/Vδ2 ratio (C) of 29 TOL and 201 STA-Liver is represented.

We also employed immunophenotype analysis to determine the presence of donor-derived lymphocytes in the peripheral blood of seven TOL recipients. Donor/ recipients mismatched HLA class-I and class-II monoclonal antibodies were used, to assess their presence in CD4+, CD8+ and γδ T cells, NK and NKT cells. Studied recipients showed <1% of γδ T cell positively stained for the donor type-HLA (Table 5). The reported frequencies were not found to be significant when compared to background.

**Table 5. Percentage of donor origin cells among PBMC subsets from 7 TOL liver recipients.**

| | Donor HLA [%(SEM)] |
|---|---|
| | TOL |
| γδ TCR | 0,61 (0,23) |
| CD4+ | 0,31 (0,17) |
| CD8+ | 0,22 (0,10) |
| NK | 1,32 (0,60) |
| NKT | 1,26 (0,43) |

Despite these results, we can not exclude the presence of microchimerism at peripheral blood level, as flow cytometry might not be sensitive enough to detect it.

### 1.3.2 Effects of persistent viral infections on γδ T cell subset distribution

It has been described that persistent and/or past exposure to viral infections could account for altered distribution of γδ T cells. Therefore, we investigated the serum prevalence of different virus in a large cohort of age- and sex matched STA-Liver recipients.

We correlated the frequency of γδ, Vδ1, Vδ2 T cells and Vδ1/Vδ2 ratio with seropositivity for CMV, HSV, EVB and HCV.

- 70 HCV-positive recipients (69% male, mean age 62 years) exhibited a trend towards a decreased number of Vδ2 T cells and increased number of Vδ1

compared to 110 HCV-negative STA-Liver (66% male, mean age 55 years). That resulted in a significantly increased Vδ1/Vδ2 ratio (p= 0.0046; Figure 4A) in HCV-positive recipients.

- CMV seropositivity (112 individuals; 74% male, mean age 57 years) was associated with an expansion of Vδ1 T cells (3.22% versus 1.02%; Figure 4C), an increased Vδ1/Vδ2 ratio (p=0.006; Figure 4B) and also a higher amount of total γδ T cells (p=0.007; Figure 4D) compared with the CMV-negative group (13 individuals; 66% male, mean age 53 years).

- EBV and HSV status did not influence the number of peripheral blood γδ T cell subsets. As expected, in both cases the seronegative cohort was markedly small, due to the overall presence of these viruses.

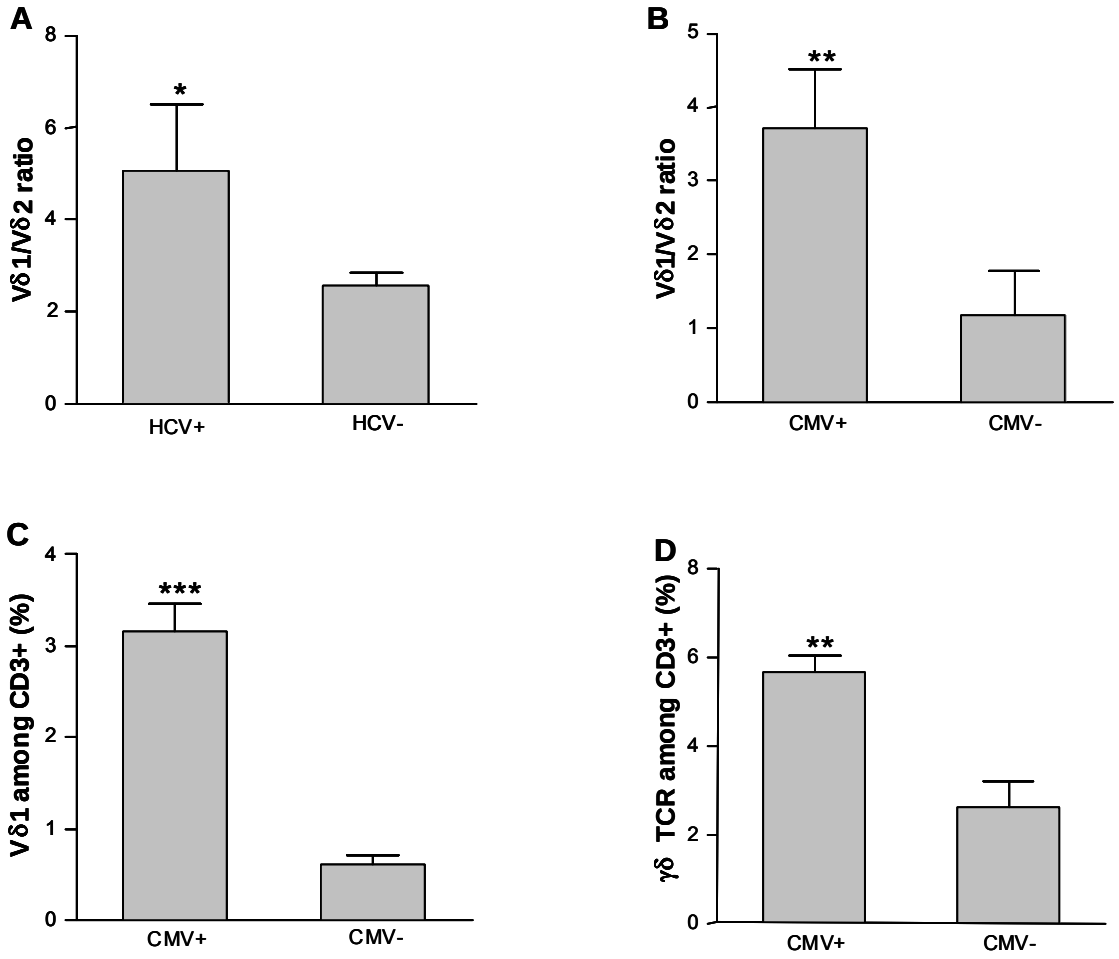


**Figure 4. Peripheral blood γδ T cells, Vδ1 and Vδ2 subsets and the subsequent ratio are**

**quantitatively altered in HCV-positive and CMV-positive in a set of liver recipients receiving maintenance IS.** (A) Vδ1/Vδ2 ratio from HCV-positive and HCV-negative STA-Liver, B) CMV-positive versus CMV-Negative liver recipients computed Vδ1/Vδ2 ratio, C) Relative amount of Vδ1 subset among CD3+ and (D) γδ T cell population among CD3+. Bar plot shows mean (±SEM). (*)=P-value<0.05, (**)=P-value <0.01, (***)=P-value<0.001 (t-test was employed for HCV cohort and Mann-Whitney test for CMV group).

Additionally, we divided the studied cohort according to CMV and HCV status in order to examine whether the reported γδ T cells alterations were attributable to CMV seropositivity. Our results showed that 91% and 90% of the HCV-positive and the HCV-negative were also seropositive for CMV. This indicates that the differences observed in Vδ1/Vδ2 ratio are not related to CMV status dissimilarities.

### 1.3.3 Analysis of the Vδ1 TCR CDR3 repertoire

We studied the clonal diversity of the Vδ1 TCR in six TOL, six STA-Liver and six CONT age- and sex matched, through cloning and sequencing of the Vδ1 CDR3.

The CDR3 repertoire analysis from the TOL liver recipients revealed a global tendency towards a skewed TCR repertoire. We found an elevated mean of 58.3% of repetitive sequences among TOL, consisting of clonotypes harboring identical nucleotide sequences (Table 6). This result contrasted with a rate of 30% and 31.6 % repetitive sequences in STA-Liver and CONT, respectively. The repetitive sequences were unique to each individual and were not found when conducting a BLAST search at the NCBI GenBank database (private sequences).

We determined whether the Vδ1 T cell subpopulation shared recurrent CRD3 aminoacid motifs indicative of antigen-driven selection. Therefore we translated

all Vδ1 TCR nucleotide sequences from TOL, STA-Liver and CONT into the corresponding aminoacid. We could not find a common CDR3 motif (Table 6).

**Table 6. Aminoacid sequences of repeated polyclonal Vδ1 TCR CDR3 clonotypes in 6 TOL, 6 STA-Liver and 6 CONT.**

| Status | Vδ1 | N-D-N | Jδ1 | Colony frequency | CDR3 length |
|---|---|---|---|---|---|
| TOL | CALG | DGSGVL | DKLIFGKG | 10/33 | 13 |
| | CALGE | KEWELLGDN | TDKLIFGKG | 7/33 | 18 |
| TOL | CALG | DPPNLGGYP | YTDKLIFGKG | 27/40 | 18 |
| | CALGE | VVGPTVGDLHH | TDKLIFGKG | 4/40 | 22 |
| TOL | CALGE | PYINAFLLTGGFDLKVP | YTDKLIFGKG | 6/35 | 27 |
| | CALGE | LTPTFLLLALGAS | DKLIFGKG | 4/35 | 21 |
| TOL | CALG | DSTDGEWGGL | YADKLIFGKG | 10/33 | 19 |
| | CALGE | PPPSYESQCWGIGPLCG | TDKLIFGKG | 10/33 | 26 |
| | CALG | ASTFLLWGIRT | YTDKLIFGKG | 4/33 | 20 |
| TOL | CALG | GPTSYRIFSYWGIGW | TDKLIFGKG | 13/27 | 23 |
| | CALGE | PGFLRFYWGIR | TDKLIFGKG | 5/27 | 20 |
| TOL | CALG | DPLSRSTGGYRRGQA | DKLIFGKG | 8/33 | 22 |
| | CALGE | PFLGPT | KLIFGKG | 6/33 | 13 |
| STA-Liver | CALG | VYKEGLNWGIRKYLS | DKLIFGKG | 6/34 | 22 |
| | CALGE | PYRPAEGENP | YTDKLIFGKG | 5/34 | 20 |
| STA-Liver | CALG | DRLWGPGPLALTAQ | LFFGKG | 13/34 | 19 |
| STA-Liver | CALG | DPGGKTATGGL | YTDKLIFGKG | 6/31 | 20 |
| STA-Liver | CALG | NSHPTGYWGILRW | TDKLIFGKG | 4/25 | 21 |
| | CALG | TQIPRRVSGDHVRSWVGDML | TDKLIFGKG | 4/25 | 28 |
| STA-Liver | CALG | DTSLPTLTGGYPTRP | LIFGKG | 5/19 | 19 |
| STA-Liver | CALGE | HDPPWGIS | TDKLIFGKG | 3/19 | 17 |
| | CALGE | RRGYLK | YTDKLIFGKG | 3/19 | 16 |
| CONT | --------- | ---------- | ---------- | 0/26 | -- |
| CONT | CALG | SHHGSSSKYWGV | YTDKLIFGKG | 6/23 | 19 |
| CONT | CALGE | LPPGD | YTDKLIFGKG | 5/20 | 15 |
| | CALG | GPLPPLGWGIRG | YTDKLIFGKG | 3/20 | 21 |
| CONT | CALG | NTYRRWGIGETF | TDKLIFGKG | 6/20 | 20 |
| | CALG | LSTVGIRTYWGIFVG | TDKLIFGKG | 3/20 | 23 |
| CONT | CALGE | SLPTNGIRGSRP | LIFGKG | 2/14 | 18 |
| | CALGE | PVRTSFSWDTRQMF | FGTG | 2/14 | 18 |
| CONT | CALGE | PRRRRYSGGSV | TDKLIFGKG | 2/14 | 20 |
| | CALGE | LRPGSYALLGTPLSSWDTRQMF | FGTG | 2/14 | 26 |

Taken together, TOL recipients exhibited a significantly biased Vδ1 repertoire compared to STA-Liver (p= 0.009) and CONT (p=0.035) (Figure 5).

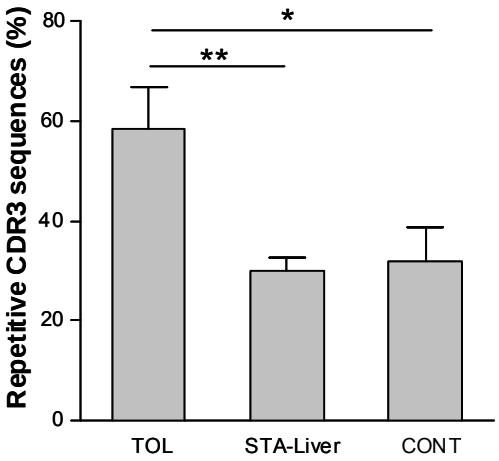


**Figure 5**: **TOL recipients exhibit a more restricted Vδ1-TCR CDR3 repertoire than STA-Liver and CONT.** The bar plot represents the mean (±SEM) frequency of repetitive CDR3 sequences exceeding 10% of totally analyzed amplicons (at least 14 per sample). (*)=*P*-value<0.05, (**)=*P*-value<0.01 (*t*-test).

## 1.4 DISCUSSION

Research and validation of clinically applicable biomarkers of operational tolerance constitutes a pre-requisite for the implementation of tolerogenic therapies. Liver transplantation possesses a privileged immunostatus, given that operational tolerance occurs in approximately 20% of stable liver recipients. Therefore, clinical liver tolerance has been widely studied. Two different laboratories, reported an altered distribution of the γδ T cell population consisting of an expansion of Vδ1 T cells accompanied by a shift of Vδ1/Vδ2 ratio [66, 143]. These observations encouraged us to focus on the role that γδ T cell subsets play in the context of organ transplantation and their contribution to operational tolerance.

Hence, we investigated the repertoire, and functional and phenotypic characteristics of these lymphocytes, since they had not been previously explored in detail in clinical liver transplantation.

The analysis of γδ T cells and their main subsets from liver and kidney transplant recipients revealed an expansion of Vδ1 T cells regardless of the type of transplanted allograft, which was also independent of whether IS was maintained or completely withdrawn. Moreover, Vδ2 T cells were diminished in liver transplanted cohorts. Thus, the whole γδ T cell population is increased in the context of transplantation, and this increment seems to cause an important shift in the Vδ1/Vδ2 ratio in TOL liver recipients. However, none of these populations could be considered as a useful tolerance biomarker, as they are not able to accurately discriminate between TOL and STA-Liver recipients. By employing a high Vδ1/Vδ2 ratio threshold, we could only classify a small fraction of TOL recipients correctly.

We observed that Vδ1 and Vδ2 are phenotypically and functionally different T cell subsets (Table 1). The Vδ1 subset resembled terminally differentiated lymphocytes ($T_{EMRA}$) expressing higher levels of perforin, and of activator killer-like receptors such as NKG2C and KLRF1. This phenotype parallels the characteristics described on effector memory RA αβ CD8+ T cells ($T_{EMRA}$) [146] which have been identified in kidney recipients responding to CMV infection [147]. On the other hand, Vδ2 T cells exhibited an effector memory phenotype ($T_{EM}$), with increased production of IFNγ. Curiously, molecules involved in immunoregulatory pathways were not expressed (Foxp3) or present at similar and low levels (GITR and PD-1).

Additionally, Vδ1 T cells displayed a skewed CDR3 repertoire, defined as multiple repeated sequences within each individual that is reminiscent of previous clonal expansions driven by peripheral antigens. It has been reported that, the Vδ1 population is quantitatively altered during cell stress [148], infection with intracellular bacteria (*Mycobacteria, Listeria, Borrelia*) and viruses (HIV, CMV) [149, 150]. However, it remains unclear whether Vδ1 T cells recognize and expand in response to these pathologic situations, or if they are triggered by endogenous gene products.

As previously reported, we found a clear expansion of peripheral blood Vδ1 T cells correlating with CMV seropositivety [151]. Moreover, we also detected a less marked but significant alteration within the γδ T cells directly associated to HCV infection, resulting from an increase in Vδ1 T cells and a significant decrease in Vδ2 T cells. These findings support the hypothesis that, in transplant recipients persistent viral infections could constitute the main force shaping the repertoire of peripheral blood γδ T cells; providing a rationale for

deeply exploring the influence of this population in the pathogenesis of CMV and HCV in clinical transplantation.

Our study does not clarify the reasons explaining why operationally tolerant liver recipients exhibit a significant alteration in the distribution of γδ T cell subsets. Hypothetically, the immune reconstitution after complete IS discontinuation could specifically influence Vδ1 T cells distribution. Alternatively, Vδ1 TCR ligands could be differentially expressed between tolerant and stable liver recipients, resulting in Vδ1 T cell proliferation. Since our findings showed that Vδ1 TCR repertoire is more restricted in TOL than in STA-Liver recipients, this supports the latter hypothesis.

## 1.5 CONCLUSIONS

- Most transplant recipients exhibit an increased number of γδ T cells in peripheral blood and an altered distribution of the 2 main γδ T cell subsets (Vδ1 and Vδ2).

- This phenomenon appears to be influenced by the exposure to persistent viral infections such as HCV and CMV.

- The altered distribution of peripheral blood γδ T cell subsets in liver recipients is stable over a fixed period of time.

- In tolerant liver recipients the increase in the number of Vδ1 T cells is more pronounced than in other recipients, and these cells exhibit a skewed Vδ1 TCR CDR3 repertoire.

- Neither the quantification of peripheral blood γδ subsets nor their phenotypic cell surface characteristics allow for an accurate discrimination between operationally tolerant and liver recipients requiring maintenance immunosuppression.

## 2. Transcriptional and phenotypic analysis of kidney recipients receiving either cyclosporin A or sirolimus monotherapy

## 2.1 MAIN GOALS

Operational tolerance is considered rare in kidney transplantation. The half-life of renal allografts is conditioned by the establishment of chronic allograft nephropathy, which derives from drug-related nephrotoxicity and chronic rejection. The development of safer immunosuppressive strategies is a desirable objective. mTOR inhibitors, like sirolimus, were clinically introduced as a group of immunosuppressants potentially capable to replace CNIs.

Standardized IS maintenance treatments in kidney recipients include two or three different drugs. Renal transplanted patients included in our study were atypical, as they were either receveing CsA or sirolimus monotherapy. This fact allowed us to establish the *in vivo* effects of each drug.

The principal goals of this study were:

1) To assess the phenotypic patterns of blood mononuclear cells from kidney recipients on IS monotherapy, employing multiparameter flow cytometry.

2) To determine the impact of sirolimus and CsA monotherapy on a transcriptional level, performing gene expression profiling and posterior functional analysis of these data.

3) To investigate the prevalence of tolerance-related transcriptional biomarkers in stable kidney recipients on monotherapy.

## 2.2 MATERIAL AND METHODS

### 2.2.1 Patients

The study was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks. All patients who participated in this study gave informed consent.

Heparinized blood samples from two groups of kidney recipients from the Renal Transplant Unit, Hospital Clinic Barcelona were included in the study:

- CSA: 13 stable kidney recipients under CsA monotherapy

- SRL: 24 stable kidney recipients under sirolimus monotherapy, which was either started de novo or by converting patients with CNI based immunosuppressive protocols.

All patients received a kidney from a deceased donor, and fulfilled the following criteria:

1. Recipients of a renal transplant (deceased donor) with more than one year of post-transplant follow-up.
2. Stable renal function (no significant variations in both plasmatic creatinemia and proteinuria over the previous 12 months).
3. Immunosuppressive monotherapy with either CsA or sirolimus for at least six months (at the time of analysis).
4. No acute rejection episodes over the previous 12 months.
5. No active neoplasia or infectious diseases.

**Table 2: Demographic data from stable kidney recipients and healthy individuals included in the study.**

| Patient group | Number | Age [A] | Gender | Time from transplantation (years) [A] | Treatment | Creatinemia at inclusion (mg/dL)[A] | Proteinuria at inclusion (mg/24h)[A] | A+B+DR incompatibility |
|---|---|---|---|---|---|---|---|---|
| SRL | 24 | 61 (8.9) | 46% Male | 9.75 (2.6) | Sirolimus | 1.43 (0.35) | 710 (769) | ≤2: 17 ≥3: 7 |
| CSA | 13 | 61.1 (14) | 54% Male | 16.4 (3.3) | CsA | 1.4 (0.6) | 519 (452) | ≤2: 5 ≥3: 6 NA 2 |

[A] Mean (SD)

Blood samples from seven operationally tolerant and seven age-matched non-tolerant kidney recipients recruited from ITERT, Nantes, France were employed for quantitative RT-PCR experiments.

- Tolerants: kidney graft recipients with stable graft function (blood creatinemia<1.7 mg/dL, proteinuria<1000 mg/24 h) for at least one year after complete cessation of immunosuppressive therapy (range: 2-13 years). Immunosuppression was stopped due to non-compliance. No kidney biopsies were available from these recipients.

- Non-tolerants: kidney recipients under standard IS with deteriorating kidney graft function (serum creatinine >1.7 mg/dL and/or proteinuria>1000 mg/24h), that exhibited a transplant glomerulopathy according to the up-dated Banff classification criteria and/or an active humoral component as demonstrated by the presence of graft C4d deposits and circulating anti-donor antibodies.

Additionally, peripheral blood samples from nine age-matched healthy individuals (CONT-K) were included for the microarray experiments.

### 2.2.2 Peripheral blood immunophenotyping

#### Surface and intracellular cell staining

Flow cytometry immunophenotyping was performed from whole blood following the same procedures and reagents described in 1.2.2.

Monoclonal antibodies that were tested: CD3, CD4, CD8, CD16, CD25, CD28, CD56, CD19, CD45RA, CD62L, CCR7, $\gamma\delta$ TCR, $\alpha\beta$ TCR (BD Biosciences); V$\delta$1 TCR (ThermoScientific); V$\delta$2 TCR (Immunotech) and Foxp3 (eBioscience).

We particularly assessed the proportion and absolute value of CD4+CD25$^{high}$CD62L+, CD4+CD25$^{high}$Foxp3+, $\gamma\delta$ T cells, V$\delta$1 and V$\delta$2 T cells, CD19+ B cells, NK, NKT suppressive and cytotoxic CD8+ T cells.

### 2.2.3 Process and analysis of gene expression data

**Microarrays: preparation and sample hybridization**

The assay was conducted using blood samples from 24 SRL and 13 CSA kidney recipients. Additionally, nine CONT-K were included in this experiment.

RNA was extracted as previously described using TRIzol reagent. After RNA quantification using Agilent 2100 BioAnalyzer, genomic DNA was removed by DNase treatment (DNase I recombinant; Roche, Mannheim, Germany). First-strand cDNA was synthesized employing polydT oligonucleotide and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Reaction was performed as specified in the technical data sheet.

After *in vitro* reverse transcription, resulting cRNA samples were labeled and hybridized according to manufacturer instructions onto Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix Inc., Santa Clara, CA) containing 54675 probes for 47000 transcripts.

**Data normalization and analysis**

Tested samples were normalized using the GC content adjusted-robust multi-array (GC-RMA) algorithm [152]. This is a model-based background adjustment for oligonucleotide expression arrays that computes expression values from probe intensity values incorporating probe sequence information.

Next, a conservative probe-filtering step was assessed; excluding those probes not reaching a $\log_2$ expression value of five in at least one sample. A total of 22586 probes were selected.

*SAM* analysis was employed to identify genes differentially expressed between SRL and CSA groups and between SRL and CONT-K among the 22586-probe set.

**Functional analysis of gene expression data**

The GSEA method was employed to assess the deregulated sets of genes associated with specific functional pathways [134, 153]. This computational method determines whether an *a priori* defined gene set shows statistically significant concordant differences between two biological states. This method uses a variation of a Kolmogorov-Smirnov statistic to provide an enrichment score for each gene set.

Analyzed genes comprised the filtered probe set ranked according to *SAM*.

Consulted gene sets databases to infer involvement in biologic process were obtained from:

- Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database
http://www.genome.jp/kegg/pathway.html

- The Protein Analysis Through Evolutionary Relationships (PANTHER) classification scheme
http://www.pantherdb.org/pathway/

Blood cell lineage specific transcripts reported in Haematology Expression Atlas [154] were employed to generate gene containing transcripts considered unique for the following PBMC subsets:

- CD4+ and CD8+ T lymphocytes
- CD14+ monocytes
- CD19+ B lymphocytes
- CD56+ NK cells
- CD66b+ granulocytes

Additionally, Ingenuity Pathway Analysis Toxicology (IPA-Tox™; http://www.ingenuity.com) was utilized to explore in detail the pharmacological response of PBMCs to either CsA or sirolimus treatment. IPA-Tox is an analytic

tool that employs molecular toxicity pathways and related gene sets to deliniate transcriptional responses to xenobiotic insults.

### 2.2.4 Quantitative RT-PCR

A gene signature of “operational tolerance” defined by Brouard *et al.*, [43] using microarrays was assayed by quantitative RT-PCR (qPCR) employing mRNA from both the 24 SRL and the 13 CSA.

The gen set comprised 40 out of the 49 genes previously identified. Primers and probe sets were manually redesigned to achieve the best correspondence between the microarray and qPCR data. Two housekeeping genes were also included.

Gene set:

| | | | |
|---|---|---|---|
| AKR1C1 | CHECK1 | NR2F1 | RGN |
| AKR1C2 | DEPDC1 | PARVG | RHOH |
| AREG | ELF3 | PCP4 | SLC29A1 |
| AURKA | GAGE7 | PLEKHC1 | SP5 |
| BTLA | HBB | PLXNB1 | SPON1 |
| BUB1B | IGFBP3 | PODXL | SYNGR3 |
| C1S | LTB4DH | PPAP2C | TACC2 |
| CCL20 | MS4A1 | RAB30 | TLE4 |
| CDC2 | MTHFD2 | RASGRP1 | TMTC3 |
| CDH2 | NCAPH | RBM9 | ZWILCH |

Housekeeping genes:

| | |
|---|---|
| HPRT1 | GADPH |

The expression pattern of these target genes was measured by qPCR employing the ABI 7900 Sequence Detection System (ABI PRISM 7900 user bulletin, PE Applied Biosystems, Foster City 2:11-24, 1997). All qPCR experiments were performed in duplicates.

Levels of mRNA target genes were normalized to HPRT1 (the most stable of the housekeeping genes).

Data was presented as relative expression between cDNA of target samples and a calibrated sample according to ΔΔCt method. This quantitation approach involves comparing the Ct values of the samples of interest with a control. In all cases the Ct values are normalized to an endogenous housekeeping gene. Amplification efficiencies of the target genes and the calibrator must be similar.

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$$

$\Delta Ct_{sample}$ is the Ct value for each normalised sample
$\Delta Ct_{reference}$ is the Ct value for the calibrator also normalised

Predictive Analysis of Microarrays (*PAM*) was employed for classifying the status of SRL and CSA recipients as either tolerant or non-tolerant.

- The training set included seven tolerant and seven Non-tolerant kidney recipients.
- The test set comprised all samples from CSA and SRL groups (n=37).
- A 10-fold cross-validation was performed on the training set, selecting the threshold associated with the lowest error rate and filtering the noisiest genes.
- This threshold was used for class prediction on the test set (CSA+SRL).

## 2.3 RESULTS

Parts from the results presented here are being published in the following article: *Comparative transcriptional and phenotypic peripheral blood analysis of kidney recipients under Cyclosporin A or sirolimus monotherapy* (Brouard *et al.*, 2010, submitted).

### 2.3.1 Immunophenotypic results

Phenotypic differences in PBMC subsets of operationally tolerant kidney [155, 156] and liver [66] recipients have been recently reported, upon comparison to chronic rejectors or non-tolerant recipients, respectively.

We collected peripheral blood and performed combined monoclonal antibody staining in 24 SRL and 13 CSA kidney recipients. We searched for differences in the total cell count of leukocyte populations according to clinical diagnosis data. This showed that SRL patients harbored decreased total numbers of lymphocytes (p=0.0178) and basophiles (p=0.0146) compared to CSA recipients.

Furthermore, we analyzed memory, naïve and activation markers together with various lymphocyte populations. Within the T cell population, patients under sirolimus showed statistically higher percentages of $\alpha\beta$ T cells (p=0.0202) and lower percentages of $\gamma\delta$ T cells (p=0.0026) than CSA patients. Among the V$\delta$1 and V$\delta$2 T cells, SRL recipients exhibited a significantly decreased percentage of V$\delta$2 T cells (p=0.045). For the V$\delta$1/V$\delta$2 ratio and V$\delta$1 T cell populations no difference was observed (Table 7). Additionally, SRL group harbored statistically higher percentages of CD4+ effector memory ($T_{EM}$) CD4+CD45RA-CCR7- (p=0.0164, Figure 6A). They also expressed a reduced amount of naïve

CD4+CD45RA+CCR7+ (p=0.0112, Figure 6B) and central memory ($T_{CM}$) CD4+CD45RA-CCR7+ (p=0.0114, Figure 6C) lymphocytes compared to CSA. Additionally neither the frequencies nor the absolute numbers of B cells (CD19+), NK cells (CD3-CD56+) and NKT cells (CD3+CD56+) significantly differed between the two groups of recipients. SRL and CSA groups exhibited equivalent numbers of CD4+ and CD8+ T cells (Table 7).

**Table 7: Peripheral blood phenotypical profile of 24 and 13 stable kidney recipients under sirolimus and CsA monotherapy, respectively**.

| | **SRL [%(SEM)]** | **CSA[%(SEM)]** | |
|---|---|---|---|
| | | | *P* value |
| CD4+ | 56.70 (2.70) | 54.54 (3.88) | ns |
| CD8+ | 37.43 (2.61) | 37.79 (3.43) | ns |
| **CD4+CD45RA-CCR7- ($T_{EM}$)** | 49.01 (4.31) | 32.16 (4.32) | 0.0164 |
| **CD4+CD45RA-CCR7+ ($T_{CM}$)** | 23.46 (3.05) | 28.99 (1.97) | 0.0114 |
| **CD4+CD45RA+CCR7+ (Tnaïve)** | 21.51 (2.76) | 34.39 (4.16) | 0.0112 |
| CD8+CD45RA-CCR7- ($T_{EM}$) | 47.28 (4.52) | 45.62 (5.20) | ns |
| CD8+CD45RA-CCR7+ ($T_{CM}$) | 2.99 (0.74) | 4.19 (0.77) | ns |
| CD8+CD45RA+CCR7+ (Tnaïve) | 14.47 (3.23) | 17.94 (3.72) | ns |
| **αβ TCR** | 94.11 (0.54) | 88.21 (1.97) | 0.0202 |
| **γδ TCR** | 3.39 (0.39) | 5.85 (0.73) | 0.0026 |
| Vδ1 TCR | 1.48 (0.29) | 2.20 (0.52) | ns |
| **Vδ2 TCR** | 1.65 (0.25) | 3.34 (0.75) | 0.0450 |
| Vδ1/Vδ2 | 2.53 (0.99) | 1.81 (0.65) | ns |
| CD3-CD56+ (NK cells) | 13.06 (1.20) | 10.38 (1.18) | ns |
| CD3+CD56+ (NKT cells) | 8.70 (1.57) | 6.03 (0.88) | ns |
| CD19+ (B cells) | 6.82 (1.01) | 5.99 (0.95) | ns |
| **CD4+CD25$^{high}$CD62L+** | 7.29 (0.53) | 3.38 (0.41) | 0.0001 |
| CD4+CD25+Foxp3+ | 6.48 (0.70) | 4.38 (0.50) | ns |
| CD8+28+ (Tc) | 10.47 (0.90) | 11.49 (1.16) | ns |
| CD8+CD28- (Ts) | 15.87 (2.40) | 12.21 (2.31) | ns |

ns: non significant

In addition, we detected an augmented population of CD4+CD25$^{high}$CD62L+ T cells in the SRL group (p=0.0002, Figure 6D). We also observed that the CD4+CD25$^{high}$Foxp3+ T cell population was significantly incremented in the SRL (p= 0.004, Figure 6E) in comparison to CSA group. No statistical

differences were detected between the two groups of recipients regarding CD8+ T cell subsets (Table 7).

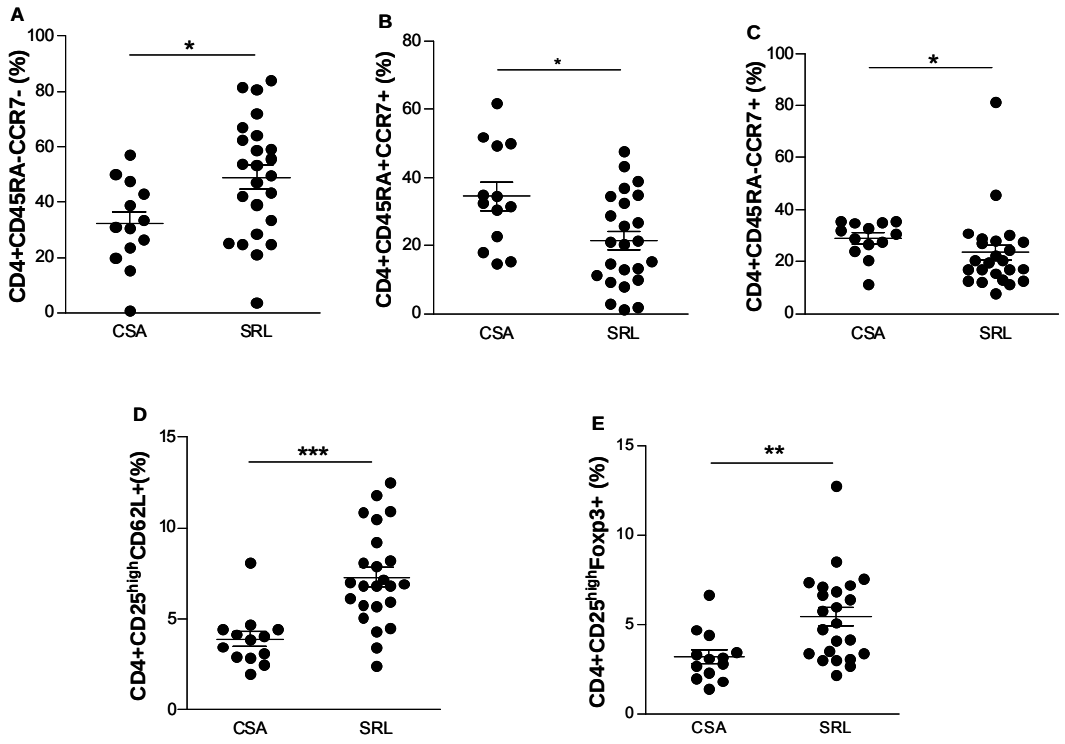


**Figure 6: CSA and SRL recipients differ in CD4+ memory/naïve T populations and regulatory T cells.** Comparison of the relative numbers of effector memory ($T_{EM}$) (A), naïve (B) and central memory ($T_{CM}$) (C) CD4+ T cells. Peripheral blood differences of CD4+CD25$^{high}$CD62L+ (D) and CD4+CD25$^{high}$Foxp3+ (E) T cells detected between CSA and SRL treated cohorts. Scattered dot plots represent mean (±SEM) values. (*)= P-value<0.05, (**)=P-value <0.01, (***)=P-value<0.001.

### 2.3.2 Blood transcriptional profile

To investigate the impact of the immunosuppressive regimens on the gene expression of kidney recipients, we used peripheral blood from SRL, CSA and CONT-K individuals to perform *Affymetrix* microarray assays. Data was normalized using the GC-RMA algorithm and filtered. Following this, a comparative data analysis was conducted employing *SAM* (FDR<5%). *SAM*

analysis yielded a total of 468 up-regulated and 586 down-regulated genes in the SRL compared to the CSA (Figure 7).

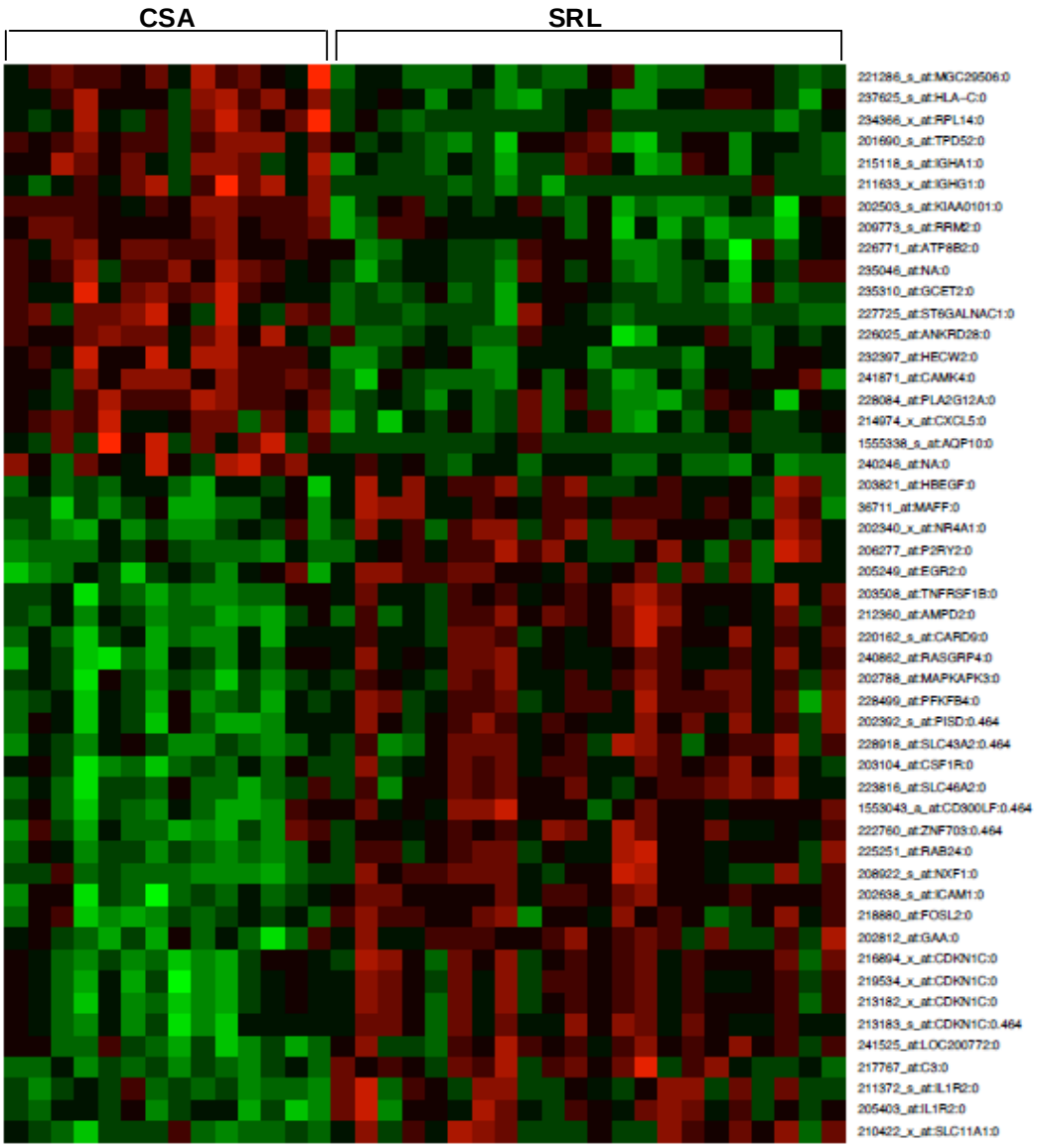


**Figure 7: Whole genome expression profiling of PBMC samples reveals transcriptional differences between SRL and CSA samples.** Expression profiles of the 50 most significant genes among the 1054 genes identified by *SAM* (FDR<5%). Results are expressed as a matrix view of gene expression data (heat map) where rows represent genes and columns represent hybridized samples. Red pixels correspond to an increased abundance of mRNA in the indicated blood sample, whereas green pixels indicate decreased mRNA levels. The colour intensity denotes the standardized ratio between each value and the average expression of each gene across all samples.

We further interpreted the gene list provided by *SAM* employing the Gene Set Enrichment Analysis (GSEA) method, and we used KEGG and Panther as database gene sets.

The analysis revealed genes involved in with mTOR signaling and a wide representation of pro-inflammatory pathways significantly enriched in the SRL group compared to the CSA recipients (Table 8).

These over-represented gene clusters positively associated with the SRL group included:

- angiotensin-II signaling
- cytokine and chemokine signaling
- MAPK signaling pathway
- TLR signaling pathway

**Table 8. Gene sets significantly enriched in the SRL group compared with CSA group.** Reported gene sets showed a p-value<0.01 and FDR <25%. No pathways were enriched in the CSA group with this significance value.

| KEGG Pathways | Nominal p-value | FDR q-value | Representative genes with highest enrichment scores |
|---|---|---|---|
| Toll-like receptor signaling pathway | 0.000 | 0.026 | *TLR5, CD14, IL1B, CD86, NFKB2, TLR4, TLR2, IL8* |
| MAPK signaling pathway | 0.000 | 0.029 | *NR4A1, RASGRP4, IL1R2, MAPKAPK3, CD14, MPK7, TNFRSF1A* |
| Ephithelial cell signaling in *H.pylori* infection | 0.000 | 0.053 | *HBEGF, LYN, TCIRG1, ATP6V0C, NFKB2* |
| mTOR signaling pathway | 0.001 | 0.042 | *TSC2, STK11, LYK5, AKT1, EIF4BP1, ULK1* |
| Acute myeloid leukemia | 0.003 | 0.042 | *TCF7L2, RARA, SPI1, NFKB2, CEBPA* |
| Adipocytokine signaling pathway | 0.005 | 0.054 | *TNFRSF1B, RXRA, NFKB2, TNFRSF1A, NFKBIE* |
| Snare interactions in vesicular transport | 0.004 | 0.052 | *STX11, STX5, STX6, STX3, STX10* |
| Notch signaling pathway | 0.008 | 0.076 | *NOTCH1, PSEN1, CTBP2, NOTCH2, NCOR2* |
| Insulin signaling pathway | 0.000 | 0.073 | *PKM2, RAF1, ARAF, ACACA, TSC2, SOCS3, AKT1* |
| GNRH signaling pathway | 0.002 | 0.081 | *HBEGF, PLCB1, MAPK7, PTK2B, PLCB2, PRKCD* |
| **Panther Pathways** | **Nominal p-value** | **FDR q-value** | **Representative genes with highest enrichment scores** |
| Angiotensin II stimulated signaling through G proteins and b-arrestin | 0.000 | 0.021 | *ARRB2, RHOC, RHOB, PLCB1, PLCB2* |
| Inflammation mediated by chemokine and cytokine signaling pathway | 0.000 | 0.018 | *RELB, PTGS2, STAT3, RHOB, IFNGR2, IL8, IFNAR1, SOCS3* |
| Parkinson disease | 0.000 | 0.021 | *MAPK7, FGR, LYN, HCK, ADRBK2* |
| MAPK pathway | 0.000 | 0.027 | *MAPKAPK3, MAPKAPK2, EIF4E2, MAP3K5, IL1R1, MAP3K11* |
| RAS pathway | 0.000 | 0.026 | *MAPKAPK3, RHOC, RHOB, MAPKAPK2, PAK1, RAF1, RRAS* |
| P53 glucose deprivation pathway | 0.009 | 0.046 | *TP53, TSC2, AKT1, STK11, IGBP1, FRAP1* |
| Angiogenesis | 0.000 | 0.052 | *MAPKAPK3, TCF7L2, RHOC, RHOB, NOTCH1, PAK1* |
| Toll-like receptor signaling pathway | 0.005 | 0.059 | *PTGS2, TLR1, CD14, NFKB2, TLR4, RELB, RELA, NFKBIE* |
| Endothelin signaling pathway | 0.001 | 0.061 | *PTGS2, ARAF, MAPK7, ADCY7, PRKAR2A, MAP2K2* |
| WNT signaling pathway | 0.000 | 0.058 | *ARRB2, TCF7L2, TLE3, PLCB1, PLCB2* |

Several of the previously identified pro-inflammatory pathways were also upregulated in SRL recipients when compared to CONT-K (*e.g.*, TLR signaling pathway, adipocytokine signaling pathway, MAPK signaling pathway). Additional pathways found to be significantly associated with SLR group in this analysis were ubiquitin proteasome, TGF-β, PI3K and apoptosis signaling (Table 9).

**Table 9. Functional pathways enriched in SRL as compared to CONT-K.** Reported gene sets showed a p-value<0.01 and FDR<25%. In the CONT-K, no pathways showed this significance value.

| KEGG Pathways | Nominal p-value | FDR q-value | Representative genes with highest enrichment scores |
|---|---|---|---|
| Porphirin and chlorophyll metabolism | 0.001 | 0.028 | *BLVRA, EPRS,FTH1,ALAS2,COX10* |
| Ubiquitin mediated proteolysis | 0.000 | 0.035 | *UBE2E2, UBE2D2, CUL1, UBE2E1, UBE2D1* |
| Oxidative phosphorylation | 0.000 | 0.049 | *UQCRB, SDHC, ATP6V0C, COX10, NDUFV3* |
| O-glycan biosynthesis | 0.001 | 0.040 | *GALNT1, B4GALT5,C1GALT1, GCNT3, GALNT10* |
| Axon guidance | 0.000 | 0.035 | *PP3R1, DPYSL2, GNAI3, PAK1, KRAS, CDC42* |
| Neurodegenarative diseases | 0.001 | 0.035 | *PINK1, BCL2L1, SNCA, HSPA5, APP, NR4A2* |
| Adipocytokine signaling pathway | 0.001 | 0.044 | *STAT3, SOCS3, NFKBIA, CPT2, ACSL3* |
| Tight junction | 0.002 | 0.077 | *CSDA, GNAI3, PTEN, RAB13, EPB41L3, RRAS* |
| Toll-like receptor signaling pathway | 0.000 | 0.069 | *TLR5, IL1B, IFNAR1, NFKBIA, TLR4, PIK3CG* |
| Ephithelial cell signaling in *H.pylori* infection | 0.004 | 0.070 | *PAK1, ATP6V0C, LYN, HBEGF, CDC42* |
| WNT signaling pathway | 0.003 | 0.071 | *TCF7L2, PPP3R1, SMAD2, VANGL1, CUL1* |
| MAPK signaling pathway | 0.000 | 0.065 | *PPP3R1, STK3, DUSP3, ARRB1, DUSP1* |
| Apoptosis | 0.002 | 0.070 | *PPP3R1, CYCS, IL1B, BCL2L1, PRKACA* |
| Alzheimer disease | 0.010 | 0.066 | *APH1A, IL1B, SNCA, APP, GSK3B, PSEN1* |
| Pancreatic cancer | 0.004 | 0.064 | *STAT3, RALA, RALBP1, SMAD2, BCL2L1, KRAS* |
| ERBB signaling pathway | 0.007 | 0.068 | *CDKN1A, PAK1, HBEGF, KRAS, PRKCB1, PIK3CG* |
| Regulation of actin cytoskeleton | 0.003 | 0.100 | *PAK1, WASL, RRAS, PPP1R12A, MRLC2* |
| **Panther Pathways** | **Nominal p-value** | **FDR q-value** | **Representative genes with highest enrichment scores** |
| Ubiquitin Proteasome Pathway | 0.000 | 0.001 | *PSMD1, UBE2E2, UBE2D2, UBE2D, PSMC2* |
| TGF-beta signaling pathway | 0.000 | 0.002 | *RIT1,SMAD1, FOXK2, SMAD2, JUNB,SMAD3, RAB10* |
| Parkinson Disease | 0.000 | 0.002 | *PSMA2, CUL1, LYM, PSMA4, MPAK7, ADRBK2, SNCA* |
| PI3 Kinase pathway | 0.000 | 0.023 | *FOXK2, GNAI3, RRAS, FOXJ2, PDPK1, KRAS, PIK3CG* |
| Apoptosis signaling pathway | 0.000 | 0.056 | *BCL2A1, ATF6, CYCS, ATF3, BCL2L1, NFKBIA, TNFSF10* |
| Axon guidance mediated by semaphorins | 0.006 | 0.054 | *DPYSL2, PAK1, AKAP13, PAK2, PAF4, CDK5* |

Furthermore, we assessed the contribution of several PBMC subsets to the distinctive gene expression observed between SRL and CSA samples. In this case, GSEA analysis was performed on the basis of the Haematology Expression Atlas, as a source of cell lineage specific gene lists.

We identified that transcripts specific for either CD14+ or CD56+ cells were statistically over-represented in the SRL group (p<0.001 and FDR<0.1% and

p=0.03 and FDR 2%, respectively). Among the CSA, we identified up-regulated transcripts specifically associated with CD4+ T cells and CD19+ cells ($p<0.001$ and FDR<0.1% in both subsets).

Finally, to further understand the biological responses of PBMCs to either CsA or sirolimus, we re-analyzed the differentially expressed genes employing the toxicity list capability of Ingenuity Pathway Analysis. Among previously defined pharmacological responses, this application identified NFkB signaling pathway as the top “toxicological” response contained within the expression dataset (p=0.000943).

### 2.3.3 qPCR experiments

We performed qPCR to measure the expression of 40 genes which had been previously selected as a signature of operational tolerance in a set of TOL kidney recipients [43].

Employing *PAM* we performed a selection of variables to obtain an optimal gene classifier which included 26 out of the original 40 genes. To estimate the proportion of potentially tolerant individuals among the studied cohorts, we conducted *PAM* analysis to tentatively classify the 37 studied samples into tolerant and non-tolerant categories. An independent group of seven tolerant and seven non-tolerant constituted the training set, and the CSA and SRL patients comprised the test set.

The selected genes showed an excellent performance in the training set by correctly classifying all tolerant specimens and six of seven non-tolerant samples (overall success 93.7%).

Among the test set, four out of 37 samples were predicted as potentially tolerant (three CSA and one SRL) (Table 10). The 4 patients classified as potentially tolerant could not be discriminated from the remaining 33 recipients on the basis of clinical or immunophenotypic characteristics.

**Table 10. Tolerance prediction based on qPCR gene expression for both the training set and the test set.** The calculated probability of TOL and Non-TOL is shown. The maximum predictive value (indicating positive prediction) is 1 and the minimum 0.

| TRAINING SET | | | | TESTING SET | | | |
|---|---|---|---|---|---|---|---|
| Observed | Predicted | TOL probability | Non-TOL probability | GROUP | Predicted | TOL probability | Non-TOL probability |
| TOL | TOL | 1,000 | 0,000 | SRL | TOL | 0,940 | 0,060 |
| TOL | TOL | 1,000 | 0,000 | SRL | Non-TOL | 0,688 | 0,312 |
| TOL | TOL | 0,999 | 0,001 | SRL | Non-TOL | 0,624 | 0,376 |
| TOL | TOL | 0,884 | 0,116 | SRL | Non-TOL | 0,575 | 0,425 |
| TOL | TOL | 0,819 | 0,181 | SRL | Non-TOL | 0,545 | 0,455 |
| TOL | TOL | 0,804 | 0,196 | SRL | Non-TOL | 0,481 | 0,519 |
| TOL | TOL | 0,508 | 0,492 | SRL | Non-TOL | 0,180 | 0,820 |
| Non-TOL | TOL | 0,818 | 0,182 | SRL | Non-TOL | 0,136 | 0,864 |
| Non-TOL | Non-TOL | 0,411 | 0,589 | SRL | Non-TOL | 0,130 | 0,870 |
| Non-TOL | Non-TOL | 0,393 | 0,607 | SRL | Non-TOL | 0,128 | 0,872 |
| Non-TOL | Non-TOL | 0,008 | 0,992 | SRL | Non-TOL | 0,105 | 0,895 |
| Non-TOL | Non-TOL | 0,000 | 1,000 | SRL | Non-TOL | 0,094 | 0,906 |
| Non-TOL | Non-TOL | 0,000 | 1,000 | SRL | Non-TOL | 0,077 | 0,923 |
| Non-TOL | Non-TOL | 0,000 | 1,000 | SRL | Non-TOL | 0,065 | 0,935 |
| | | | | SRL | Non-TOL | 0,063 | 0,937 |
| | | | | SRL | Non-TOL | 0,038 | 0,962 |
| | | | | SRL | Non-TOL | 0,037 | 0,963 |
| | | | | SRL | Non-TOL | 0,036 | 0,964 |
| | | | | SRL | Non-TOL | 0,033 | 0,967 |
| | | | | SRL | Non-TOL | 0,025 | 0,975 |
| | | | | SRL | Non-TOL | 0,022 | 0,978 |
| | | | | SRL | Non-TOL | 0,018 | 0,982 |
| | | | | SRL | Non-TOL | 0,008 | 0,992 |
| | | | | SRL | Non-TOL | 0,004 | 0,996 |
| | | | | CSA | TOL | 1,000 | 0,000 |
| | | | | CSA | TOL | 0,921 | 0,079 |
| | | | | CSA | TOL | 0,880 | 0,120 |
| | | | | CSA | Non-TOL | 0,677 | 0,323 |
| | | | | CSA | Non-TOL | 0,569 | 0,431 |
| | | | | CSA | Non-TOL | 0,557 | 0,443 |
| | | | | CSA | Non-TOL | 0,496 | 0,504 |
| | | | | CSA | Non-TOL | 0,313 | 0,687 |
| | | | | CSA | Non-TOL | 0,311 | 0,689 |
| | | | | CSA | Non-TOL | 0,250 | 0,750 |
| | | | | CSA | Non-TOL | 0,173 | 0,827 |
| | | | | CSA | Non-TOL | 0,146 | 0,854 |
| | | | | CSA | Non-TOL | 0,040 | 0,960 |

## 2.4 DISCUSSION

CsA improved the outlook in renal allograft treatment when it was introduced into the clinic in 1983 [157] as one-year survival rates significantly increased. However, nephrotoxicity arising from long-term treatment with CNIs may contribute to chronic allograft dysfunction [158], and demands alternative treatment with reduced toxicity.

mTOR inhibitors, like sirolimus, were introduced as potent immunosuppressants capable of replacing CNIs as they target different key molecules and have completely different mechanisms of action, and it has been observed that conversion from CNIs to sirolimus in kidney transplant recipients with chronic allograft dysfunction improved creatinine clearance in the short term. However, this strategy was associated with the appearance of proteinuria leading to high discontinuation rates [159] and dyslipidemia [160]. Moreover, several inflammatory lesions affecting mucosa, skin or lungs have been correlated to sirolimus therapy [161]. The pathogenesis of these various inflammatory disorders is still not well understood. mTOR inhibitors *in vitro* appeared to play a role in the innate immune cells by impairing DC differentiation, maturation, function and survival [14], and promoting pro-inflammatory cytokines (via NFκB) in monocytes and macrophages [162]. In contrast, other *in vitro* studies suggested that sirolimus contributes to the generation of high numbers of CD4+CD25+ T cells, and these Tregs appeared to maintain their suppressive activity and levels of Foxp3 [163]. *In vivo* data suggested that kidney recipients under sirolimus preserve the frequency of circulating Tregs whereas CNIs decrease their numbers [164, 165], but a suppressive capability was not confirmed .

Despite all generated data, the molecular pathways involved in downstream effects of mTOR inhibition remain incompletely elucidated, particularly in the clinic. As it is common to administer several immunosuppressive drugs simultaneously, this hampers the precise delineation of the mechanisms associated with a unique immunosuppressant. This study allowed for the first time to analyze and compare the impact of sirolimus and CsA on gene expression and phenotypic patterns of blood mononuclear cells from stable kidney recipients on maintenance monotherapy.

The SRL group showed a phenotypic profile characterized by increased percentages of CD4+CD25$^{high}$CD62L+. As CD25 can be also expressed in activated/memory T cells, we evaluated intracellular Foxp3 expression by flow cytometry, since this transcription factor is considered a reliable marker of CD4+CD25+ Tregs [78]. We detected a significant elevation of Foxp3 expression within the CD4+CD25$^{high}$ population in SRL compared to CSA group. As previously reported [164], patients under sirolimus display higher amounts of Foxp3+ Tregs. Thus, it would be desirable to further investigate the suppressive activity of these potentially regulatory T cells. Additionally, it seems likely that the calcineurin-inhibitor effect that blocks IL-2 transcription, could negatively influence the acquisition of CD25 molecules and, consequently, the surviving and function of Tregs in CsA treated recipients [165, 166].

In contrast, SRL kidney recipients showed increased numbers of CD4 $T_{EM}$ cells. Interestingly, it has been reported that sirolimus has immunostimulatory effects on the generation of memory CD8 T cells in a murine model following acute lymphocytic choriomeningitis virus infection [167]. The presence of these donor-

antigen reactive T cells it is expected to increase the likelihood of immune-mediated rejection. Therefore, this finding would limit the expected tolerogenic properties that have been attributed to sirolimus

Furthermore, results emerging from peripheral blood gene expression analysis indirectly highlighted the effect of sirolimus on the innate immune system of kidney recipients. The data analyses performed employing functional enrichment strategies revealed that, the SRL group exhibited an over-representation of pro-inflammatory pathways upon comparison to the CSA cohort and healthy individuals (Table 8 and 9).

In agreement with these experimental data, when we employed the IPA-Tox function from Ingenuity Pathway Analysis, we observed that NFκB signaling pathway was the xenobiotic response most highly associated with the SRL *versus* CSA differential gene expression pattern. The transcriptome of SRL recipients was also characterized by up-regulation of monocyte and NK cell lineage specific transcripts, in contrast to CSA recipients who displayed up-regulation of B cell and CD4+ T cell specific transcripts. Interestingly, this signature was observed despite no increase of the number of these cells *in vivo*, suggesting a “real” footprint of these cell populations on the SRL-associated expression dataset. According to various reports [162, 168], it has been described a pro-inflammatory action due to mTOR inhibition after TLR stimulation in freshly isolated human monocytes and myeloid DCs. This resulted in augmented production of IL-6, IL-12, IL-23 and TNF$\alpha$ and reduced production of IL-10. This pro-inflammatory response is mediated by an increased activity of NFκB transcription factor and decreased activity of Stat3 [162]. We conclude that our *in vivo* gene expression data are consistent with the above cited *in vitro*

results. This also indicates that the pro-inflammatory bias induced by sirolimus is even detectable in whole PBMCs collected from stable kidney recipients with no clinical evidences of systemic inflammation.

mTOR signaling was also identified among the functional pathways over-represented in SLR recipients. The upregulated genes contained in this pathway included both effectors (*AKT1*) and inhibitors (*TSC2, EIF4EBP*) of mTOR signaling, which does not fit current models of mTOR signaling transduction. Under chronic SRL treatment compensatory mechanisms might try to restore the inhibited mTOR pathway with corresponding downregulation of inhibitors and upregulation of effectors. It is difficult however to estimate the downstream net effect on a complex signal transduction pathway employing transcriptional data only. Our data clearly warrant a more detailed study on sorted PBMC populations with direct measurement of the phosphorilation status of key mediators of mTOR signaling.

As previously mentioned, data suggest that mTOR inhibition on effector T cells and Tregs could favor its ability to promote tolerance. This is mostly based on data generated in experimental animal models in which, in contrast to CsA, sirolimus promotes allograft tolerance or at least does not hamper the induction of tolerance when administered in combination with tolerogenic reagents [14]. Having determined in our study that the use of sirolimus monotherapy is associated with both expansion of Tregs and upregulation of pro-inflammatory genes, we decide to investigate the overall effect of this agent compared to CsA on tolerance-associated biomarkers. Therefore we determined the expression

of set of transcriptional biomarkers previously identified in a cohort of operationally tolerant kidney recipients [43]. Only three CSA and one SRL recipients out of 37 recipients were predicted as potentially tolerant. Hence, neither sirolimus nor CsA monotherapy could be associated with a transcriptional profile of operational tolerance, confirming the low prevalence of renal allograft tolerance (<1%) [169].

In conclusion, the overall effects of sirolimus when administered in monotherapy to human kidney recipients are dominated by innate immune cells and NFkB-related pro-inflammatory events. While sirolimus treatment is associated with a larger pool of circulating potentially regulatory T cells, it does not appear to confer a more “tolerogenic” environment than that provided by CNIs. These need to be confirmed in the context of prospective randomized studies.

## 2.5 CONCLUSIONS

- Stable kidney recipients on sirolimus monotherapy harbor an increased population of CD4+CD25$^{high}$Foxp3+ Treg cells compared to kidney recipients treated with CsA.

- The peripheral blood gene expression profile of kidney recipients receiving sirolimus is characterized by the enrichment of several pro-inflammatory pathways (like MAPK and TLR signaling) together with genes involved in mTOR signaling.

- Sirolimus treated recipients display increased levels of monocyte and NK cell specific transcripts, while recipients on CsA monotherapy exhibit upregulation of genes specific for CD4 T cells and B cells.

- Analysis of the differential gene expression between sirolimus and CsA treated recipients employing Ingenuity Pathway Analysis (IPA-Tox) identifies, among previously defined pharmacological responses, NFκB signaling pathway as the top “toxicological” pathway contained within the differentially expressed dataset.

- The use of a previously identified signature of operational tolerance in kidney transplantation fails to detect significant differences between recipients treated with CsA and those treated with sirolimus.

- These results do not support the notion that sirolimus is a drug more permissive to the development of allograft tolerance than calcineurin inhibitors.

# III. CONCLUDING REMARKS

Despite vigorous investigation in organ transplantation over the past decades, critical questions remain unanswered. Among them, a major limitation of the field is the incomplete understanding of the mechanisms of rejection and tolerance. This results in our inability to predict the immunological outcome of a transplanted organ and to titrate immunosuppressive therapy according to each patient's needs. The studies presented here address the relevant subject of whether blood can be employed in clinical transplantation to identify biomarkers of transplantation tolerance.

In our first study we decided to focus on a very specific lymphocyte subset, $\gamma\delta$ T cells, associated with operational tolerance in liver transplantation. Our study revealed that the population of circulating $\gamma\delta$ T cells is very heterogeneous, with different subsets displaying very different phenotypic and functional characteristics, and that most transplant recipients (either liver or kidney) exhibit an altered repertoire of $\gamma\delta$ T cell subsets. Despite the results of previous reports, however, our study failed to identify a robust association between the numbers and phenotypic traits of $\gamma\delta$ T cells and the development of tolerance to liver allografts. Furthermore, our results indicated that the altered $\gamma\delta$ T cell repertoire of transplanted recipients was clearly linked to the history of past infections (particularly CMV but also HCV). These results highlight the difficulties of conducting this kind of immunological research in transplanted recipients, who are individuals commonly exposed to many external factors that can act as confusing variables in any analysis.

Our second attempts to answer the question of whether it is possible to detect in blood of kidney recipients markers characteristic of chronic sirolimus and CsA usage, and whether these markers can be employed to advance our knowledge on the overall biological effects of these drugs. We could confirm *in vivo* that sirolimus treated recipients exhibited an increased expression of Foxp3+ Tregs as compared with patients under CsA. However, this increase in Tregs was accompanied by an increase in memory T cells and by a clearly detectable pro-inflammatory bias in the PBMC transcriptome. Due to the deleterious effect of memory T cells and inflammation on tolerance acquisition, our results do not give credit to the general assumption, based on experimental animal models, that sirolimus is more permissive for tolerance than calcineurin inhibitors

Overall, our studies emphasize the limitations and challenges, but also the opportunities, of biomarker discovery research in organ transplantation. The next step is now to conduct such studies within carefully designed prospective clinical trials to be able to identify clinically useful tests and decision rules that can guide the clinician in the search for rational means to prescribe immunosuppressive drugs.

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# VIII. APPENDIX

Original articles:

- Brouard S, **Puig-Pey I**, Lozano JJ, Pallier A, Braud C, Giral M, Guillet M, Campistol JM, Soulillou J-P, Sanchez-Fueyo A. Comparative transcriptional and phenotypic peripheral blood analysis of kidney recipients under cyclosporine A or sirolimus monotherapy (submitted).

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