UNIVERSITY OF BARCELONA

DIVISION OF CLINICAL SCIENCE

FACULTY OF MEDICINE

DONOR-SPECIFIC MEMORY/EFECTOR AND REGULATORY T CELL RESPONSES AS BIOMARKERS IN KIDNEY TRANSPLANTATION

Doctorand: ORIOL BESTARD MATAMOROS

Directors: Professor JOSEP Mª GRINYÓ BOIRA

Dr. JOSEP Mª CRUZADO GARRIT

Josep Mª GRINYÓ I BOIRA, Doctor in Medicine and Surgery, Chief of the Nephrology Department at the University Hospital of Bellvitge and Professor at the Medicine Department at the Barelona University and **Josep Mª CRUZADO I GARRIT**, Doctor in Medicine and Surgery and staff member of the Nephrology Department at the University Hospital of Bellvitge**.**

CERTIFY

That Oriol Bestard Matamoros, graduate in Medicine and Surgery, has carried out under our direction the research work to elaborate his Doctoral Thesis untitled "**donor-specific memory/efector and regulatory t cell responses as biomarkers in kidney transplantation"**, and through this writing they authorize its presentation to achieve the Degree of Doctor in Medicine and Surgery.

This is made evident to all effects in Barcelona, the 15th June 2009.

Josep Mª GRINYÓ I BOIRA
Director de la Thesis

Josep Mª CRUZADO I GARRIT
Codirector of the Thesis

To my father, with whom, unfortunately, I was not able to share this work. I am certain he would have turned into music and a smile, like he used to do with everything.

To my mother, for the strength and love she has always given us.

ACKNOWLEDGMENTS

This doctoral thesis has come out as a result of the conjunction of the effort and inestimable help, in different areas, of several people. Their contribution, in a way or another, has allowed me to carry out this work, in which enthusiasm, dedication and, above all, the desire to learn have been the premises prevailing all this time.

I would like to point out and thank the Director of this thesis, Doctor Josep Mª Grinyó, for having given me the chance and confidence to elaborate this research work. His outstanding professional and personal quality, as well as his constant and never-ending capacity to motivate, have made all this years of my Nephrology and renal transplantation training much easier.

To Dr. Josep Mª Cruzado, the thesis co-director, regarding his constant help either intellectual and personal. His clairvoyance and analytical capacity have been fundamental to carry out this work.

I would like to specially thank Professor Petra Reinke and Professor Hans-Dieter Volk from the "La Charité" Hospital in Berlin, with whom I had the pleasure to work during one and a half year at their center, making me feel like another member of their team. Their working capacity and knowledge have been short to essential to develop this thesis.

To the colleagues and friends at the Nephrology department, Drs. Salvador Gil-Vernet, Daniel Serón, Joan Torras, Alberto Martínez-Castelao, Francesc Moreso, Xavier Fulladosa, Miquel Hueso, Rafael Poveda, Teresa Gonzalez and Lluís Carreras for having taught me so much regarding the different approaches towards Nephrology and also life during these years.

To my residence colleagues Dr. Inés Rama and Dr. Meritxell Ibernón, for the amazing residence years shared during these intense training courses.

To the outstanding **grup d'assajos clínics**, Anna Caldés, Maria Sarrias, Carol Polo and Eulàlia Molinas, without them all the research work would have been useless.

To Dra. Mariona Mestre and Dr. Jordi Bas from the Immunology Department of the Hospital of Bellvitge, for having received me and helped all along the year spent at their lab.

To Dr. Marta Carrera and Dr. Montse Gomà, from the Patohlogy Department, for their very valuable and constant help at all the histological analysis.

To the whole group of the Experimental Nephrology Laboratory, Dr. Nuria Lloberas, Dr. Immaculada Herrero, Marcel·la Franquesa, Linda Cassis and Nuria Bolaños for their sincere help at different laboratory tasks.

To my dear friends Richard Mast, Joan B. Gornals, Toni Llabrés, Sergi Cànovas and Carles Castilla, for being there whenever needed.

To my brothers Maurici and Esteve, unwarily omnipresent at my side and always making me remember who I am and where I come from.

To my grandparents, a never-ending source of good advises.

INDEX

I. INTRODUCTION

As the incidence of chronic renal failure (CRF) rises in the Western world, so does the need for renal replacement therapy (1). Kidney transplantation is the treatment of choice in patients who develop end-stage CRF, offering greater survival (2), better quality of life and lower cost than other renal replacement therapies (3).

A great deal has been achieved since the first successful kidney transplant in 1954. Originally practiced only in highly selected cases, kidney transplantation is now performed routinely as treatment of choice in patients with CRF. In fact, short-term results of kidney transplantation have improved significantly in the last fifteen years, reaching a graft survival rate of almost 95% during the first year after transplantation (www.unos.org). The significant progress made in the understanding of the mechanisms of the immune response has allowed the production of increasingly selective immunosuppressive drugs aimed at specific targets in the activation of the immunological response (Figure 1). Among them, we find the calcineurin inhibitors cyclosporin and tacrolimus and the antiproliferative mycophenolate mofetil developed in the 1980s and 1990s; the m-TOR inhibitors (mammalian target of rapamycin), and the new formulations of mycophenolic acid as well as the new monoclonal and polyclonal antibodies which have been used as induction therapy since the year 2000. These drugs have allowed a significant reduction in the episodes of acute cellular rejection, which have fallen from a rate of 30-40% to a current rate between 10-15%. Nonetheless, the inexorable appearance of chronic graft dysfunction, giving rise to non-specific histological lesions such as interstitial fibrosis and tubular atrophy, and patient death essentially due to cardiovascular disease and cancer (both paradoxically related to the chronic use of these powerful immunosuppressive therapies) have meant that,

contrary to expectations, the long-term survival of the renal graft has not increased in the last 15 years. In fact, approximately 50-60% of renal grafts are lost around 10 years after transplantation (4), a situation that has enormous clinical, economic and emotional consequences for the patient and is immensely costly in terms of social and health resources as well: for instance, the resumption of dialysis, the return to the waiting list for retransplantation and the increased risk of death. Therefore, in spite of the introduction of new immunosuppressive treatment strategies in the last twenty years, no significant improvement in long-term graft survival has been achieved.

In recent years, increasing support has emerged inside the scientific community for attempts to prepare or program the immunological system to achieve a state of anti-donor hypo-responsiveness or tolerance, in order to be able to reduce immunosuppressive treatment safely – or even, in selected cases, to eliminate it totally. To achieve this aim, it is vital to have biological markers and immune-monitoring tools which are sensitive and specific enough to allow us to know the functional state of the alloimmune response at different stages during renal transplantation.

The immune system is programmed epigenetically to defend the organism from external pathogens (antigens), but to tolerate or recognize its own antigens. From this perspective, kidney transplant represents an insult to the immunological system which will inevitably provoke its activation and response throughout the entire lifetime of the transplant. The effect of this complex and highly specialized process on the graft will depend on the degree of activation of the immunological response to the alloantigens involved.

The advent of chronic graft dysfunction is related to the multiple insults which the organ continually undergoes and which eventually lead, at the histological level, to the

appearance of non-specific, generalized fibrosis. Classically, all these factors are categorized according to whether they are related or not with the activation of the immunological response. On the one hand, among those not directly related to the activation of the alloimmune response are preexisting lesions of the donor, acute tubular necrosis, damage due to ischaemia-reperfusion, high blood pressure, vesico-ureteral reflux and repeated urine infections and damage due to the nephrotoxic effect of the calcineurin inhibitors (CNI) (5). All these etiologies are clincally identifiable, and therefore potentially modifiable, in daily clinical practice. On the other hand, histological lesions may also be produced by the activation of the donor-specific immune response. All these pathogenic factors combine in the non-specific histological lesion currently known as interstitial fibrosis and tubular atrophy (IF/TA) (6). However, this condition may or may not be accompanied by lesions which indirectly suggest a possible immunological origin of the damage. In fact, certain highly specific structural lesions such as the appearance of double contours at the level of the glomerular basal membrane or the multilamination of the tubular basal membrane together with diffuse deposits of the C4d complement fraction in the peritubular capillaries suggest histologic damage caused by a humoral immunological mechanism. In contrast, lesions caused by a cell effector mechanism are few in number or highly unspecific, basically associated with the presence of mononuclear infiltrates (6).

In current clinical practice, the monitoring of the donor-specific alloimmune response centers exclusively on the humoral effector mechanism, via the detection of circulating alloantibodies, mainly against the major histocompatibility complex antigens (HLA antigens). Paradoxically, however, the T-lymphocyte-mediated effector mechanism, which is indeed the target of the majority of immunosuppressive drugs, is not evaluated

at any stage of the transplant. The diagnostic study of graft dysfunction, both acute and chronic, is based essentially on the evaluation of clinical parameters such as renal function (changes in serum creatinine or glomerular filtrate and proteinuria levels), plasma though levels of the immunosuppressive drugs, and the histological study of the graft. Noteworthy, none of these parameters allow us to establish reliably the state of the donor-specific immune response in a functional and dynamic fashion.

So, the search for sensitive and specific immunological biomarkers that can dynamically represent the state of the donor-specific alloresponse at different stages of the transplant is vitally important to the establishment of an individualized therapeutic strategy in which immunosuppressive treatment can be safely increased or reduced as appropriate. Currently, many efforts are centered on the development of new approaches which allow the prediction of the post-transplant graft evolution and which identify the main factors that trigger the dysfunction. Among them are the specific characterization of circulating antibodies in peripheral blood using various diagnostic techniques (7) and the use of soluble or cell surface markers (both proteins and genes) in peripheral blood and urine (8). More recently, several groups have researched certain genetic profiles and/or patterns of protein expression at different biological levels (blood, urine and even in the graft tissue) as markers of histological damage of immunological or non-immunological origin (9, 10). Nonetheless, due to the technical and methodological complexity of these new approaches and the low number of patients evaluated, most of them are still at the research stage and are not yet used in daily clinical practice.

Achieving a state of immunological tolerance or hyporesponsiveness is considered classically as the preservation of graft function over time without concomitant

immunosuppressive treatment. Unlike the kidney, heart, gut and pancreas, liver transplant seems to be immunologically privileged, as is suggested by the survival analysis and the very low incidence of rejection episodes which, in addition, have a very limited influence on graft evolution (11, 12). This difference in immunological tolerance seems due, above all, to the different degrees of immunogenicity of the tissues due to the presence of cells with a high expression of HLA molecules and also, due to their antigen-presenting capacities (13, 14).

Achieving a state of tolerance of a renal graft is currently possible, as has been shown by several experimental models in rodents and larger mammals such as pigs, dogs and non-human primates (15-21). For this reason, it appears that avoiding graft rejection without maintenance immunosuppressive treatment is not biologicaly impossible. Nonetheless, in humans the achievement of graft tolerance has been proven in only a very small number of patients (<1%). Moreover, as well as rare, it is unpredictable, although recent data suggest that it may be predicted by particular gene profiles (22). In fact, the achievement of a tolerogenic state has been shown to be driven by a wide variety of immunological processes acting simultaneously, among them modulations in the frequency of effector cell precursors, the efficiency of the antigen presentation, the activation threshold of effector cells, the alteration in the cell traffic and the appearance of mechanisms that regulate the immunological response. Therefore, precise knowledge and monitoring of the mechanisms of the alloimmune response would help to identify patients with immunological profiles that predispose to better or worse graft acceptance.

Mechanisms of the alloimmune response

The alloimmune response is initiated via T lymphocyte antigen recognition through the HLA molecules of the major histocompatibility complex on professional antigen-presenting cells (APC). This antigen recognition is performed through two different pathways: a direct pathway, in which the recipient's T lymphocytes recognize the allopeptides on the donor's professional APC, and through an indirect pathway, in which the recipient's T lymphocytes recognize the allopeptides on the recipient's APC after they have been processed and presented on the cell surface along with the class II HLA molecules. The type of antigen presentation that occurs will have a key role in the subsequent immunological response: the direct pathway is associated with a stronger and faster immunological response, but the indirect pathway has more long-term implications due to the absence of the donor's professional APC, thus producing a longlasting immunological response that endures over time (23). Nonetheless, recent studies suggest that both antigen presentation pathways may persist and be involved in the development of the IF/TA in the graft in the long term. Herrera et al. (24) showed that the recipient's dendritic cells, when co-cultured with allogenic dendritic cells or even endothelial cells, are able to acquire substantial levels of donor class I and II MHC-peptide complexes and then migrate to lymph nodes and prime T cells by both allorecognition pathways. This is type of antigen allorecognition is called third or semi-direct pathway (figure 2).

Once antigen recognition has taken place, the effector mechanisms of the immunological response are activated. These highly specialized mechanisms vary according to the local cytokine milieu generated after specific antigen recognition, and according to the biological origin of the APC and the types of specialized cell subpopulation present. The classical paradigm of the effector mechanisms of the

alloimmune response is determined by the differentiation of the CD4+ T lymphocyte into a cellular pathway (Th1) or a humoral pathway (Th2) (25). In the first case, the Th1 effector response activates the T lymphocytes, converting them into the main effector cells producing proinflammatory cytokines such as interferon-gamma (IFN-γ) and perforins. In the second case, the Th2 effector pathway activates the B lymphocytes causing a humoral response or a response mediated by antigen-specific antibodies via B-cell differentiation in plasma cells.

It is well known that in order to achieve full activation of *naive* CD4+ T lymphocytes (LTnaïve) and subsequently proliferate, expand clonally and exert an effector response, three stimulatory signals must be generated. The first one is produced after the alloantigen recognition by the T cell receptor (TCR) through HLA molecules on the professional APC. The second one is produced by costimulatory molecules between the T lymphocytes and the APC: the most characterized are the CD28 and CD154 (CD40 Ligand) surface receptors and the B7 (CD80/CD86) and CD40 complexes. This second signal is produced exclusively by professional APCs. The absence of this second signal makes the lymphocytes anergic and induces their programmed death, or apoptosis. Once the signal of antigen recognition has been received via the TCR, it induces the appearance of cytokine receptors and other growth factors on the cell surface which allow the clonal expansion of both cytotoxic and helper antigen-specific T lymphocytes. The cytokine with the best known effect is IL-2, which interacts via its receptor (IL-2R or CD25). This interaction is known as the third signal of T-cell activation.

Apart from these classical effector responses that result in graft rejection, the appearance of a suppressor or tolerogenic response (Th3) mediated by a subpopulation of lymphocytes known as regulatory T cells (Treg), seems to be taking on an

increasingly important role in the induction and maintenance of antigen-specific tolerance. Under certain highly specific biologically favorable conditions, this cell subpopulation is able to suppress or inhibit both cellular and humoral effector responses and promotes graft acceptance over time (26-30).

Recently (31), another T lymphocyte subset population has been described: CD4+ (Th17), which appear in a highly specific environment rich in IL-23, TGF-β and IL-6. This T-cell subset population, which produces proinflammatory cytokines sucha as IL-17, IL-22 and TNF-α, is responsible for inducing a variety of autoimmune disorders and seems to play an important role in the development of episodes of acute rejection by inducing granulopoiesis, and its migration towards inflamatory tissues, above all in situations in which the Th-1 IFN-γ response is inhibited. In addition, this pathway seems to have the opposite effect to the generation of Tregs in the presence of IL-6 (figure 3).

Memory/effector T-cell immune response

Inside the T lymphocyte population we also find the memory/effector T lymphocytes (LTm). This T-cell subset population plays a key role in the development of the antigen-specific alloresponse which leads to both acute and chronic rejection of the renal graft. After a second exposure to the same antigen, this T-cell subset population produces a much faster, more vigorous and effective response than that produced by the LTnaïve subpopulation. The highly specific biological characteristics of the LTm provide the organism with highly beneficial protection against pathogens. However, in the transplant setting, the presence of these LTm primed specifically against donor antigens is potentially harmful. Nonetheless, the impact of this lymphocyte subpopulation in the area of transplantation has been underestimated; it has only recently been recognized that controlling the response triggered by this T-cell subpopulation is considerably more difficult than controlling the LTnaive subset.

Oustandingly, the biological profile of this T-cell subpopulation makes them particularly effective. These cells have a specific localization, either in the lymphatic tissues (in the case of central LTm) or circulating on the periphery (peripheral or effector LTm). In addition, this antigen-specific T-cell subpopulation has less need of the second signal of costimulation for its complete activation. These cells have also been shown to exhibit a different kinetic action: rapid secretion of cytotoxic cytokines such as IFN-γ, rapid proliferative capacity, greater response to a lower dose of antigen exposure and a direct cytolytic effect (32, 33). Most importantly, unlike LTnaïve cells, LTm cells have the capacity to lyse cells directly after the new antigen recognition without the need to differentiate or proliferate clonally (34). Moreover, LTm can be activated and differentiated into cytotoxic effector cells by non-professional antigen-presenting cells such as endothelial cells (35), a fact that makes them even more

dangerous (table 1). With the appearance of new immunosuppressive drugs, the clinical importance of this cell subpopulation has increased significantly given the reports of their greater resistance to specific immunosuppressants: some studies suggest that calcineurin-inhibitors, especially tacrolimus, exert a greater inhibitory effect on LTm, in terms of both proliferation and cytokine secretion, than the mTOR-inhibitors, mycophenolic acid and azathioprin (36).

It is known that this T-cell subpopulation is generated after a first exposure to alloantigens. In the case of transplantation, its presence is to be expected in patients with prior blood polytransfusions (37), a history of pregnancy (38), previous transplants (39), cross-reaction with viral or bacterial antigens (heterologous immunity) and even the time on hemodialysis implies a greater risk of cell sensitization. Therfore, its presence is implicitly associated with a higher risk of acute rejection and poorer renal graft function, (40).

This specific T-cell compartment has been studied with a range of monitoring techniques, primarily tests of cytotoxic T cell precursors using limiting dilution analysis (LDA) (41), detection of secreted cytokines with flow cytometry (42, 43) or the Trans-vivo analysis of human delayed-type hypersensitivity (44). However, all these techniques are very laborious and have demonstrated little sensitivity and reproducibility for immune-monitoring this lymphocyte subpopulation.

One of the most sensitive and precise techniques for functional monitoring of the antigen-specific effector/memory T-cells is the Elispot IFN-γ immunoassay (45). This technique detects the frequency of IFN-γ secretion of each stimulated cell in a short-term mixed lymphocyte culture (20-22 hours). Using a high resolution image analysis program, the number of spots (IFN-γ) secreted is counted and compared with the number of stimulated cells specific for the antigens exposed in the mixed cell culture.

In kidney transplantation, the monitoring of this lymphocyte subpopulation using the Elispot IFN-γ technique has been performed mainly in the initial stage of the transplant. The presence pre-transplantation of high frequences of donor-specific IFN-γ producing T cells circulating in peripheral blood, activated by the direct antigen presentation pathway, has been associated with a higher risk of acute cellular rejection post-transplant. In addition, their detection during the first weeks of the transplant has also been associated with a poorer graft function after six months (46, 47). Interestingly, the presence of these highly alloreactive LTm does not always correlate with the degree of humoral sensitization, suggesting a certain independence of action between the cellular effector mechanisms activated by the direct antigen presentation pathway and the humoral effector mechanisms (48, 49).

So, it appears that this memory/effector T-cell subset population, evaluated using the Elispot technique, may be a relatively sensitive biomarker for monitoring the degree of donor-specific cellular alloreactivity in kidney transplantation.

Regulatory T cell immune response

Challenging the classical effector paradigm of the lymphocyte effector response Th1/Th2/Th17 described above, the immune system also contains a suppressor T lymphocyte subpopulation named regulatory T cells (Treg). This lymphocyte subpopulation (Treg) has a suppressive capacity; it is able to control or inhibit immune effector responses to specific antigens and is therefore a mechanism of self-regulation, facilitating the induction of central and peripheral tolerance. In recent years this lymphocyte subpopulation has acquired growing importance in the field of auto and alloimmunity. In fact, we know that the immune system in normal conditions produces this type of regulatory lymphocyte subpopulation endogenously, as a cell constituent of its own.

Tregs have a highly specialized function in suppressing the immunological response; in fact, alterations in the number and function of these cells have been shown to trigger autoimmune or inflammatory diseases in both animals and humans (50). Tregs play a crucial role in multiple immunological scenarios: in the prevention of processes of an autoimmune origin such as diabetes type 1 (51, 52) and immunoinflammatory processes (53), regulating the immune response to viral and parasitary infections (54, 55), in the maintenance of materno-fetal tolerance (56) and inhibiting anti-tumoral immunity (57). Specifically in the field of transplantation, the role of this T-cell subpopulation is attracting interest, since they appear to be able to induce and maintain a state of antigen-specific hyporesponse (58).

Though a number of cell lineages have been reported for Tregs, the best known are the ones that express the "master gene" that identifies them: the intranuclear transcription factor forkhead boxp3 (FoxP3). In fact, in humans and mice all known cells with regulatory activity contain non-mutated versions of this gene (59). Nonetheless, in

humans some cells without regulatory capacity may be able to express this gene transitorily after being activated through antigen recognition by the TCR (60).

Tregs can be divided into two main subpopulations; the more numerous, and the better known, are the ones that are formed naturally in the thymus and enter the peripheral circulation in the form of mature functional cells ("naturally occurring" CD4+CD25+FoxP3+ Treg or nTregs) (61, 62) and the CD4+ Tregs induced on the periphery from lymphocyte precursors CD4+CD25- or expanded from the lymphocyte subpopulation CD4+CD25+ thanks to the effect of certain soluble factors such as TGF-β or IL-10 (63, 64). The differentiation of the T lymphocyte into this regulatory functional phenotype depends on the different factors that are produced after the antigen recognition by the T cell receptor: the local cytokine milieu, the origin of the APC and the presence of pre-existing Tregs. Furthermore, this cell subpopulation may express other molecules such as the GITR (glucocorticoid-induced TNFR family related gene), CTLA-4 (cytotoxic T lymphocyte antigen) and a low amount of CD127. However, the essential factor for the maintenance of the regulatory/suppressive effect of this lymphocyte subpopulation is the expression of the transcription factor FoxP3 which is maintained due to persistent and constant antigen exposure. One of the most important characteristics of these cells is their antigen specificity (65). This phenomenon is essential for inducing a state of antigen-specific tolerance.

These suppressive properties with such selective antigen specificity make this cell subpopulation particularly attractive, not only as a biomarker of a pro-tolerogenic immunological state but also for use in the future as a potential immunosuppressive agent in transplantation. In fact, in murine models of skin and heart transplantation, the administration of Tregs previously sensitized against donor antigens via direct and

indirect antigen presentation pathways has proved able to prevent both acute and chronic graft rejection (66).

In kidney transplant, high levels of mRNA-FoxP3 in urine during episodes of acute rejection have proved to be a favorable marker for the reversibility of the rejection episode and graft survival, indirectly suggesting that this cell subpopulation exerts a protective role infiltrating the renal graft and thus promoting immunological acceptance (67). In fact, various experimental studies of skin, heart and kidney transplantation have shown the positive role of cellular infiltrates with Tregs in the graft for graft acceptance (68-70). These findings suggest that not all lymphocyte infiltrates in the graft have the same biological significance.

The differentiation between pro-inflammatory and pro-tolerogenic cellular infiltrates acquires special relevance in the histopathological entity known as *subclinical acute rejection*. This entity is defined as the presence of exactly the same histological patterns as those observed during episodes of clinical acute rejection, but in patients with stable renal function. The prognostic significance of subclinical acute rejection is controversial, especially with regard to the analysis of the effect of treatment of these infiltrates (71-73). Therefore, differentiating between these protective or inflammatory infiltrates would theoretically allow the establishment of different therapeutic strategies in each case.

Interestingly, some immunosuppressive agents also seem to exert a different effect on this T-cell subpopulation. CNI drugs, because of calcineurin's inhibitory effect on the synthesis of IL-2, inhibit the formation and expansion of these cells. The lack of IL 2 impedes the overexpression of its receptor (CD25), a key factor for the survival of the Tregs both *in vitro* (74) and *in vivo* (75). In contrast, although m-TOR inhibitory drugs inhibit the transducer signal from the bond of IL-2 to its receptor, have the capacity to

expand and maintain this cell subpopulation in peripheral blood, probably because this inhibition is restricted exclusively to the activated effector lymphocyte subpopulation (76). In the same way, polyclonal anti-lymphocyte antibodies such as r-ATG (rabbit anti thymocyte globulin) have favorable properties as they permit the induction and expansion *in vitro* of functional Tregs in the peripheral blood, basically via the conversion of T lymphocytes CD4+CD25- to CD4+CD25^{high} in the periphery (77).

Therefore, the functional examination of this lymphocyte subset population in kidney transplantation may also help to identify patients who are most likely to accept the graft and in whom immunosuppressive treatment could therefore be safely minimized.

II. HYPOTHESIS

The hypothesis of this doctoral thesis is that the immune-monitoring of the donor-specific memory/effector T-cell and FoxP3+ regulatory T cell subset populations in kidney transplantation could be useful biomarkers of the anti-donor alloimmune response and thus be related to the graft function and to the histological damage of the graft, both in protolerogenic protocols as well as during the follow up of all kidney transplanted patients.

III. OBJECTIVES

1. The first objective of this thesis is to evaluate whether immune-monitoring of donor-specific memory/effector t cells circulating in peripheral blood and activated by the two alloantigen presenting pathways (direct and indirect) using the IFN-γ Elispot assay in longlasting kidney transplant patients (at least for more than 2 years), is a reliable biomarker to discriminate those patients with immune-mediated graft dysfunction.

2. The second objective of this work is to evaluate if the presence of regulatory T cells (CD4+CD25+FoxP3+) within cellular graft infiltrates in patients with the diagnoses of subclinical acute rejection at 6 month after the transplant, is a reliable biomarker to differenciate harmful from protective cellular graft infiltrates.

3. The third objective is to assess the immune mechanisms by which through an immunosuppressive strategy with immunomodulatory properties based in T-cell depletion using low doses of thymoglobulin and maintenance immunosuppression with sirolimus and mofetil mycophenolate, avoiding steroids and calcineurin-inhibitors, is able to induce a donor-specific hyporesponsiveness state after transplantation. In order to achieve this goal, an accurate monitoring of the main effector anti-donor alloimmune responses at different time-points after the transplant is carried out; the cellular alloresponse using the IFN-γ Elispot assay, the humoral alloresponse assessing the presence of circulating donor-specific alloantibodies (DSA) and presence of C4d at the level of the pertubular capilaries in the graft and lastly, the regulatory alloresponse evaluating the presence and functional behaviour of the FoxP3+Tregs both in peripheral blood and directly in the graft.

IV. STUDIES

V. RESULTS

Study 1.

Circulating alloreactive T cells correlate with graft function in longstanding renal transplant recipients.

J Am Soc Nephrol. Jul; 19(7):1419-1429, 2008.

In 34 patients with kidney transplant of more than two year's evolution, the presence of donor-specific memory/effector T cells circulating in peripheral blood activated by both antigen presentation pathways (direct and indirect) was evaluated using IFN-γ ELIspot assay. Their presence or absence was correlated with the respective clinical evolution of each patient. The presence of circulating donor-specific alloantibodies was also evaluated in all patients selected.

The direct antigen presentation pathway was performed *in vitro* using donor antigen-presenting cells present in peripheral blood. In contrast, the indirect pathway was mimicked *in vitro* using allopeptides of the respective donors obtained by mechanical fragmentation of mononuclear peripheral cells. Compared with other methods, which use specific peptides of the HLA system, this technique obtains a much more accurate antigen profile of the donor, as the whole of the alloantigen spectrum is present.

Clinical variables:

- Patients with an episode of acute cellular rejection (n=16) presented a significantly poorer renal function than those without (n=18; serum creatinine 21.±1 *versus* 1.5±0.4 mg/dL; p=0.007). Furthermore, patients with episodes of late acute rejection (>3 months, n=12) also had significantly poorer renal function than the rest (serum

creatinine 22.±2.2 *versus* 1.5±0.5 mg/dL; p=0.0019). Those with more than one episode of acute rejection (n = 10) showed a trend towards worse serum creatinine (2.1±1.2 *versus* 1.6±0.5 p=0.051) than the rest (n = 24), (table 2 in the article).

In the univariate analysis, no clinical variables such as the donor-recipient age, sex, time since transplant, the number of donor-receptor HLA mismatches and the different type of immunosuppressive treatment predicted renal function (either serum creatinine, glomerular filtration rate (GFR) or proteinuria).

Immunological monitoring:

- First, we checked the validity of the technique for reproducing the indirect antigen presentation pathway *in vitro* (figure 7 in the article). After performing a mixed culture for six days of T lymphocytes from a healthy individual and allopeptides obtained from the mechanical fragmentation of the membrane proteins of antigen-presenting cells from another healthy individual with complete HLA class I and II missmatch (A, B and DR) (checking for the presence of HLA molecules in supernatants using Western-blot), the T lymphocytes were again extracted from the culture and exposed to the same allopeptides from the same individual to assess the degree of memory immune response. This memory lymphocyte line presented large-scale T cell proliferation (basically at the expense of the CD4+ T-cell subset population) and cytokine secretion of IFN-γ after being restimulated with the same allopeptides with which they had been previously primed.

- Thirty-four patients were evaluated by the direct antigen presentation pathway, and 33 by the indirect pathway. In 20/34 (58%) patients had detectable direct Elispot frequencies. Regarding the indirect pathway, 20/33 (60%) of patients had positive Elispot responses. The degree of response differed considerably between the two

presentation pathways: the direct frequencies were approximately three times higher than the indirect frequencies (figure 1 in the article). Though 14 patients presented memory alloreactivity via both pathways and eight presented no alloresponse of any kind, no relationship was found between the two pathways in the univariate analysis.

1. Direct pathway (DP)

- The direct pathway was correlated positively with serum creatinine and inversely with the GFR (r=0.551, p=0.001 and r=-0.34, p=0.042, respectively). No correlation was found between the direct pathway and the presence of proteinuria (estimated semiquantitatively), (figure 2 in the article).

- The patients with more than one episode of acute rejection and those with episodes of late acute rejection (>3m) had significantly higher direct frequencies (figure 2 B in the article).

- Fourteen out of 34 patients (41.2%) were donor-specific hyporesponders by the direct pathway. They presented a significantly better GFR than donor-specific non-hyporesponders (59.7 ± 17 *versus* 41.2 ± 16 ml/min; p=0.006), (figure 3 in the article). In addition, the higher the number of HLA mismatches (A, B and DR) between recipient and the donor, the higher the number of donor-specific IFN-gamma frequencies were detected (figure 4 in the article).

2. Indirect pathway (IP)

- This pathway was not correlated with the renal function or with history of acute rejection, but it was correlated with the presence of proteinuria. In fact, the presence of proteinuria was significantly associated with a high level of indirect alloreactivity (29.2

± 24.3 *versus* 12.3 ± 38.1 spots in patients with and without proteinuria respectively; p=0.022), (figure 5 in the article).

- Unlike the direct pathway, the presence of indirect alloreactivity was correlated positively with time after transplantation; that is, the longer the time after transplant, the higher the indirect pathway alloreactivity (r=0.351, p=0.045), (figure 5 B in the article).

3. Global cellular alloreactivity

- Forty-two per cent of the patients studied had positive responses of donor-specific alloreactive memory/effector T cells in both pathways (DP+/IP+), 24% presented no sign of alloreactivity in either pathway (DP-/IP-), 15.1% were positive only in the direct pathway (DP+/IP-) and 18% presented alloreactivity only in the indirect pathway (DP-/IP+).

- Highly alloreactive patients in both pathways presented significantly worse serum creatinine than those with alloreactivity undetected in either pathway and those with only indirect alloreactivity (2.1±1 *versus* 1.1±0.3 mg/dL; p=0.004 and 2.1±1 *versus* 1.3±0.3 mg/dL; p=0.023). Patients with direct alloreactivity only, presented no differences in renal function with respect to those with high alloreactivity by both pathways (figure 6 in the article).

4. Humoral alloreactivity

- Only one patient (with indirect alloreactivity) presented circulating donor-specific alloantibodies after the transplant.

Multivariate analysis

In the multivariate analysis, all the variables that had been associated with renal function (serum creatinine) and presence of proteinuria in the univariate analysis were studied (table 3 in the article). In all of them, only the achievment of a state of hyporesponse by the direct pathway was independently correlated with renal function (β coefficient =-0.505, p=0.003). With regard to the presence of proteinuria, indirect alloreactivity was the only variable that correlated independently (RR=1.047, p=0.04).

Study 2.

Presence of FoxP3+ regulatory T cells predicts outcome of subclinical rejection of renal allografts.

J Am Soc Nephrol. Oct; 19(10):2020-2026, 2008.

In 37 patients who had received a kidney transplant and diagnosed with subclinical acute rejection by protocol biopsy six months post-transplantation (stable renal function defined as variability of <15% of the renal function in the two weeks before and after the biopsy and histological signs of borderline changes and/or tubular-interstitial acute rejection Banff grade ≥1), we determined the presence of Tregs FoxP3+ cells among the cellular infiltrates, their proportion among the rest of the T-cell subset population (CD4+, CD8+ and CD25+), and its impact on the clinical evolution of these patients.

- Of the 37 patients, twelve (32.5%) presented no evidence of Tregs among the cellular infiltrates, whereas 25 (67.5%) did present Tregs. Most of these cells were CD4+CD25+. The percentage of Tregs in the global lymphocyte T population (Treg/CD3+) varied from 0.7% to 52%.

- A history of induction therapy with a T-cell depleting agent (thymoglobulin) and maintenance immunosuppression with sirolimus were the only clinical variables significantly associated with the presence of Tregs among the tubular-interstitial infiltrates (table 2 in the article). In addition, only the immunosuppressive combination of thymoglobulin and sirolimus (rATG+SRL) was significantly associated with the presence of Tregs in the infiltrates. In contrast, patients who were receiving calcineurin-inhibitors (CNI) or who had not received induction therapy presented significantly lower infiltration by this T-cell subset population.

- No clinical or demographic variables were associated with the presence of this Treg population. Nor were history of acute rejection, renal function/proteinuria, acute or chronic scores of the Banff classification in the different renal histology compartments nor the types of lymphocyte aggregates (nodular or diffuse) associated with the presence of Tregs cells among the infiltrates.

- Patients with Tregs cells among the cellular infiltrates presented a better evolution of the renal function (serum creatinine and GFR) at two and at three years after the transplant than those without (figures 2 and 2 B in the article). In addition, the percentage of Tregs/CD3+ among the infiltrates was correlated positively with the GFR two years after transplant (r=0.36, p=0.03), (figure 2C in the article). Interestingly, analysing the proportion of Tregs with regard to the total lymphocyte infiltrate (CD3+), the patients with lower degrees of chronic lesion (Banff tubular-interstitial scores <2) were the ones with a highest proportion of Tregs (14.2±16.2 *versus* 8.3±8.1%, p=0.035).

- Patients receiving SRL presented a significantly better renal function than those receiving CNI two and three years after transplant (GF 78.3±21 *versus* 55.1±18.2 ml/min [p=0.003] and 89.7±11 *versus* 56.1±18.2 ml/min [p=0.003] respectively), (figure 3 in the article). Nonetheless, analysing the impact of Tregs exclusively among patients treated with CNI, the patients with presence of Tregs among the infiltrates also presented a significantly better renal function two and three years after kidney transplant (GFR 63±17.1 *versus* 44.5±5 ml/min [p=0.02] and 62±20 *versus* 52.3±16.6 ml/min [p=0.04], respectively), (figure 3 B in the article).

- In the Kaplan-Meyer analysis, considering patients with GFR < 40ml/min (obtained with ROC curve analysis), as event variable, patients with Tregs among the infiltrates had a significantly lower risk of reaching this GFR level during follow-up than those without Tregs. Immediately afterwards, both in the univariate and multivariate analysis,

serum creatinine six months post-transplant and the presence of Tregs in the infiltrates were the only independent variables that predicted maintenance of a GFR $\geq$ 40ml/min (figure 4 in the article).

Study 3.

Achieving donor-specific hyporesponsiveness is associated with FoxP3+ regulatory T cell recruitment in human renal allograft infiltrates.

J Immunol. Oct; 179(7):4901-4909, 2007.

Twenty renal transplant patients with low immunological risk were administered a CNI and steroid-free immunosuppressive regimen, based on induction therapy with low doses of thymoglobulin and maintenance treatment with sirolimus (SRL) and mycophenolate mofetil (MMF). During the first two years post-transplant the cellular and humoral donor-specific immune responses in peripheral blood were evaluated, as well as the evolution of the different lymphocyte subset populations and the degree of cell apoptosis. Histological damage was studied in protocol biopsies performed six months after the transplant; in all of them, phenotypical characterization of the cellular infiltrates was also performed.

A. Clinical variables

- The mean follow-up period was 34 months (range: 21-47 months). The survival rates of patient and graft were 95 and 85% respectively. The incidence of biopsy-proven acute rejection (BPAR) was 35% (12/20); three (15%) were diagnosed as *borderline changes* (table II in the article). All the rejections were sensitive to steroids. At the end of follow-up, there were no statistically significant differences with regard to renal function or levels of proteinuria between patients with and without BPAR (figure 1 in the article).

- After 34 months of follow-up, 70% of the patients remained CNI-free and 35% steroid-free. Two patients were on SRL monotherapy.

- One patient presented viremia due to cytomegalovirus and was treated with gancyclovir without further complications. Another developed a testicular seminoma 27 months after transplantation, which was controlled by chemotherapy with excellent clinical response. One patient on CNI died due to an extrahospital pneumonia 42 months post-transplantation.

B. Immunological monitoring in peripheral blood

1. Lymphocyte subpopulations and cell apoptosis

- After transplantation there was a significant depletion in all the lymphocyte subpopulations (T, B and Natural Killer) during the first two months, which recovered slowly but steadily. Two years post-transplantation, the total T lymphocyte count (CD3+) and the CD4+ subset were still significantly below baseline levels. In contrast, the total number of CD8+, CD19+ and CD56+ had recovered (figure 2 in the article).

- After the transplant, the only lymphocyte subpopulation which increased significantly with respect to baseline was the CD4+CD25+^{high}. After two years, the patients who had reached a state of cellular donor-specific hypo-response presented a significantly greater presence of Tregs (CD4+CD25+^{high}FoxP3+) in peripheral blood (55±9% *versus* 28±13%, p=0.005) than the others. This finding was associated with the maintenance of immunosuppressive treatment with SRL (48±11% *versus 3*8±13%, p=0.005), (figure 4 in the article).

- After the profound initial lymphocyte depletion, on day 5 post-transplantation a peak of apoptosis was found, and restricted to the activated subpopulation CD8+ (HLA DR+), (figure 3 in the article).

2. Cellular alloreactivity monitoring (Elispot IFN-γ)

- The presence of high donor-specific alloreactivity pre-transplantation was associated with a greater incidence of acute rejection (p=0.02) (figure 5 in the article). During the first six months after transplantation a generalized hypo-response was produced in most patients. Six months after transplantation, nine patients acquired a state of donor-specific hypo-response and this immunological state was maintained until two years after transplantation (figure 6 in the article).

- Patients who were donor-specific hyporesponders six months after transplantation presented a significantly better renal function than non-hyporesponders; at 24 months a trend towards statistical significance remained (figure 7 in the article).

- In order to confirm that this immune state of donor-specific hypo-response was achieved thanks to the donor-specific suppressive effect of the Tregs in these patients, the functional tests were repeated *in vitro,* this time removing the CD4+CD25+^{high} cells from the mixed culture by means of cell-sorting (figure 8 in the article*).* The depletion of the CD4+CD25+^{high} population in patients with donor-specific hyporesponse caused a recovery of the cellular alloresponse. In contrast, the depletion of this T cell subpopulation in responders caused a state of hypo-response, suggesting that in this patient subgroup, these CD4+CD25+^{high} cells were not Tregs but activated T lymphocytes. The anti-specific nature of the suppressive effect of this Treg subpopulation was antigen-specific as confirmed by the appearance of immune suppression only when these cells were added to an allogenic mixed culture with the presence of donor cells to which these Tregs had been exposed previously.

3. Monitoring of the humoral alloresponse

- None of the 20 patients presented circulating donor-specific antibodies in the year after the transplant, and none presented C4d deposits at the level of the peritubular capillaries in the protocol biopsies.

C. Histological analysis in protocol biopsies after 6 months

- Patients who had reached the state of donor-specific hypo-response presented a lower degree of chronic lesion in the histological study. In contrast, patients with a higher degree of chronic histological damage (Banff>Ia) had not achieved this immune privilege status (table IV in the article).

- Among the cellular infiltrates in the tubules and interstitium, the presence of Tregs among the global T lymphocyte population (CD3+) was significantly greater in donor-specific hyporesponders than in non-hyporesponders (figure 9 in the article).

- None of these patients presented C4d deposits in the peritubular capillaries in the respective protocol biopsies.

VI. DISCUSSION

The donor-specific alloimmune response is present in varying degrees of activation in each individual in the different periods of the transplant. In fact, the acceptance or rejection of the renal graft will depend on whether it is the effector or the regulatory mechanisms of the alloimmune response that predominate. This study has analysed new biological markers that can identify the dominant immune state in the renal transplant patient at the different stages of transplant development and has explored the extent to which certain immunosuppressive strategies are able to modulate this immunological response to the renal graft.

Here, it is shown how the functional monitoring of two lymphocyte subpopulations, the highly alloreactive IFN-γ-secreting effector/memory T lymphocytes and the regulatory T lymphocytes expressing the intranuclear transcription factor FoxP3+, both with very specific immunological functions, seem to be useful immunological biomarkers of rejection or acceptance of the kidney transplant.

To date, the evaluation *in vitro* of the degree of donor-specific immunological alloresponse in the transplant has mainly been performed using techniques of cellular proliferation and cytotoxic precursors to measure naïve and memory lymphocyte populations at the same time. Here, in contrast, we show that the antigen-specific effector/memory T cell alloresponse can be accurately measured at different stages in the development of the transplant using the IFN-γ ELIspot assay, which shows an excellent correlation with the functional evolution of the kidney graft. Recent studies have explored whether the cellular alloresponse during the different stages of the transplant should be monitored by studying independently the different T-lymphocyte subpopulations activated via the two antigen presentation pathways currently known,

the direct and the indirect pathways (78-80). In this study we show that with the IFN-γ ELIspot assay it is possible to accurately mimic *in vitro* the degree of teh anti-donor alloreactivity of the effector/memory T-lymphocyte subpopulations activated by the two antigen presentation pathways. What is more, in contrast to previous studies which monitored direct and indirect cellular alloreactivity simultaneously in renal transplant patients (78), here, for the first time, we use allogenic fragments of donor cells instead of allogenic synthetic peptides in order to cover the whole spectrum of epitopes present in the respective donors. The advantage of this method is that it provides a more accurate *in vitro* reproduction of the real situation, as it includes all the repertoire of epitopes, including also the minor MHC/HLA complex missmatches.

Interestingly, this study shows that the presence of donor-specific memory/effector T cells primed by the direct pathway, both before and after transplantation, can be detected in a large number of patients, and that these cells appear to play a key role in both acute and chronic graft dysfunction. On the one hand, the detection of this T-cell subpopulation before the transplant indicates a prior T lymphocyte allosensitization to donor antigens, which is associated with a higher risk of acute cellular rejection post-transplantation. Interestingly, this finding is independent of the degree of humoral sensitization. On the other hand, and in contrast to the evidence reported in the literature to date (81), here we show that the presence of this T-lymphocyte subpopulation primed persistently via the direct antigen presentation pathway can still be detected in a significant number of patients (more than 50%) more than two years post-transplantation. This phenomenon persists even in patients receiving immunosuppressive maintenance treatment and in the case of the theoretical disappearance of the donor's professional APCs. The long-term activation of the direct

pathway clearly differentiated between patients with preserved renal function and those with graft dysfunction. This indirectly suggests that these patients are more susceptible to exposure to structural damage to the graft mediated by a cellular effector immune mechanism. This in a way raises doubts about the empirical management of the immunosuppressive treatment currently applied in clinical practice (in which the immunosuppressive dose is steadily reduced after transplantation, on the assumption that the effector immune response decreases with time in all transplanted patients). The results of this study suggest the need to determine the donor-specific immune response in order to be able to adjust the immunosuppressive treatment over time.

There are a number of biological explanations for the persistence of T lymphocyte activation via the direct pathway long after the transplant; for instance, the fact that the effector/memory T cells have less need for costimulation and a lower antigen requirement in order to remain permanently activated, or the fact that this direct antigen presentation can be carried out (24) via the recipient's own APC which are able to acquire class I and II HLA molecules on their surface (a process known as the third pathway or the *semi-direct pathway* of antigen presentation), or the fact that the lymphocytes may even be activated by other cell populations in the graft such as endothelial cells. Classically, the number of HLA mismatches between donor and recipient has been associated with poorer long-term results, especially among hypersensitized patients (82). In the present study we found that a poor HLA compatibility between donor and recipient leads to a greater donor-specific cellular alloreactivity post-transplant, emphasizing to an extent the long-term impact of the degree of HLA compatibility in kidney transplantation.

This study also underlines the importance of the indirect alloreactivity. As reported in the literature, indirect T-lymphocyte activation seems to acquire more functional

relevance as time passes after the transplant. This finding is corroborated in our study, in which we observed greater indirect alloreactivity in patients of longer duration. This phenomenon might be due to the constant cell replacement that takes places after the graft, bringing with it a continuous processing of allogenic molecules by the APCs.

In a similar way to direct T-lymphocyte activation, the presence of indirect cellular alloreactivity also seems to play a pathogenic role in the development of the structural damage in the graft (83-87). This hypothesis emerges from the observation of a significantly greater presence of proteinuria in patients with high indirect alloreactivity. Several experimental studies have shown how in the donor-specific humoral immunological response, the change of isotype from IgM to IgG is mediated by the indirect antigen presentation pathway (88, 89). This fact may lead to speculation that the development of the specific lesion called transplant glomerulopathy, a histopathological entity caused by a humoral immunological mechanism, may be originated initially by the activation of the indirect antigen presentation pathway.

So it seems evident that knowledge of the degree of donor-specific effector/memory alloreactivity via the two antigen presentation pathways *in vitro* using a sensitive test such as the Elispot IFN-γ may well help to identify patients at risk of suffering structural damage in the graft of immunological origin.

Like the monitoring of the effector/memory cell alloreactivity, the study of the regulatory lymphocyte T subset expressingteh transcription factor FoxP3+ ($CD4+CD25^{high}Foxp3+$) has a very important role in the regulation of the immunological response, and so it may also be relevant as a functional biomarker in kidney transplantation in some specific clinical situations. Here we show that this regulatory lymphocyte subpopulation is able to proliferate and appear in significant quantities after transplantation both in peripheral blood and directly in the renal graft

showing lymphocyte aggregates in the tubules and the interstitium, characteristically in patients with excellent graft evolution over time.

The infiltration of mononuclear cells in the tubulo-interstitial level of the graft in kidney transplant patients with strictly stable renal function is classically labeled as subclinical acute rejection, as it mimics the histological pattern found in patients with clinical acute rejection. The impact of this histopathological entity on the evolution of the graft is quite controversial (71-73, 90-93). The interpretation of the functional status of these asymptomatic cellular infiltrates is made difficult by the lack of functional information on this cell population infiltrating the graft, with the result that researchers have attributed the same aggressor effector mechanism to all of them. In this study we show, for the first time in humans, that the presence of the Treg Foxp3+ subpopulation among these mononuclear infiltrates in patients diagnosed with subclinical acute rejection in protocol biopsies six months after the transplant predicts a significantly better functional evolution of the renal graft over time, and that the absence of Tregs among these lymphocyte infiltrates reflects a significantly greater risk of progressive graft dysfunction. In addition, patients with a higher proportion of Tregs among these mononuclear infiltrates present a better structural preservation of the graft, in accordance with the recently updated Banff '07 criteria (6).

Various experimental studies have shown that the infiltration of cells with a regulatory phenotype (FoxP3+) in stable renal grafts (66, 68-70) exerts a protective effect. This probably reflects a process of specific antigen recognition directly in the graft by these Tregs, in order to be able to exercise their antigen-specific suppressor function.

This hypothesis is supported by the fact that the patients who present Tregs among the asymptomatic mononuclear infiltrates in the tubules and interstitium have been able to achieve donor-specific hypo-responsiveness in the peripheral blood, an immunological

state achieved thanks to the antigen-specific suppressive effect of this same regulatory T-cell subset population. Interestingly, this immune privilege attained by this group of patients, far from being a simply static biological phenomenon, has an extremely dynamic role and exerts a major functional impact: the presence of Tregs in the renal graft is associated with a significant long-term improvement in renal function.

It is vitally important to distinguish between this phenomenon, identified in patients with stable renal function, and the presence of the same types of tubulo-interstitial mononuclear infiltrates in patients undergoing acute or subacute graft dysfunction due to episodes of acute rejection. In this regard, various studies in the literature have shown discrepancies regarding the impact of the Tregs among cellular infiltrates in patients with clinical acute rejection (94, 95). The most plausible explanation of this phenomenon, which is well documented in the literature, is the fact that under severe inflammatory conditions the Tregs lose their capacity to suppress the immunological response (96, 97). It is also important to bear in mind that in humans the transcription factor FoxP3 may be expressed in a very transitory way in CD4+CD25+ activated T lymphocytes after the antigen recognition through the TCR, but without presenting this antigen-specific suppressive activity. This might lead to a false phenotypical identification of Tregs, fundamentally in situations of high alloreactivity. Therefore, it appears that during the episodes of acute graft rejection the Tregs are basically exercising a reactive or compensatory function in response to the destructive effect of the effector lymphocyte population, rather than exercising a pro-tolerogenic role.

The biological characteristics of this regulatory lymphocyte subpopulation confer on them a special sensitivity for differentiating and proliferating under the effect of specific immunosuppressive agents (98, 99). Several studies *in vitro* show that SRL and thymoglobulin have a positive effect on the differentiation and proliferation of this

regulatory lymphocyte subpopulation (77, 100). In accordance with these experimental findings, our study shows that the effect of these two immunosuppressive drugs favors the appearance of Tregs directly infiltrating the graft, although it should be borne in mind that some patients receiving immunosuppressive treatment with CNI may also present Tregs among the cellular infiltrates in the tubules and interstitium, thus maintaining the beneficial impact on the functional evolution of the graft in the period after the transplant.

With the availability of these immunological biomarkers, it becomes possible then to design and evaluate immunosuppressive strategies trying to achieve donor-specific hypo-responsiveness and thus, improving long-term graft survival. In this study we show that with an immunosuppressive protocol based on low doses of thymoglobulin and maintenance with SRL and MMF, without the use of steroids or CNI, donor-specific hypo-responsiveness can be achieved and maintained over time in a substantial proportion of patients.

This protocol shows that patients without effector/memory T-cell alloreactivity before transplantation are the ones most likely to benefit from implementation of this immunosuppressive strategy from the beginning of the transplant, as they present a significantly lower risk of acute cellular rejection. Even if acute rejection does occur under this immunosuppressive strategy, this fact is not an obstacle to reaching this state of immune privilege either at six months or two years after the transplant. This suggests that highly T-cell alloreactive patients would probably benefit more from an initial immunosuppressive regimen under CNI agents or simply adding low doses of steroids to the maintenance treatment with SRL and MMF.

Under this immunosuppressive protocol, achieving donor-specific hypo-responsiveness has a dramatic functional effect, as it is associated with a significant improvement in the

structural preservation of the graft and therefore with a better renal function six months and two years post-transplantation, thus confirming the results obtained in the cross-sectional study. Significantly, we also show that the donor-specific hypo-responsiveness achieved by these patients under this immunosuppressive protocol is caused by the donor-specific suppressive effect of TregsFoxp3+ cells present in peripheral blood oand also directly forming aggregates in the renal graft. This is shown very clearly in this study by the removal of the regulatory lymphocyte subpopulation from the donor-specific hyporesponders in the functional tests by means of cell-sorting, which converted these patients become donor-specific responders. In contrast, this phenomenon does not occur in donor-specific responders, who become hyporesponders with the withdrawal of this lymphocyte subpopulation in the functional tests. This shows that in this group of responders, this lymphocyte subpopulation comprises, activated effector T lymphocytes rather than Tregs. Most importantly, this privileged immune status mediated by the suppressive effect of the Tregs is extremely specific, as it only suppresses the effector immunological response to the respective donors.

In conclusion, in this study we show that it is the biological homeostasis obtained between these two lymphocyte populations with opposite immunological functions and influenced by the various immunosuppressive treatments that allows the achievement of a better or worse acceptance of the renal graft and maintenance over time. This stresses the importance of knowing the functional status of the donor-specific alloimmune response in each patient and at different stages in the transplant, so as to individualize the immunosuppressive approach and to be able to interpret accurately the functional evolution of the renal graft.

VII. CONCLUSIONS

1. The presence of high T-cell alloractivity against the donor at different time-points of renal transplantation, mainly activated by the direct pathway of antigen presentation, assessed by the presence of highly alloractive donor-specific memory/effector T-cells circulating in peripheral blood using the IFN-γ Elispot assay, is able to discriminate those patients exposed to an immune-mediated graft injury after renal transplantation.

2. The donor-specific T-cell alloimmune response activated by the indirect pathway of antigen presentation seems to acquire more functional relevance as time passes after the transplant and is associated with presence of proteinuria

3. The presence of the FoxP3+ regulatory T-cell subset population, seems to have a relevant role both for the recognition and for the acceptance of alloantigens, through their specific capacity to suppress alloimmune responses. Even in controversial clinicopathological entities such as subclinical acute rejection, the presence of Tregs seems to be able to differenciate those cases with favourable clinical evolution from those with a progressive loss of graft function.

4. The functional immune-monitoring of both T-cell subset population; memory/effector T cells and Tregs, seems to be useful as a biomarker, in order to accurately stratify the different immunological risk and thus, allow to individualize the type and immunosuppressive load to give to every kidney transplant patient.

5. The induction and maintenance of a donor-specific hyporesponsive state is feaseble avoiding calcineurin-inhibitor drugs in some kidney transplant patients and this state seems to be driven by the antigen-specific suppressive activity of FoxP3+Tregs

6. The use of these immunologic biomarkers at different time-points of renal transplantation may facilitate the development of new immunosuppressive protocols with the main goal of inducing donor-specific hyporesponsive states.

VIII. REFERENCES

1. US Renal Data System: US Renal Data System: Excerpts from the USRDS 2003 Annual Data Report: Atlas of End-Stage Renal Disease in the United States. Am J Kidney Dis. 42:s1–s230, 2003.

2. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, Held PJ, Port FK. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med. 341:1725-30, 1999.

3. Laupacis A, Keown P, Pus N, Krueger H, Ferguson B, Wong C, Muirhead N: A study of the quality of life and cost-utility of renal transplantation. Kidney Int. 50:235–242, 1996.

4. Meier-Kriesche HU, Schold JD, Kaplan B: Long-term renal allograft survival: Have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant. 4: 1289 –95, 2004.

5. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. N Engl J Med. 346:580-90. Review, 2002.

6. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, Drachenberg CB, Gibson IW, Grimm PC, Haas M, Lerut E, Liapis H, Mannon RB, Marcus PB, Mengel M, Mihatsch MJ, Nankivell BJ, Nickeleit V, Papadimitriou JC, Platt JL, Randhawa P, Roberts I, Salinas-Madriga L, Salomon DR, Seron D, Sheaff M, Weening JJ. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 7:518-526, 2007.

7. Antibody monitoring: a solid approach to predicting clinical outcome. Zachary AA. Transplantation. 86:768, 2008.

8. Elevation of CXCR3-binding chemokines in urine indicates acute renal allograft dysfunction. Hu H, Aizenstein BD, Puchalski A, Burmania JA, Hamawny MM, Knechtle SJ. Am J Transplant. 4:432-437, 2004.

9. Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, Nicolle L, Karpinski M, Jeffery J, Nickerson P: Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *J Am Soc Nephrol*. 15:219–227, 2004.

10 Schaub S, Wilkins JA, Nickerson P: Proteomics and renal transplantation: Searching for novel biomarkers and therapeutic targets. Contrib Nephrol. 160:65–75, 2008.

11. United Network for Organ Sharing: Data, Richmond, VA, United Network for Organ Sharing. Available at: www.unos.org/data. Accessed December 14, 2006.

12. Lerut J, Sanchez-Fueyo A: An appraisal of tolerance in liver transplantation. Am J Transplant. 6:1774 –1780, 2006.

13. Donohoe JA, Andrus L, Bowen KM, Simeonovic C, Prowse SJ, Lafferty KJ: Cultured thyroid allografts induce a state of partial tolerance in adult recipient mice. Transplantation. 35:62 –67, 1983.

14. Crispe IN, Giannandrea M, Klein I, John B, Sampson B, Wuensch S: Cellular and molecular mechanisms of liver tolerance. Immunol Rev. 213 : 101 –118, 2006.

15. Calne RY, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, Binns RM, Davies DA: Induction of immunological tolerance by porcine liver allografts. Lancet. 223:472–476, 1969.

16. Horner BM, Cina RA, Wikiel KJ, Lima B, Ghazi A, Lo DP, Yamada K, Sachs DH, Huang CA: Predictors of organ allograft tolerance following hematopoietic cell transplantation. Am J Transplant. 6:2894–2902, 2006.

17. Kuhr CS, Allen MD, Junghanss C, Zaucha JM, Marsh CL, Yunusov M, Zellme E, Little MT, Torok-Storb B, Storb R: Tolerance to vascularized kidney grafts in canine mixed hematopoietic chimeras. Transplantation. 73:1487–1493, 2002.

18. Storb R, Yu C, Wagner JL, Deeg HJ, Nash RA, Kiem HP, Leisenring W, Shulman H: Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. Blood. 89:3048–3054, 1997.

19. Strober S, Modry DL, Hoppe RT, Pennock JL, Bieber CP, Holm BI, Jamieson SW, Stinson EB, Schroder J, Suomalainen H: Induction of specific unresponsiveness to heart allografts in mongrel dogs treated with total lymphoid irradiation and antithymocyte globulin. J Immunol.132:1013 –1018, 1984.

20. Kean LS, Gangappa S, Pearson TC, Larsen CP: Transplant tolerance in non-human primates: Progress, current challenges and unmet needs. Am J Transplant 6 : 884 –893, 2006.

21. Kirk AD: Crossing the bridge: Large animal models in translational transplantation research. Immunol Rev 196 : 176 –196, 2003.

22. Martínez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, Benítez C, Pons JA, Parrilla P, Ramírez P, Bruguera M, Rimola A, Sánchez-Fueyo A. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. J Clin Invest. 118:2845-2857, 2008.

23. Sayegh MH, Carpenter CB:Role of indirect allorecognition in allograft rejection. Int Rev Immunol. 13:221-229, 1996.

24. Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, Lechler RI. A novel pathway of alloantigen presentation by dendritic cells. J Immunol. 173:4828-4837, 2004.

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

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

27. Waldmann, H., S. Cobbold. Regulating the immune response to transplants: a role for CD4+ regulatory cells?. Immunity 14: 399-406, 2001.

28. Zheng, X. X., A. Sanchez-Fueyo, C. Domenig, T. B. Strom. The balance of deletion and regulation in allograft tolerance. Immunol. Rev. 196: 75-84, 2003.

29. Davies, J. D., L. Y. Leong, A. Mellor, S. P. Cobbold, H. Waldmann. T cell suppression in transplantation tolerance through linked recognition. J. Immunol. 156: 3602-3607, 1996.

30. Wood, K. J., S. Sakaguchi. Regulatory lymphocytes: regulatory T cells in transplantation tolerance. Nat. Rev. Immunol.3:199-210, 2003.

31. Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23+17 equal rejection?. Transplantation. 84:1071-1074. Review, 2007.

32. Dutton, R. W., L. M. Bradley, S. L. Swain. T cell memory. Annu. Rev. Immunol. 16: 201-223, 1998.

33. Freitas, A. A., B. Rocha. Population biology of lymphocytes: the flight for survival. Annu. Rev. Immunol. 18: 83-111, 2000.

34. Dummer, W., B. Ernst, E. LeRoy, D. Lee, C. Surh. Autologous regulation of naive T cell homeostasis within the T cell compartment. J. Immunol. 166: 2460-2468, 2001.

35. Vella, J. P., M. Spadafora-Ferreira, B. Murphy, S. I. Alexander, W. Harmon, C. B. Carpenter, M. H. Sayegh. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. Transplantation. 64: 795-800, 1997.

36. Jones DL, Sacks SH, Wong W. Controlling the generation and function of human CD8+ memory T cells in vitro with immunosuppressants. Transplantation.82:1352-1361, 2006.

37. Kaminski ER, Hows JM, Goldman JM, Batchelor JR. Lymphocytes from multi-transfused patients exhibit cytotoxicity against autologous cells. Br J Haematol. 81: 23, 1992.

38. van Kampen CA, Versteeg-van der Voort Maarschalk MF, Langerak-Langerak J, van Beelen E, Roelen DL, Claas FH. Pregnancy can induce long-persisting primed CTLs specific for inherited paternal HLA antigens. Hum Immunol. 62: 201, 2001.

39. Pelletier RP, Hennessy PK, Adams PW, Orosz CG. High incidence of donor-reactive delayed-type hypersensitivity reactivity in transplant patients. Am J Transplant. 2: 926, 2002.

40. Augustine JJ, Poggio ED, Clemente M, Aeder MI, Bodziak KA, Schulak JA, Heeger PS, Hricik DE. Hemodialysis vintage, black ethnicity, and pretransplantation antidonor cellular immunity in kidney transplant recipients. J Am Soc Nephrol. 18:1602-1606, 2007.

41. Longitudinal study of the frequency of cytotoxic T cell precursors in kidney allograft recipients. Mestre M, Massip E, Bas J, Alsina J, Romeu A, Castelao AM, Buendia E, Grinyo JM. Clin Exp Immunol. 104:108-114, 1996.

42. Volk HD, Kern F. Insights into the specificity and function of (allo)antigen-reactive T cells. Am J Transplant. 1:109-114, 2001.

43. Couzi L, Thiebaut R, Carron JC et al. Immunological monitoring of calcineurin inhibitors for predicting cytomegalovirus infection in kidney transplant recipients. Transplantation. 86: 1060-1067, 2008.

44. Carrodeguas L, Orosz CG, Waldman WJ, et al. Trans vivo analysis of human delayed-type hypersensitivity reactivity. Hum Immunol. 60: 640, 1999.

45. Enzyme-linked immunosorbent assay spot detection of interferon-gamma and interleukin 5-producing cells as a predictive marker for renal allograft failure. Tary-Lehmann M, Hricik DE, Justice AC, Potter NS, Heeger PS. Transplantation. 66:219-224, 1998.

46. Heeger PS, Greenspan NS, Kuhlenschmidt S, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. J Immunol. 163: 2267, 1999.

47. Nickel P, Presber F, Bold G, Biti D, Schönemann C, Tullius SG, Volk HD, Reinke P. Enzyme-linked immunosorbent spot assay for donor-reactive interferon-gamma-producing cells identifies T-cell presensitization and correlates with graft function at 6 and 12 months in renal-transplant recipients. Transplantation. 78:1640-1646, 2004.

48. Andree H, Nickel P, Nasiadko C, Hammer MH, Schönemann C, Pruss A, Volk HD, Reinke P. Identification of dialysis patients with panel-reactive memory T cells before kidney transplantation using an allogeneic cell bank. J Am Soc Nephrol. 17:573-580, 2006.

49. Augustine JJ, Poggio ED, Clemente M, Aeder MI, Bodziak KA, Schulak JA, Heeger PS, Hricik DE. Hemodialysis vintage, black ethnicity, and pretransplantation

antidonor cellular immunity in kidney transplant recipients. J Am Soc Nephrol. 18:1602-1606, 2007.

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

51. Shevach, EM, et al. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev. 212:60–73, 2006.

52. Sakaguchi, S, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev. 212:8–27, 2006.

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

54. Belkaid, Y; Blank, RB; Suffia, I. Natural regulatory T cells and parasites: a common quest for host homeostasis. Immunol Rev. 212:287–300, 2006.

55. Rouse, BT; Sarangi, PP; Suvas, S. Regulatory T cells in virus infections. Immunol Rev. 212:272–286, 2008.

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

57. Beyer, M; Schultze, JL. Regulatory T cells in cancer. Blood. 108:804–811, 2006.

58. Long E, Wood KJ. Understanding FOXP3: progress towards achieving transplantation tolerance. Transplantation. 84:459-461. Review, 2007.

59. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. Nature Rev Immunol. 3:199, 2003.

60. Mantel PY, Ouaked N, Ruckert B, et al. Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol. 176: 3593, 2006.

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

62. Cupedo T, Nagasawa M, Weijer K, et al. Development and activation of regulatory T cells in the human fetus. Eur J Immunol. 35: 383, 2005.

63. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest. 116: 2423, 2006.

64. Karim M, Kingsley CI, Bushell AR, et al. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. J Immunol. 172: 923, 2004.

65. Sánchez-Fueyo A, Sandner S, Habicht A, Mariat C, Kenny J, Degauque N, Zheng XX, Strom TB, Turka LA, Sayegh MH. Specificity of CD4+CD25+ regulatory T cell function in alloimmunity. J Immunol. 176:329-334, 2006.

66. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, van Meerwijk JP. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. Nat Med. 14:88-92, 2008.

67. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, Hartono C, Li B, Sharma VK, Seshan SV, Kapur S, Hancock WW, Schwartz JE, Suthanthiran M.Messenger RNA for FOXP3 in the urine of renal-allograft recipients.N Engl J Med. 353:2342-2351, 2005.

68. Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. J Exp Med. 195:1641-2646, 2002.

69. Lee I, Wang L, Wells AD, Dorf ME, Ozkaynak E, Hancock WW. Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. J Exp Med. 201:1037-2044, 2005.

70. Brown K, Moxham V, Karegli J, Phillips R, Sacks SH, Wong W. Ultra-localization of Foxp3+ T cells within renal allografts shows infiltration of tubules mimicking rejection. Am J Pathol. 171:1915-2922, 2007.

71. Nickerson P, Jeffery J, Gough J, Grimm P, McKenna R, Birk P, Rush D. Effect of increasing baseline immunosuppression on the prevalence of clinical and subclinical rejection: a pilot study. J Am Soc Nephrol. 10:1801-1805, 1999.

72. Scholten EM, Rowshani AT, Cremers S, Bemelman FJ, Eikmans M, van Kan E, Mallat MJ, Florquin S, Surachno J, ten Berge IJ, Bajema IM, de Fijter JW. Untreated rejection in 6-month protocol biopsies is not associated with fibrosis in serial biopsies or with loss of graft function. J Am Soc Nephrol. 17:2622-2632, 2006.

73. Beneficial effects of treatment of early subclinical rejection: a randomized study. Rush D, Nickerson P, Gough J, McKenna R, Grimm P, Cheang M, Trpkov K, Solez K, Jeffery J. J Am Soc Nephrol. 9:2129-2134, 1998.

74. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS: Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. Blood. 108:390-399, 2006.

75. Suzuki H, Kundig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, et al.: Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. Science. 268:1472–1476, 1995.

76. Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E, Roncarolo MG, Battaglia M.Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+CD25+FOXP3+ regulatory T-cells. Diabetes. 57:2341-2347, 2008.

77. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N: A novel mechanism of action for anti-thymocyte globulin: Induction of CD4+CD25+Foxp3+ regulatory T cells. J Am Soc Nephrol. 17:2844–2853, 2006.

78. Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: potential novel assay for prediction of outcomes for renal transplant recipients.Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH. J Am Soc Nephrol. 13:252-259, 2002.

79. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, Sayegh MH: Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. Transplantation 64: 795–800, 1997.

80. Ciubotariu R, Liu Z, Colovai AI, Ho E, Itescu S, Ravalli S, Hardy MA, Cortesini R, Rose EA, Suciu-Foca N: Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J Clin Invest 101: 398–405, 1998.

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

82. Opelz G, Wujciak T, Döhler B, et al. HLA compatibility and organ transplant survival. Collaborative Transplant Study. Rev Immunogenet. 1: 334, 1999.

83. Vella, J. P., F. M. Spadafora, B. Murphy, S. I. Alexander, W. Harmon, C. B. Carpenter, M. H. Sayegh. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. Transplantation. 64:795, 1997.

84. Hornick, P. I., P. D. Mason, R. J. Baker, M. Hernandez-Fuentes, L. Frasca, G. Lombardi, K. Taylor, L. Weng, M. L. Rose, M. H. Yacoub. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. Circulation. 101:2405, 2000.

85. Ciubotariu, R., Z. Liu, A. I. Colovai, E. Ho, S. Itescu, S. Ravalli, M. A. Hardy, R. Cortesini, E. A. Rose, N. Suciu Foca. Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J. Clin. Invest. 101:398, 1998.

86. SivaSai, K. S., M. A. Smith, N. J. Poindexter, S. R. Sundaresan, E. P. Trulock, J. P. Lynch, J. D. Cooper, G. A. Patterson, T. Mohanakumar. Indirect recognition of donor HLA class I peptides in lung transplant recipients with bronchiolitis obliterans syndrome. Transplantation 67:1094, 1999.

87. Valujskikh, A., D. Matesic, A. Gilliam, D. Anthony, T. Haqqi, P. Heeger. T cells reactive to a single immunodominant self-restricted allopeptide induce skin graft rejection in mice. J. Clin. Invest. 101:1398, 1998.

88. Steele D, Laufer T, Smiley, Ando Y, Grusby M, Glimcher L, Auchinloss H: Two levels of help for B cell alloantibody production. J Exp Med. 183:699 –703, 1996.

89. Bradley JA, Mowat AM, Bolton EM: Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. Immunol Today 13 : 434 –438, 1992.

90. Moreso F, Ibernon M, Gomà M, Carrera M, Fulladosa X, Hueso M, Gil-Vernet S, Cruzado JM, Torras J, Grinyó JM, Serón D.Subclinical rejection associated with chronic allograft nephropathy in protocol biopsies as a risk factor for late graft loss.Am J Transplant. 6:747-752, 2006.

91. Seron D, Moreso F, Ramón JM, Hueso M, Condom E, Fulladosa X, Bover J, Gil-Vernet S, Castelao AM, Alsina J, Grinyó JM: Protocol renal allograft biopsies and the

design of clinical trials aimed to prevent or treat chronic allograft nephropathy. Transplantation 69: 511–514, 2000.

92. Choi BS, Shin MJ, Shin SJ, Kim YS, Choi YJ, Kim YS, Moon IS, Kim SY, Koh YB, Bang BK, Yang CW: Clinical significance of an early protocol biopsy in living-donor renal transplantation: Ten-year experience at a single center. Am J Transplant 5: 1354–1360, 2005.

93. Roberts IS, Reddy S, Russell C, Davies DR, Friend PJ, Handa AI, Morris PJ: Subclinical rejection and borderline changes in early protocol biopsy specimens after renal transplantation. Transplantation 77:1194–1198, 2004.

94. Veronese F, Rotman S, Smith RN, Pelle TD, Farrell ML, Kawai T, Benedict Cosimi A, Colvin RB.Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. Am J Transplant. 7:914-922, 2007.

95. Grimbert P, Mansour H, Desvaux D, Roudot-Thoraval F, Audard V, Dahan K, Berrehar F, Dehoulle-Poillet C, Farcet JP, Lang P, Le Gouvello S.The regulatory/cytotoxic graft-infiltrating T cells differentiate renal allograft borderline change from acute rejection.Transplantation. 83:341-346, 2006.

96. Koulmanda M, Budo E, Bonner-Weir S, Qipo A, Putheti P, Degauque N, Shi H, Fan Z, Flier JS, Auchincloss H Jr, Zheng XX, Strom TB. Modification of adverse inflammation is required to cure new-onset type 1 diabetic hosts.Proc Natl Acad Sci USA. 104:13074-1379, 2007.

97. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR, Bäckström BT, Sobel RA, Wucherpfennig KW, Strom TB, Oukka M, Kuchroo VK. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation.Nat Med. 13:423-431, 2007.

98. Segundo DS, Ruiz JC, Izquierdo M, Fernández-Fresnedo G, Gómez-Alamillo C, Merino R, Benito MJ, Cacho E, Rodrigo E, Palomar R, López-Hoyos M, Arias M.Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients.Transplantation. 82:550-557, 2006.

99. CD4+ CD25+ FOXP3+ regulatory T cells increase de novo in kidney transplant patients after immunodepletion with Campath-1H. Bloom DD, Chang Z, Fechner JH, Dar W, Polster SP, Pascual J, Turka LA, Knechtle SJ. Am J Transplant. 8:793-802, 2008.

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

IX. TABLES AND FIGURES

Table 1. Main differences between naive and memory T lymphocytes.

	Naive T cell	**Memory T cell** **Central / Effector**
Phenotype	$CD45RO^{low}$	$CD45RO^{high}$ $CCR7^{high}CD62L^{high}$ / $CCR7^{low}CD62L^{low}$
APC	Professional	Non Professional
Response to low Ag exposure	Weak	Strong
Effector function	None (IL 2→ proliferation)	Cytokin release (IFN-γ, Granzym-B), Direct citolysis
Kinetics	Slow (days)	Rapid (hours)
Localization	Lymphoid tissue	Lymphoid and non-lymphoid tissue

APC: Antigen presenting cell. Ag: Antigen

Figure 1. Therapeutic targets of immunosuppressants.

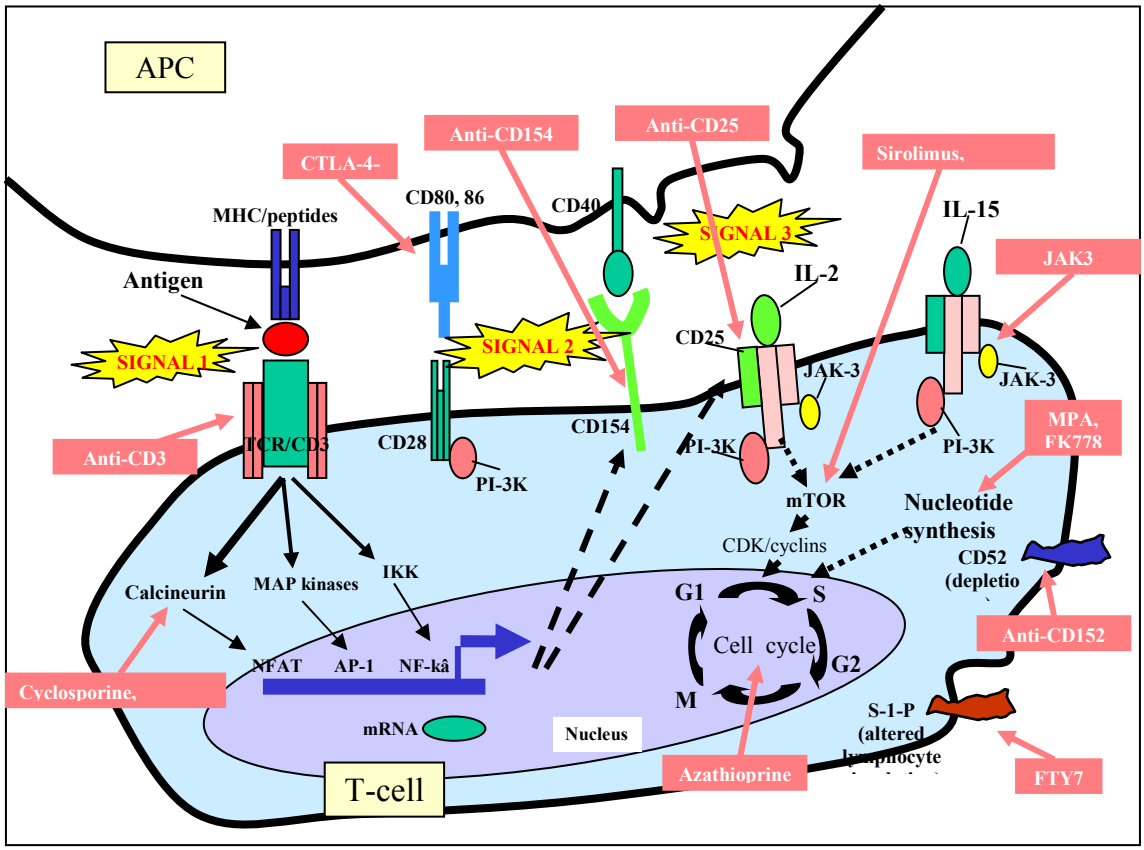
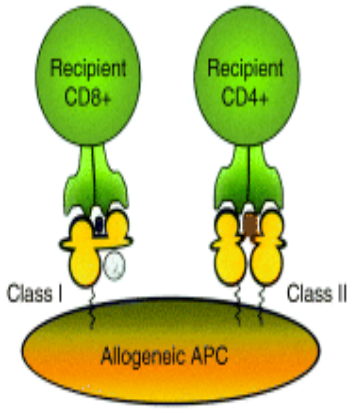
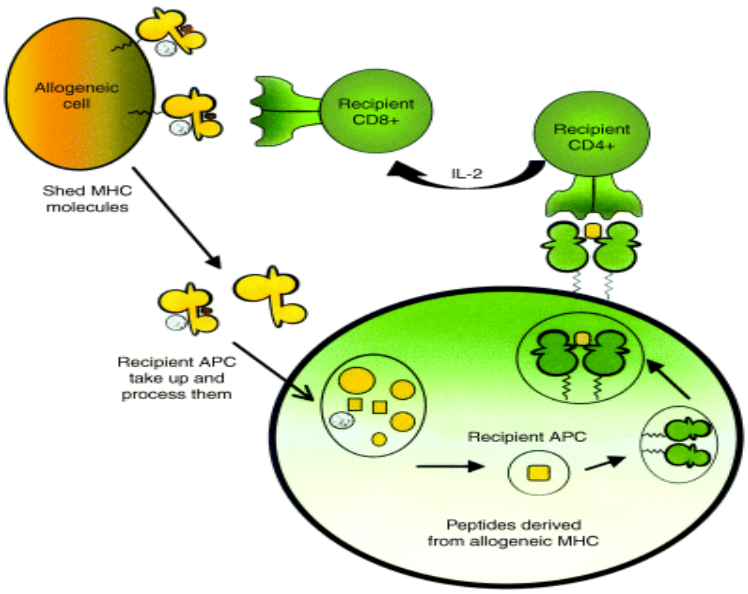


Figure 2. Pathways of alloantigen presentation

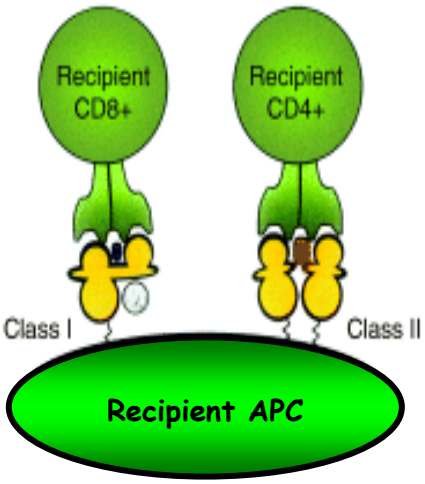
a. Direct pathway



b. Indirect pathway



c. Semi-direct or third pathway



Figur3 3. Alloimmune response