

THE CHALLENGE OF CLINICAL TRIALS AIMING AT TRANSPLANT TOLERANCE

Lucienne Chatenoud and Sylvaine You

Faculté Paris Descartes, INSERM U580, Hôpital Necker-Enfants Malades, Paris France

For several years research on strategies aiming at induction of transplant tolerance has been conducted in the experimental field with very few attempts of clinical translation. This was mainly due to the wealth of clinical and biological immunosuppressive agents which were made available during the 80s and the 90s raising hope in the clinic that with adequate combinations of these drugs, in spite of the need for chronic treatment, long-term allograft survival could be achieved with minor toxicity. Unfortunately, results argue against such conclusion. In general practice, the very potent and broad immunosuppressive protocols used are certainly effective at preventing acute rejection but not chronic deterioration of graft function. In addition, with these treatments patients are exposed to higher risk of infections and tumours. From a more fundamental point of view this observation certainly questions the long-held dogma that acute rejection is the main factor leading to chronic allograft dysfunction. This further points to the pressing need of getting further insights into the pathophysiology of what is generally defined as “chronic rejection”, a broad clinical definition certainly encompassing distinct entities.

Taking the lessons from these assessments as a whole one must admit that without any doubt major progress was made, as present strategies effectively cope with acute rejection. Yet the situation is far from ideal when one considers the morbidity and mortality associated with long-term outcome of transplant patients. Whatever we may do to improve immunosuppression it will always be a “non-specific” approach, unrelated to the alloantigens involved, exposing over long term to an unacceptable overall depression of immune responses. In this complex situation inducing “operational tolerance” represents the only sensible solution. Thus presently, the transplant community should envision this aim not only as a rewarding scientific goal but as a pressing clinical need. This is the challenge the Riset consortium proposed to address. Twenty six European groups involved in experimental and clinical transplantation gathered and it is interesting to briefly retrace the salient steps of the work they performed as well as how the clinically oriented part of the program (Work package 3) was redirected along the way based on the results obtained.

AN INITIAL AMBITIOUS GOAL

The program was launched with three hypothesis-driven pilot clinical investigations aiming at inducing “operational transplant tolerance” defined as a state of lasting antigen-specific unresponsiveness in the absence of generalised immunosuppression. The rationale was based on clinical transfer of pre-defined protocols which proved effective to induce transplant tolerance in the experimental setting and for which compelling evidence was accumulated to demonstrate that peripheral tolerance mechanisms, mostly based on T lymphocyte-mediated immunoregulatory circuits, played a key role in the induction and maintenance of such tolerance states.

These protocols were cell therapy-based involving the use of:

- *Anergised interleukin (IL-)10 driven T lymphocytes (Tr1 cells) of donor origin in recipients of allogeneic bone marrow* (R. Bachetta, MG. Roncarolo; San Raffaele-TIGET center, Milano, Italy) (1, 2). The patients received HLA-haploidentical hematopoietic stem cells on day 0 and an infusion of IL-10-anergised donor T cells by one month later. This protocol was already ongoing when RISET was launched. Thus, a total of 7 patients were included in this trial and 4 were part of RISET, meaning that the clinical and biological data recovered from those patients are included in the RISET data base. No immunosuppression was given at the time of the transplant or the infusion of Tr1. Overall the treatment proved safe: the low dose of CD3⁺ anergised cells infused gave rise in some patients to a moderate acute graft versus host disease (GvHD) which in all cases was responsive to therapy. It is important to emphasise that in the context of the presently available literature these results are particularly promising in such a difficult clinical setting that is, HLA haploidentical transplantation carried out without immunosuppression. In parallel, the low frequency of infectious episodes observed, the absence of Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) replication, together with the complete remission of the disease represent the proof of concept of the good efficacy of the adoptive transfer of Tr1 cells.

- *Mobilised immature CD34⁺ bone marrow stem cells of donor origin in recipients of allogeneic liver transplants* (V. Donckier, M. Goldman; Université Libre de Bruxelles, Brussels, Belgium) (3). The rationale of the study was based on compelling experimental evidence to show that donor cells, when presented in adequate conditions, may render the host's immune system tolerant to donor antigens through different mechanisms such as deletion or regulation of anti-donor T cells and/or generation of suppressive cells. Several animal investigations and one recent published clinical experience performed in Boston by the

group of D. Sachs in living related kidney transplant recipients have shown that the infusion of donor marrow cells, after adequate conditioning of the recipient, may lead to long-term and robust, transplantation tolerance (4). In these protocols, tolerance is associated with a state of, at least transient, mixed chimerism. The possibility of using, instead of bone marrow, CD34⁺ stem cells and the access to less toxic myeloablative treatments paved the way to broader clinical indications. In the context of living-related transplantation, hematopoietic CD34⁺ stem cells can be easily obtained from donor peripheral blood after mobilization with granulocyte colony-stimulating factor (G-CSF). The trial performed here included patients with intra-hepatic malignancies, not complying to the criteria for cadaver liver transplantation due to high risk of cancer recurrence following conventional immunosuppression. Patients received a living-related liver graft (from a family member including spouses) followed by the infusion of mobilized donor stem cells on day 7. These cells were harvested after (G-CSF) treatment and infused in recipients conditioned with cyclophosphamide and Thymoglobulin^R. The immunosuppressive regimen included steroids for 3 days and rapamycin starting on day 0 and followed until liver function tests returned to normal, a point in time where immunosuppression was discontinued. Four patients were included. In all patients reversible acute rejection occurred when immunosuppression was discontinued. Relapse of the original tumor occurred in 2/4 patients. Global hyporesponsiveness and donor-specific hyporeactivity was assessed in 2/4 patients after discontinuation of immunosuppression.

- Monocyte-derived Transplant Acceptance Inducing cells (TAIC) of donor origin in recipients of allogeneic kidneys (F. Fändrich; University Schleswig-Holstein & Blasticon, Kiel, Germany) (5-7). TAIC are derived from human monocytes following *in vitro* culture in presence of M-CSF and interferon (IFN) γ (5-7). Two major mechanisms have been proposed to explain the therapeutic effect of TAIC based on numerous experiments in rodents, large animals (minipigs) and humans. Firstly, allogeneic *in vivo* injection of TAIC into non-immunosuppressed recipients leads to transient mixed chimerism lasting for a few weeks indicating that TAIC are not acutely rejected within the allogeneic environment. Secondly, cell-to-cell interaction between TAIC and host T-cells induces regulatory T-cells as demonstrated by the induction of CD4⁺CD25⁺ T cells in these recipients. Adoptive transfer experiments with these CD4⁺/CD25⁺ T-cells demonstrated their capacity to down-regulate alloreactive graft-specific immune responses. These experiments led to the assumption that TAIC could induce donor-specific peripheral tolerance by inducing regulatory T-cells when used in clinical trials. The first trial proposed in the context of Riset was a single centre

open-label study of the administration of TAIC and autologous regulatory cells in recipients of a living donor renal allograft. The primary objective of the study was to monitor graft survival. Five days before transplantation TAIC were administered. On the day of transplantation, patients received triple immunosuppression with tacrolimus, anti-thymocyte globulin and corticosteroids. Anti-thymocyte globulin was given postoperative only on Day 0, 1 and 2. Corticosteroids were tapered off during weeks 9 to 10. If the reduction in creatinine clearance at the last visit on week 12 was $\leq 25\%$ compared to Day 56, tacrolimus was reduced during week 13. Tacrolimus was tapered during weeks 25 to 28 if there were no histological, biological or clinical signs of rejection. A total of 6 patients were included. Acute rejection that was responsive to treatment was observed in one patient. In none of the patients GvHD was observed. In 4 of the patients a rejection episode appeared when all the immunosuppressive drugs were stopped. After adequate treatment reversion of the rejection episodes was observed and the long term follow-up evidenced a satisfactory graft survival rate. One patient showed normal graft function for 8 consecutive month after withdrawal of all immunosuppressants.

In conclusion, in these three trials a total of 14 patients were included whose clinical and biological data were collected in a RISET-dedicated data base formatted in a flexible way so that it could comply with the needs of the various protocols. Fulfilling the requirements of FP6 projects after 18 month from starting the project data were reviewed by both an external advisory board, including international renowned experts in the field, as well as by a panel gathered by the commission. The comments related to the commitment and efforts provided by the clinical teams in recruiting patients were largely positive but also pointed to two relevant issues which, if implemented, could represent a further jump for the project.

The first was to pursue the cell therapy strategies undertaken but redirecting all three protocols to attempt improving effectiveness and therefore benefit to the patients and the project.

In the case of the TIGET-Milan protocol it was decided to use IL-10 anergised T lymphocytes of donor origin raised in presence of host-derived IL-10-treated dendritic cells (DC-10). This modified approach was selected based on the experimental evidence showing that this is a more effective strategy to achieve the same goals of the original protocol (i.e. a satisfactory haematopoietic reconstitution of recipients with low risk of GvHD).

In the case of the ULB-Brussels protocol it was felt that both clinical and ethical considerations were seriously delaying good progression of the trial. It was thus suggested to

apply a scheme of early immunosuppression weaning/withdrawal in recipients of cadaver (and not living donors) liver transplants based on an early depletive therapy followed by short rapamycin-based immunosuppression. As we shall discuss below this was also intended to match the design of another trial included as part of RISET thus increasing the critical mass in terms of investigators addressing a similar question in a more focussed way.

For the University Schleswig-Holstein & Blasticon-Kiel protocol it was felt that the main question was to better assess the role of the administration of TAIC in promoting long term allograft survival under conditions of minimal maintenance immunosuppression instead of complete withdrawal.

A total of 27 patients will be included in these trials by the end of RISET.

The second recommendation put a lot of emphasis on the absolute necessity to meet, in the context of this clinical endeavour, the main objective of RISET which is to provide a set of validated monitoring tools to test for “operational tolerance” in transplanted patients. By definition validation means not only establishing a panel of tests but also and perhaps more importantly, intertwining the experience of transplant physicians and immunologists so that they may come up with some really novel ways to proceed. One must remind at this point that RISET represents a unique gathering of transplant immunologists whom based on both their experimental and clinical expertise have established a number of mechanistic cellular and genomic tools that have all been made accessible for the monitoring of patients included in the program. The practical problem that appeared was that in no case the protocols mentioned above, in spite of their intrinsic “richness” of clinical experience, would be able to provide a sufficient number of samples to the RISET core laboratories running the standardised monitoring tests to achieve a reliable, statistically significant validation of the individual methods. The only way to tackle this problem was to increase the number of patients available for monitoring meaning to increase the number of protocols within RISET. For obvious reasons, most of these new trials that are now also ongoing belong to a second category of clinical investigations aiming at **minimization of immunosuppression** which would be based, if possible, in a not too distant future on a selected panel of **biomarkers**.

REDIRECTING THE CLINICAL OBJECTIVES TO BETTER ADDRESS THE COMPLEX GOALS OF RISET

A total of 7 additional clinical trials are presently ongoing in the context of RISET; four of them are focussing, as previously mentioned, on protocols aiming drug minimisation, two are devoted to innovative strategies to promote transplant tolerance and one focuses on a more explorative aspect of kidney biopsies which appears very interesting to predict and monitor chronic kidney allograft nephropathy.

Trials on drug minimisation:

three of these studies include kidney allograft recipients and one includes recipients of liver allografts. Specific sets of markers are studied in parallel to clinical parameters in order to address the delicate question when interpreting the final data of which among these monitoring tools could be selected in future prospective clinical studies to adapt minimisation of immunosuppression to individual patients. The following studies fall into this category:

- a ***Clinical study to evaluate the efficacy in kidney allograft recipients of an immunosuppression regimen based on induction therapy combining anti-CD52 (CAMPATH-1H) and anti-TNF monoclonal antibodies followed by a maintenance regimen based on either tacrolimus or sirolimus*** (O. Viklicky, IKEM, Prague, Czechoslovakia in collaboration with P. Reinke, D. Volk, Charite hospital, Berlin Germany). The clinical centre in Prague will enroll 20 patients receiving peri-operatively 2 injections of the CD52 antibody alemtuzumab (Campath-1H) to induce massive depletion of both T and B lymphocytes in combination with a single injection of Infliximab (anti-TNF) and followed by either tacrolimus or sirolimus low-dose monotherapy. The aim of the trial is to assess whether sirolimus monotherapy in absence of any treatment with calcineurin inhibitors (CNI) and/or steroids is safe and allows to promote long term survival of well functioning kidney allografts. Infliximab is associated to the treatment regimen to decrease the risk of humoral rejection which has been one of the problems reported by various groups using CAMPATH-1H as induction therapy.

- a study on ***Minimization of immunosuppression in renal allograft recipients*** (I. ten Berge, Department of Internal Medicine, Renal Transplant Unit, Academic Medical Center, Amsterdam, The Netherlands). Renal transplant recipients are treated with an induction regimen including CD25 monoclonal antibody, corticosteroids, mycophenolic acid and cyclosporine. After 6 months, the patients are randomized in 3 treatment groups, receiving

either prednisolone and mycophenolic acid; prednisolone and cyclosporin, or prednisolone and sirolimus. Protocol biopsies are performed before transplantation and at 6 and 24 months transplantation. Mononuclear cells, serum and urine samples are collected before transplantation and at frequent intervals thereafter will be provided to the RISET core laboratories. A total of approximately 30 patients will be enrolled. In addition, this center developed over the last years an “in house” mixed lymphocyte culture (MLC)-5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) test they would like to apply in parallel and, if possible, to bring to the stage of validation with the help of the consortium. In this patient cohort, the alloimmune response before transplantation and during conversion from triple to double drug treatment is measured by the multiparameter MLC-CFSE assay, which enables to determine a combination of quantitative and qualitative properties of alloreactive T cells in one assay. In MLCs, CFSE labelled recipient cells are stimulated with donor specific or with third party cells. CD4⁺ and CD8⁺ precursor frequencies and number of divisions are deduced. The intracellular content of effector molecules is measured and responder cells are analysed for expression of cytokine- chemokine- and integrin receptors. Data will be related to the clinical course and evaluated on their usefulness as predictor of alloreactivity after minimisation of immunosuppression.

- a study addressing the *Validation of an IFN- γ ELISPOT to better adapt immunosuppression in kidney allograft recipients* (J. Grinyo, Hospital Universitari de Bellvitge, University of Barcelona, Spain)

As part of potential techniques to assess T-cell alloreactivity the interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assay has currently emerged as a highly sensitive method which may help optimising immunosuppression. In fact, previous experiences have shown the value of the presence of circulating donor-specific T cells pre-transplantation evaluated by Elispot for predicting acute rejection in renal transplanted patients (8-10). Similarly than the evaluation of panel reactive antibodies in candidates for renal transplantation, which assesses the risk for antibody-mediated graft injury, it has been shown how the screening for effector/memory T cells by a “panel of reactive T cells” or PRT using an IFN- γ Elispot, can evaluate the cellular alloimmunity, giving an additional aid for assessing the risk of rejection after transplantation. The group in Barcelona also observed in a small cohort of twenty renal transplanted patients under a calcineurin-inhibitor (CNI) and steroid-free immunosuppressive regimen that pre-transplant (IFN)- γ Elispot could also detect

patients at risk to develop acute rejection. Moreover, patients becoming donor-specific hyporesponders 6 months after transplantation had significantly better renal function than those non-donor-specific “hyporesponders”.

The search for a safe CNI-free immunosuppressive regimen remains an important objective to avoid long-term nephrotoxicity thus increasing long-term renal allograft survival. However, these approaches have usually entailed an increased risk of acute rejection which refrain their more widespread use. Another important still controversial issue is to what extent current immunosuppressive regimens are able to inhibit memory T cells, the cornerstones mediating graft rejection. In fact, lymphocyte depleting drugs such as polyclonal anti-lymphocyte globulins and alemtuzumab (CAMPATH-1) has been shown to be less able to diminish the memory T cell subset (11). However, in selective studies evaluating different immunosuppressants, it was demonstrated that although effector memory T cells were resistant to steroids, deoxyspergualin and sirolimus, tacrolimus, and to a lesser degree cyclosporine A, were able to inhibit proliferation and cytokine production upon *in vitro* stimulation. Hence, the working hypothesis is that a prospective assessment of donor-specific cellular alloreactivity using the IFN- γ Elispot assay, will be helpful to identify those patients that could benefit from a CNI-free immunosuppressive strategy.

This is a non randomized, pilot, prospective, open-label, multicenter trial including 60 patients to study whether allocation of a CNI based or CNI-free immunosuppressive regimen is feasible depending on the donor-specific cellular alloresponse as evaluated by IFN- γ ELISPOT assay in low risk renal transplant patients.

From the day of transplantation patients receive Thymoglobuline starting on the day of up to day 5 post-transplantation, mofetil mycophenolate, corticosteroids in principle for 12 months.

In addition, depending on pre-transplant IFN- γ Elispot, treatment will be:

- CNI-free (sirolimus) if the donor-specific IFN- γ Elispot is negative (Group A)
- tacrolimus if the donor-specific IFN- γ Elispot is positive (Group B)

At 6 months post-transplant: a new donor-specific IFN- γ Elispot and a renal allograft biopsy will be performed in order to adapt the immunosuppressive treatment.

Group A Negative pre-transplant donor-specific IFN- γ Elispot

- if the donor-specific IFN- γ Elispot is positive and/or there are histological signs of rejection: treatment will be switched from sirolimus to tacrolimus
- if the donor-specific IFN- γ Elispot is negative and there are no histological signs of rejection: treatment with mycophenolate and corticosteroids will be discontinued.

Group B Positive pre-transplant donor-specific IFN- γ Elispot

- if the donor-specific IFN- γ Elispot is positive and/or there are histological signs of rejection: dosage of tacrolimus is increased, mycophenolate and corticosteroids are maintained.

- if the donor-specific IFN- γ Elispot is negative and there are no histological signs of rejection: progressive discontinuation of corticosteroids will be applied for the following 3 months, tacrolimus and mycophenolate are maintained.

- a study on the *Search for the immunological signature of operational tolerance in liver transplant recipients* (A. Sanchez-Fueyo, Liver Transplant Unit, Hospital Clinic Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain).

Here the objective is to prospectively validate the diagnostic accuracy of a series of previously identified biological markers of operational tolerance ("signature of tolerance") by exploring an independent cohort of 81 liver allograft recipients receiving conventional immunosuppressive therapy. Patients are recruited from several European liver transplant units and are selected based on a set of common clinical criteria. Participating centers are: Hospital Clínic Barcelona (Dr Sánchez-Fueyo), Catholic University Louvaing (Dr Pirenne) and University Tor Vergata Rome (Dr G. Tisone). Subsequently, the plan is to include 20-30 In addition, this study recently gathered in its objective with the one described above performed by the Brussel's team (V. Donckier, M. Goldman).

Innovative approaches to promote transplant tolerance:

There are two studies in this group:

- the first concerns a *Combined Pharmacologic and T Cell-Mediated Immunosuppression approach for the Induction of Donor-Specific Unresponsiveness in Liver Transplant Recipients* (Geissler, Resenburg, Germany).

This is a pilot study which main objective is to propose a novel cell therapy strategy, based on in vitro expanded CD4+CD25^{high} regulatory T cells to establish a safe immunosuppression minimization regimen in liver transplant recipients thus setting the basis for protocols aiming at inducing immunologic tolerance (12, 13). A treatment regimen associating anti-thymocyte globulin and sirolimus will be administered in the early post-transplant period, followed by an infusion of autologous (recipients') CD4+CD25^{high} regulatory T cells. Thymoglobulin and sirolimus treatment were selected because recent

studies indicate that CD4⁺CD25⁺ regulatory T cells are resistant to the effect of these drugs. This trial includes 3 successive phases:

Phase I: 18 consecutive liver transplant recipients (including recipients of organs from both living and cadaveric donors) treated with standard immunosuppression (cyclosporine, daclizumab, prednisolone) will be sequentially monitored to assess the proportions and the functional capacity of regulatory T cells. Using the standardized monitoring tools provided by the RISET core laboratories, the analysis of blood and tissue samples recovered from these patients allowed recovering informative data to embark in the second phase of the study.

Phase II: Here the aim will be to test the feasibility and safety of a thymoglobulin/sirolimus induction protocol on 10 consecutive liver transplant recipients (from living or cadaver donors). Read-out parameters will include clinical criteria (i.e. frequency of rejection episodes requiring pharmacologic intervention, ability to taper immunosuppression, and organ-directed toxicity). Immunological monitoring performed in the RISET core laboratories will be used to define differences in terms of immune reconstitution and specific alloreactive responses in these patients. Results will be compared to those recovered from patients included in the phase I of the study.

Phase III: Once the feasibility and safety of the thymoglobulin/sirolimus immunosuppressive induction protocol is assessed (within the frame of the Phase II study), this treatment will be implemented with the cell therapy approach based on the adoptive transfer of CD4⁺CD25^{high} regulatory T cells. Drs. Edinger and Hoffmann recently described protocols for the isolation of human CD4⁺CD25^{high} regulatory T cells in GMP-conditions and they have developed efficient *in vitro* expansion protocols for this T cell subset. Upon approval of the cell therapy trial by ethical and regulatory authorities they plan to enroll a total of 6-8 patients in this pilot trial.

- the second study in this group is devoted to bone marrow transplantation and deals with the *Use of a combination of anti-CD3 and anti-CD7 immunotoxins to induce long-standing remission of severe high grade graft-versus-host disease* (I. VJM van Oosterhout, Henogen, Gosselies, Belgium)

This protocol was already part of the initial application. For logistic reasons it is only about to start; the company had to fulfil the various requirements of regulatory authorities, namely EMEA and RIVM (Dutch body responsible for clinical evaluation of immunological products) since participating clinical centres are based in Holland. The batches of the GMP products have been produced and made available to the centers. This study will be performed

in bone marrow transplant recipients presenting with severe GvHD non responsive to previous conventional therapies. The objective is to provide a novel alternative therapeutic strategy to allow successful long-term engraftment of bone marrow transplants in a particularly difficult subset of patients presenting a life-threatening condition. Results from a pilot study proved highly encouraging (14, 15)

The centres which will recruit the patients have now been selected in Maastricht, Nijmegen, Rotterdam, Utrecht. Clinical Trial Submission to relevant competent authorities and ethic committee has been completed for some of the centers and is ongoing for the others. A total of 12 patients will be enrolled.

Histological markers of chronic allograft nephropathy:

A multicenter study is leaded by E. Rondeau, Hôpital Tenon, Paris, France and S. Florquin, Amsterdam, Holland on the ***Development of validated analysis of renal transplant biopsies to identify local markers of tolerance and to predict the development of fibrosis.***

Cellular infiltrates in renal biopsies are the hallmark of rejection but are also frequently observed in biopsies of good functioning grafts. The significance of these infiltrates is still a matter of debate. Their characterization may give insight in the function of these cells and provide additive surrogate markers for immune reactivity of a renal transplant recipient towards his graft, and for renal outcome. The team would like to develop predictive diagnostic tools to assess the fibrogenic properties of the infiltrates. More specifically, they propose to investigate for the presence of epithelial to mesenchymal transition (EMT) markers expressed by tubular epithelial cells in the vicinity of the infiltrating cells. Recently, evidence was indeed provided to show that tubular epithelial cells could not only be the victim, but also actively participate to the development of fibrotic lesions : if injured, epithelial cells may either die or undergo profound phenotypic changes characteristic of mesenchymal cells and act as fibroblasts, a process called EMT. The group has recently demonstrated that some epithelial phenotypic changes (such as the de novo expression of vimentin and the translocation of beta catenin into the cytoplasm), compatible with the first steps of EMT, were frequently detected in well-functioning renal grafts at early time points following transplantation, being more obvious if severe infiltration was present (16). More importantly, they found that the early expression of these markers (at three months post-transplant) was predictive of late interstitial fibrosis and tubular atrophy (at one year), the main features of chronic allograft nephropathy.

The aim is thus to confirm that in a prospective study and in a population of kidney transplanted patients, the type of cellular infiltrates and the epithelial behaviour mirror the degree of graft acceptance, and that the biomarkers we have selected will be relevant tools to identify the tolerant or near-tolerant patients in whom immunosuppressive regimen could be minimized. Renal biopsies (200) obtained from patients included in I. Ten Berge's proposal as well as protocol renal biopsies (250 biopsies recovered at 3 and 12 months after transplantation) from patients treated at Hôpital Tenon.

WHAT SHOULD BE THE NEXT STEPS AFTER RISET

The RISET project will reach and end in March 2010. Independently from the informative data which will certainly be recovered from this extensive collaborative endeavour (analysis of a total of 272 patients plus an independent analysis of 450 kidney biopsies) one must realise the important precedent the RISET networks represents for the future. It certainly provides the proof of concept that high quality interaction can be achieved between clinicians and biologists committed to transplant immunology. It also indicates that validating tests which may turned out to be **the** clue of how of transplant patients should be managed in the future can only be achieved when a sufficient critical mass is gathered, not only in terms of various clinical centers joining their efforts but also with the active participation of laboratories combining expertise in fundamental and applied immunology.

The European Union has proven, once more, that it is certainly equipped with tools that are unique to provide not only the financial support but also the visibility needed to this perform at best this type of work.

It is the responsibility of all the European transplant community to join forces in order to allow perpetuating such initiatives.

REFERENCES

1. Bacchetta, R., M. Bigler, J. L. Touraine, R. Parkman, P. A. Tovo, J. Abrams, R. de Waal Malefyt, J. E. de Vries, and M. G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 179:493.
2. Battaglia, M., S. Gregori, R. Bacchetta, and M. G. Roncarolo. 2006. Tr1 cells: from discovery to their clinical application. *Semin Immunol* 18:120.

3. Donckier, V., R. Troisi, A. Le Moine, M. Toungouz, S. Ricciardi, I. Colle, H. Van Vlierberghe, L. Craciun, M. Libin, M. Praet, L. Noens, P. Stordeur, M. Andrien, M. Lambermont, M. Gelin, N. Bourgeois, M. Adler, B. de Hemptinne, and M. Goldman. 2006. Early immunosuppression withdrawal after living donor liver transplantation and donor stem cell infusion. *Liver Transpl* 12:1523.
4. Kawai, T., A. B. Cosimi, T. R. Spitzer, N. Tolkoff-Rubin, M. Suthanthiran, S. L. Saidman, J. Shaffer, F. I. Preffer, R. Ding, V. Sharma, J. A. Fishman, B. Dey, D. S. Ko, M. Hertl, N. B. Goes, W. Wong, W. W. Williams, Jr., R. B. Colvin, M. Sykes, and D. H. Sachs. 2008. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 358:353.
5. Fandrich, F., X. Lin, G. X. Chai, M. Schulze, D. Ganten, M. Bader, J. Holle, D. S. Huang, R. Parwaresch, N. Zavazava, and B. Binas. 2002. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. *Nat Med* 8:171.
6. Hutchinson, J. A., B. G. Brem-Exner, P. Riquelme, D. Roelen, M. Schulze, K. Ivens, B. Grabensee, O. Witzke, T. Philipp, L. Renders, A. Humpe, A. Sotnikova, M. Matthai, A. Heumann, F. Govert, T. Schulte, D. Kabelitz, F. H. Claas, E. K. Geissler, U. Kunzendorf, and F. Fandrich. 2008. A cell-based approach to the minimization of immunosuppression in renal transplantation. *Transpl Int* 21:742.
7. Hutchinson, J. A., P. Riquelme, B. G. Brem-Exner, M. Schulze, M. Matthai, L. Renders, U. Kunzendorf, E. K. Geissler, and F. Fandrich. 2008. Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation. *Transpl Int* 21:728.
8. Andree, H., P. Nickel, C. Nasiadko, M. H. Hammer, C. Schonemann, A. Pruss, H. D. Volk, and P. Reinke. 2006. Identification of dialysis patients with panel-reactive memory T cells before kidney transplantation using an allogeneic cell bank. *J Am Soc Nephrol* 17:573.
9. Nather, B. J., P. Nickel, G. Bold, F. Presber, C. Schonemann, J. Pratschke, H. D. Volk, and P. Reinke. 2006. Modified ELISPOT technique--highly significant inverse correlation of post-Tx donor-reactive IFN γ -producing cell frequencies with 6 and 12 months graft function in kidney transplant recipients. *Transpl Immunol* 16:232.
10. Nickel, P., F. Presber, G. Bold, D. Biti, C. Schonemann, S. G. Tullius, H. D. Volk, and P. Reinke. 2004. 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.
11. Brook, M. O., K. J. Wood, and N. D. Jones. 2006. The impact of memory T cells on rejection and the induction of tolerance. *Transplantation* 82:1.
 12. Hoffmann, P., T. J. Boeld, R. Eder, J. Albrecht, K. Doser, B. Piseshka, A. Dada, C. Niemand, M. Assenmacher, E. Orso, R. Andreesen, E. Holler, and M. Edinger. 2006. Isolation of CD4+CD25+ regulatory T cells for clinical trials. *Biol Blood Marrow Transplant* 12:267.
 13. Hoffmann, P., R. Eder, T. J. Boeld, K. Doser, B. Piseshka, R. Andreesen, and M. Edinger. 2006. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* 108:4260.
 14. van Oosterhout, Y. V., J. L. van Emst, H. H. Bakker, F. W. Preijers, A. V. Schattenberg, D. J. Ruiter, S. Evers, J. P. Koopman, and T. de Witte. 2001. Production of anti-CD3 and anti-CD7 ricin A-immunotoxins for a clinical pilot study. *Int J Pharm* 221:175.
 15. van Oosterhout, Y. V., L. van Emst, A. V. Schattenberg, W. J. Tax, D. J. Ruiter, H. Spits, F. M. Nagengast, R. Masereeuw, S. Evers, T. de Witte, and F. W. Preijers. 2000. A combination of anti-CD3 and anti-CD7 ricin A-immunotoxins for the in vivo treatment of acute graft versus host disease. *Blood* 95:3693.
 16. Hertig, A., D. Anglicheau, J. Verine, N. Pallet, M. Touzot, P. Y. Ancel, L. Mesnard, N. Brousse, E. Baugey, D. Glotz, C. Legendre, E. Rondeau, and Y. C. Xu-Dubois. 2008. Early epithelial phenotypic changes predict graft fibrosis. *J Am Soc Nephrol* 19:1584.