REPROGRAMMING THE IMMUNE SYSTEM FOR THE ESTABLISHMENT OF TOLERANCE

FINAL REPORT

Contract n°

LSHB-CT-2005-512090

Project acronym

RISET

Instrument

Integrated Project

Period covered: from 1 March 2005 to 31st August 2010
Date of preparation: September 2010

Start date of project: 1 March 2005

Duration: 66 months

Coordinators:
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Organisation name: Université Libre de Bruxelles

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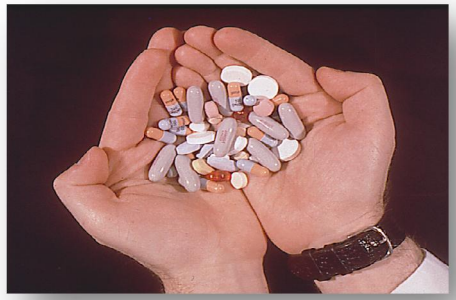
I. PROJECT EXECUTION

I.1 Objectives

The RISET acronym stands for “Reprogramming the Immune System for the Establishment of Tolerance”, a state in which the immune system becomes non-responsive to the transplant.

Transplantation dramatically improves the survival of patients with established organ failure. The transplant is literally a life saver and the short-term prospects of most transplant patients are very good. However, in the longer term many of these patients face serious health issues. A significant number of problems are caused by the cocktail of immunosuppressive drugs that transplant patients take to prevent their immune system from attacking the donor organ. These drugs are efficient in preventing or controlling early acute rejection episodes or graft versus host disease and allow for the excellent results of organ transplantation in the short term. Yet the side effects of these drugs include cancers, infections, kidney problems, cardiovascular diseases, diabetes and bone diseases, to name just a few. Currently, some patients receive more immunosuppression than required, whereas other patients develop chronic transplant damage because of insufficient immunosuppression. Tailoring immunosuppression according to the immune reactivity of each transplant recipient would therefore represent a significant advance in transplantation medicine.



The ultimate goal of RISET was to develop safe and efficient diagnostic tools and therapies to enable drug minimization in transplanted patients. To this end, the development and validation of reliable tests to predict tolerance are mandatory steps for the implementation of large-scale clinical trials. These tests are based on immunological measurements as well as genomic or proteomic assays. They are needed to lower as much as possible the risk of rejection during immunosuppression minimization or after withdrawal. The RISET consortium has successfully developed new tests and defined molecular signatures of transplantation tolerance and rejection. The critical next step is the validation of the tests and signatures to enable them to be introduced as immune monitoring tools in clinical trials and to make them acceptable as surrogate markers of graft acceptance by regulatory agencies.

I.2 Consortium

The RISET consortium started its research activities in March 2005 and included 25 partners from 11 countries. In total, more than a hundred of researchers contributed to RISET, including a panel of renowned scientists. Annual meetings of the consortium gathered together between 50 and 60 specialists of the field to discuss the progress of the project and future orientations. Over the years, RISET program and ambitions have been refined and optimised with the support of the members of its Scientific Advisory Board, Pr. Sir Peter MORRIS (University of Oxford, UK), Pr. Jeff BLUESTONE (Director of the UCSF Diabetes Center of the University of California and the NIH Immune Tolerance Network, USA) and Dr. Werner WOLF (Bio Innovations, Switzerland).

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Tc LAND Expression S.A . (FR)

PROIMMUNE Limited (UK)

HENOGEN S.A. (BE)

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Transplant centres

Organizacion Nacional de Transplantes E. MARTINEZ

I.3 A multi-steps approach

1. The development of biomarker tests predictive of transplant tolerance or "near-tolerance"

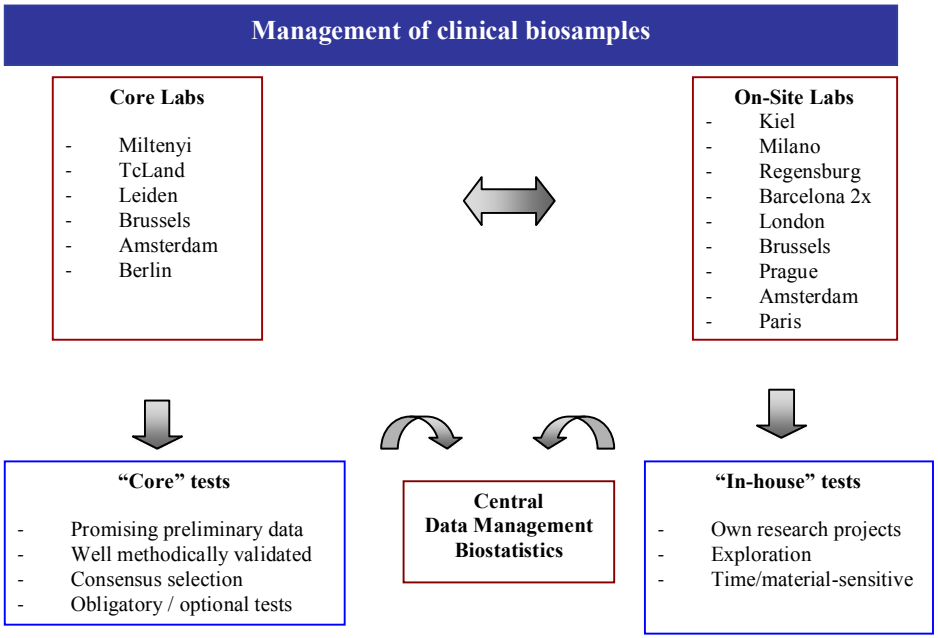
The development of assays aimed at:

- the definition of immunological pre-transplant constellations identifying patients that are not suitable for a particular tolerance induction protocol (definition of high-risk patients before transplantation).
- the early identification of failure of tolerance induction, possibly before graft deterioration occurs (definition of negative predictors early after transplantation).
- the early demonstration of tolerance after induction therapy (definition of early positive predictors of success after transplantation)
- the harmful injury/tolerisation of the anti-microbial immune response (definition of safety markers).
- the identification of tolerance state markers in long term stable recipients under immunosuppressive therapy.

The following read-out systems were used for measuring the immune reactivity in these special situations:

- ex vivo assays for the characterization of alloreactive peripheral blood T-cells by using ELISPOT, Multiplex-ELISA, CTLp, real-time RT-PCR, trans vivo DTH-assay in SCID mice
- assays for the detection of alloreactive T-cells triggered via the indirect pathway (multimer technology)
- ex vivo assays for the measurement of Treg function of peripheral leukocytes (gene expression profile, ELISPOT, DTH etc)
- analysis of TCR repertoire by TCR landscaping in PBL and biopsies
- B-cell priming (alloantibodies, B-cell ELISPOT)
- gene expression studies by real-time RT-PCR and microarray technology in blood samples
- measurement of antiviral (EBV/CMV) T-cell response of peripheral blood leukocytes, EBV/CMV/BK viral load and monocytic immunocompetence for safety reasons.

Developing diagnostic tests for transplantation tolerance and testing their performance on RISET clinical samples involved a substantial effort of methodological optimization and validation. Our initial strategy of guaranteeing quality by performing tests only by defined core units was reviewed in the course of the project. Cross-validations were organised with the objective of successfully transferring single tests from the test performing core units (reference centres) to the clinical partners.



For this purpose, 2 types of SOP were developed:

- SOP with a step-by-step description of how to collect the necessary amount of sample from a clearly defined source and how to handle, store and transfer these samples.
- SOP with detailed description of how to perform the assay, a preceding statement of its purpose, precision on the duration and indications concerning the pre-analytics.

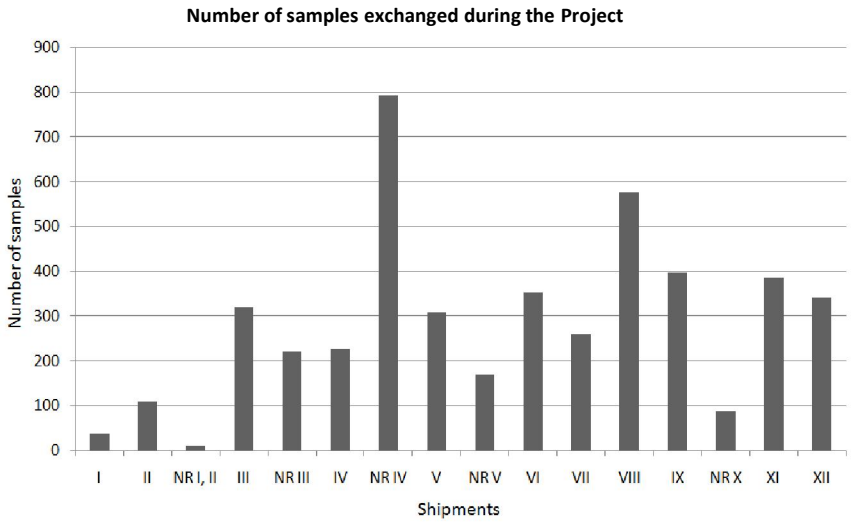
Theoretical standardization of the assays obtained by the development, evaluation and approbation of the SOPs was completed by on-site audits to ensure that standardization was effective.

Based on robust validation data and preliminary biological data, we selected the most promising candidate tests and defined a pool of assays to embed in RISET clinical studies. Our selection included safety and tolerance markers[1], as well as tests to characterize humoral and cellular sensitization[2]. Therefore, the established tests were subdivided into obligatory and optional assays: i.e. a pool of tests that needed to be applied for all patients and a pool of tests that could be applied. As only some of the assays were performed by the clinical partners themselves, sample transfer from the clinical centre to the test-performing partners was organized in collective sample shipments with centralized monitoring, allowing sample tracking via the RISET Intranet.

[1] EBV/CMV/Polyoma viral load, HLA-DR expression on monocytes, CMV/EBV spec. T-cells, gene expression profiling by real-time RT-PCR/Agilent Microarray

[2] Screening of HLA alloantibodies, IFN-gamma ELISPOT, Multiplex cytokine and CTLp assay) or particular expansions and response states (TcLandscape; HMOX-1 polymorphism)

Ten collective shipments, as well as some single shipments, made it possible to collect 4,268 biosamples during RISET.



The following tests were developed, methodologically validated, and applied to the analysis of these samples.

Safety markers to detect overimmunosuppression by novel approaches

- ✓ HLA-DR+ monocytes (Berlin)
- ✓ Anti-viral (EBV/CMV) memory T-cell response (Berlin)
- ✓ Viral load (EBV/CMV/BKV) (Berlin)

Donor-specific alloresponsiveness to detect humoral and cellular allosensitization

- ✓ High sensitivity alloantibody tests (Leiden)
- ✓ Alloreactive memory Tcells (IFNg ELISPOT) (Berlin/Barcelona)
- ✓ Alloreactive CTL precursor frequency (CTLp) (Leiden)
- ✓ Alloreactive T-cell activation by gene expression (Brussels)

Intragraft inflammation (kidney) to detect intrarenal inflammation

- ✓ Urinary IP-10 levels (Berlin)

T-cell receptor repertoire to detect (allo)antigen-driven oligoclonal expansions

- ✓ T-cell landscape (TcLand)

Immune pre-activation/silent level to measure immune activation / tolerance balance

- ✓ Toag-1 (tolerance-ass-gene-1) (Berlin/Oxford => Berlin)
- ✓ FoxP3/alpha-1,2-Mannosidase (Berlin/Oxford =>Berlin)
- ✓ TORID (tolerance-related and induced transcript) (Nantes =>Berlin)

Gene Expression Profiling to detect novel molecular signatures associated with tolerance success/failure

- ✓ Microarrays RISET (Miltenyi Biotec)

These tests demonstrated their feasibility for multicentre trials. The methodological validation proved to be successful. The results of the analyses have all been transmitted to RISET clinical partners for clinical validation. Discussions between laboratories and clinical partners were organised to review and discuss these results.
In addition, CHARITE performed the RNA isolation of all samples for the DNA microarray analysis by MILTENYI, which corresponds to more 1.140 experiments.

In parallel, exploratory research to develop new assays for the development of novel diagnostics aimed at analyzing data with a view to implement the methodical validation of novel tests. In that respect, we identified the most promising tests / markers that were feasible for the development of novel diagnostic tests within RISET:

- IL-2 mRNA quantification in MLR
- B-cell ELISPOT
- DKK-3 ELISA
- CD107a/b as replacement of the CTLp analysis
- Indirect pathway
- BcLandscaping
- Trans vivo DTH

The most important milestone for all these tests was the decision point “go/no go” based on progress, feasibility, and robustness of the assays.

2. The identification of new molecular targets for tolerance induction in pre-clinical models

Experimental studies implemented during RISET aimed at (1) gaining insight into basic mechanisms of immune regulation, allograft rejection and tolerance (cell-therapy strategy and monoclonal antibodies), (2) studying at the cellular and molecular level different models of tolerance and regulation, and (3) identifying biomarkers that could represent tools to diagnose or induce tolerance.

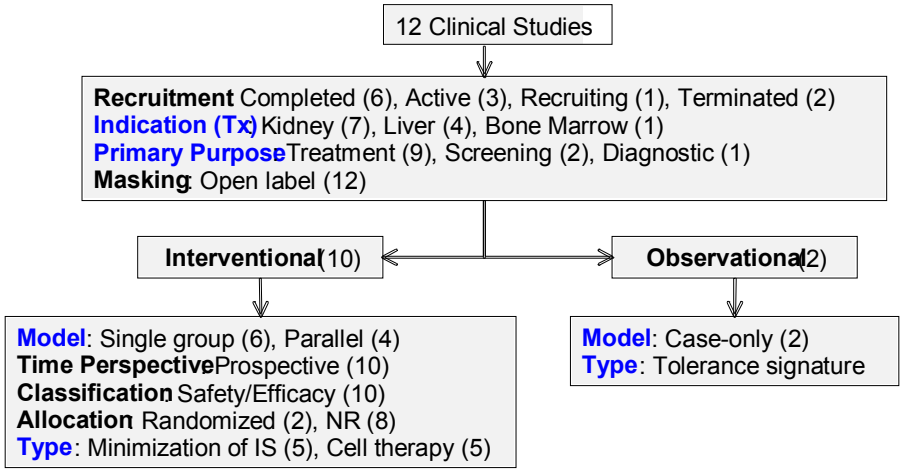
These studies were organized in 3 main layers:

- The development of pre-clinical models of tolerance through the characterization and generation of allospecific regulatory T cells and of tolerogenic dendritic cells. The latter cells involved the analysis of a common set of genes as markers for tolerance induction.
- The identification of new strategies to improve clinical protocols of transplantation using monoclonal antibody therapy.
- The identification of new strategies to prevent early endothelial cell activation and allograft rejection.

3. The participation in clinical studies including novel cell therapies to assess clinically and biologically the clinical outcome of patients enrolled in pilot clinical investigations aiming at drug minimization

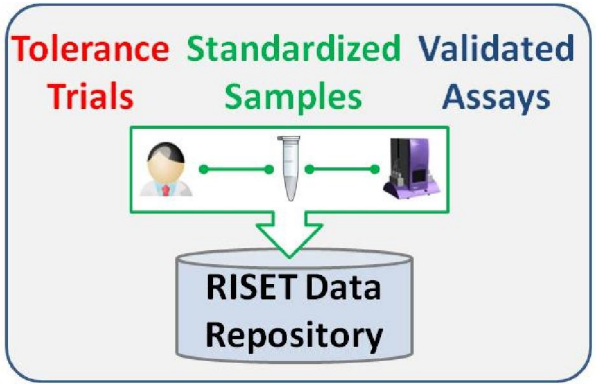
The initial objective of RISET clinical studies was to conduct hypothesis-driven pilot or medium size clinical investigations which fall into two main categories. The first is that of studies aiming at inducing "operational transplant tolerance" defined as a state of lasting antigen-specific unresponsiveness in the absence of generalized immunosuppression. The rationale was based on clinical transfer of pre-defined protocols which had proved effective to induce transplant tolerance in the experimental setting and for which compelling evidence has 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 state. As the project progressed and the results from these first studies became available, the strategies were modified to address the clinical and logistic problems posed, as well as the recommendations of our external advisory board and reviewers. Therefore, a second category of clinical investigations was launched aiming at minimization of immunosuppression on the basis of biomarkers and/or innovative therapies.

Among the 12 clinical studies, five were included over the last 18 months of the project.



4. Data management and data analysis for biomarkers qualification

In order to exploit optimally the clinical and biological data accumulated within RISET, we decided to implement solutions for the exchange and optimal use of the information collected by each partner involved in clinical investigations and/or biological assays.



Building RISET Data Repository involved standardization and formalization duties for both the biological assays and the clinical investigations. The current tool was dedicated for data storage in a perspective of Data Analysis. Integration of various sources of clinical data and of all the data produced during the experiments was the main task. The database modelling thus required merging (when possible) of the items of the various clinical studies datasets. Furthermore, recurrent formatting and importing steps of data were necessary to integrate data from the different clinical centres to fill in the database.

Major steps of the RISET Integration Process

For each clinical study

Formalizing the trial (ontology)
Identifying the relevant clinical data
Collecting the data
Setting-up an "update procedure"
Defining a closure date

For each assay

Describing the data and test parameters
Formalizing the results (data format)
Collecting the data
Setting up an import procedure
Defining the last results shipment date

Harmonizing the current patient/sample codification
Formalizing the shipment process
Formating the sampling data
Checking the "connections" between clinical and biological data

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Modeling the repository… for longitudinal data !
Implementing the relational database (SQL)
Developing : Parsing methods to integrate the data
Procedures to extract data for the statistical analyses
Consistency tests to check the "interconnections" between the data
Installing the database on the server
Back-up and security
Maintenance & Documenting

A transversal statistical analysis through the RISET biological and clinical data was then possible to be performed to identify and validate biomarkers of tolerance (longitudinal analysis, assessment of interactions between assays and similarities between clinical studies).

Due to the diversity of clinical studies, the data analysis strategy was adapted to the design of these studies and to the questions raised by the Consortium. Because the information delivered by the assays is complex and interdependent, clear knowledge formalization was needed (model above). The RISET Data Repository contains the validation data of the biomarker candidates and furthermore the data that may reveal the organization and the dynamic interactions that ruled the immune system. By investigating the correlation of the data produced by the different assays and collected during diverse clinical situations, we hypothesized that we would reveal mechanistic associations.

Data Analysis Strategy

Analyzing correlations between assays in different contexts
=> **complementarity / divergence** of assays

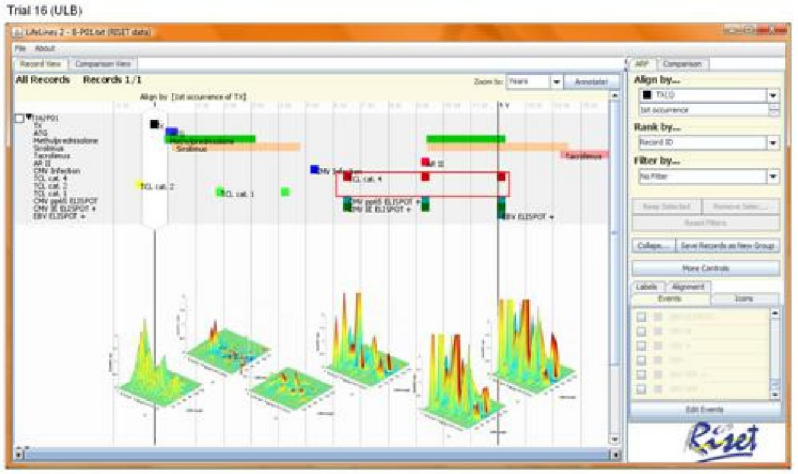
Using multifactorial analysis (PCA, CFA…) to explore
the **interaction between assays** and the **similarities between trials**

Mapping the different clinical trials in order
to find **common endpoints** and pool patients in a **"composite trial"**

Assessing biological changes over time :
Longitudinal Analyses

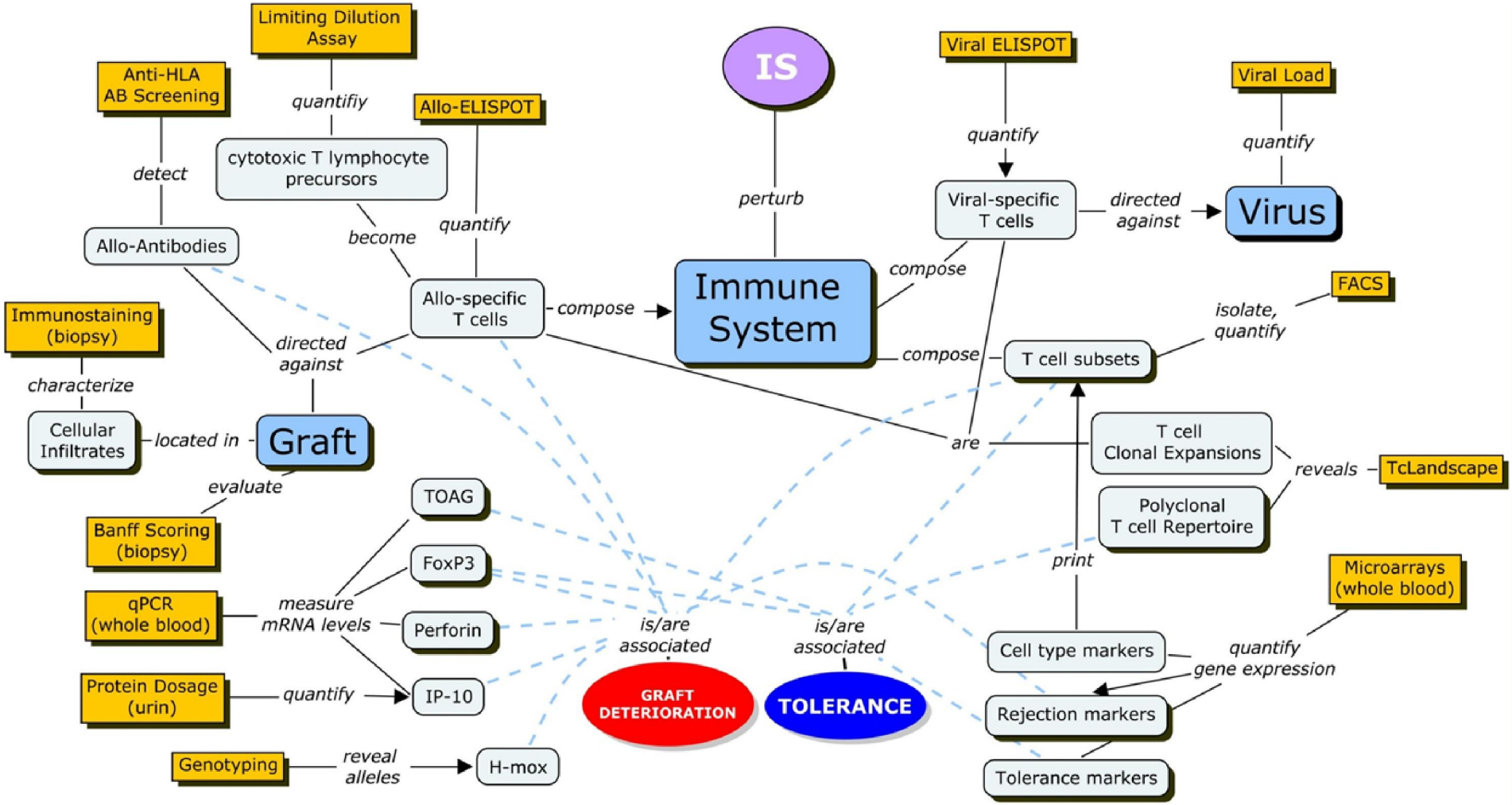
The adaptation of a program called LifeLines to meet the requirements of RISET to visualize and align events and results allowed an in-depth longitudinal analysis of RISET data.

Example of a longitudinal analysis using the LifeLines



Thanks to these efforts, RISET is achieving qualification of biomarkers, including the writing of guidelines and the design of new clinical trials (adaptive trials for small populations and with sample enrichment strategies…).

Model of clinical and experimental data collected and produced during the RISET studies



5. Designing ethical guidelines for tolerance induction protocols and educational programs on tolerance induction for patients and their families

A dedicated ethical team was an integral part of RISET not only to supervise and manage the ethical issues arising out of the consortium's research activities but also to link up with the regulatory authorities, patient associations and the public at large to provide information about research in cell and organ transplantation and tolerance induction. Several activities were undertaken which pursued different aims:

- Developing a clear understanding of the way in which transplant patients and the public at large perceive tolerance induction in the setting of transplantation
- Designing and distributing a "RISET Dissemination pack" to patient communities and clinical centres across Europe.
- Following up related ethical and regulatory developments and conducting internal assessments about the evolution of the legal framework governing clinical research in transplantation
- Designing and disseminating ethics recommendations about tolerance induction in clinical trials
- Organizing educational events about the ethical and regulatory frameworks for professionals in medical law and in transplantation as well as for young researchers
- Addressing the patients' perception of immunosuppression and other treatments accompanying transplantation
- Contributing to the development of appropriate information materials for the distribution to patients, media, schools, health professionals
- Producing a final synthesis of ethical, legal, regulatory and societal aspects related to the induction of tolerance in transplantation

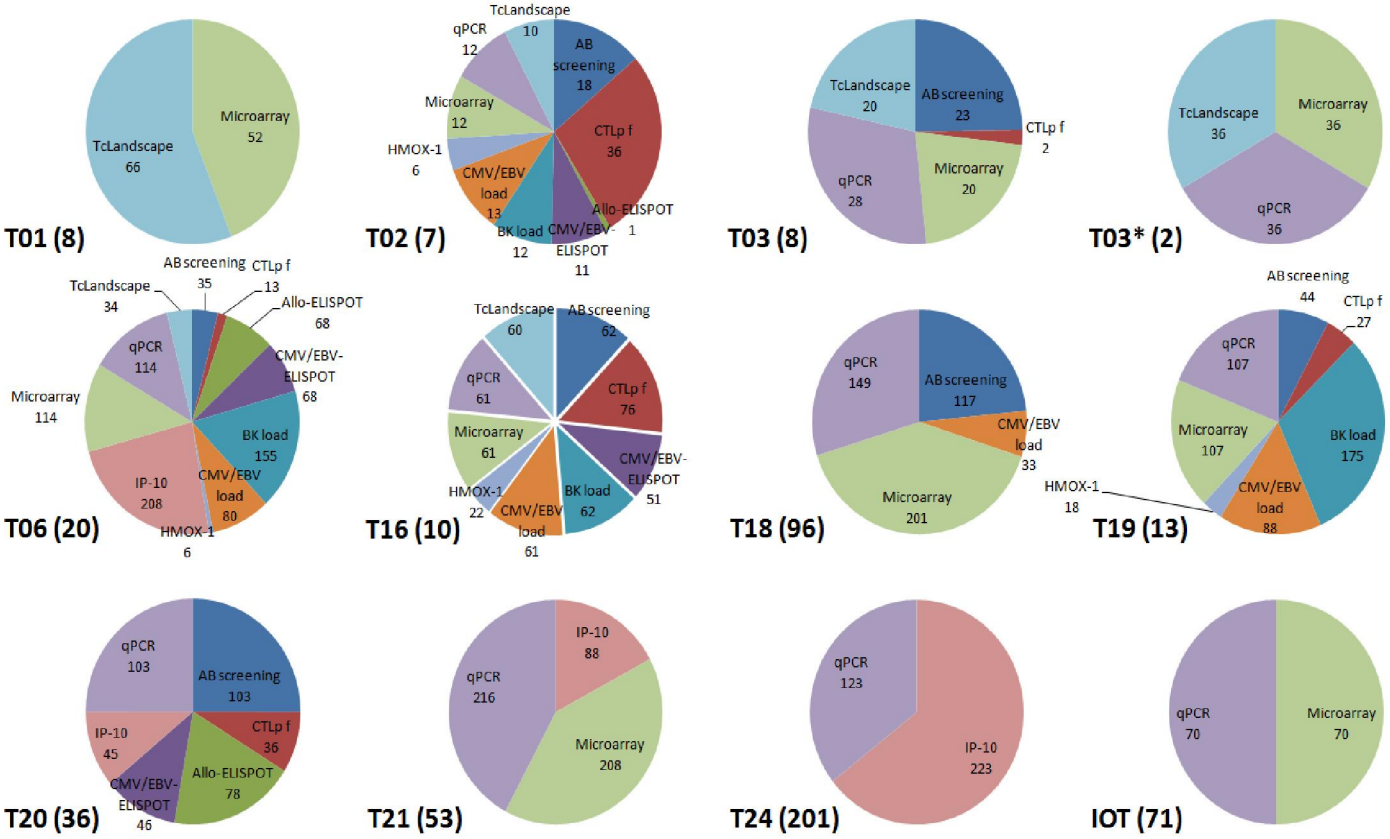
I.4 Achievements

RISET has taken important steps towards achieving transplantation tolerance by investigating innovative therapies

By June 2010, 530 patients were included in RISET clinical studies and 4,268 samples exchanged among RISET partners

Integrated efforts of clinical investigators and test performing centres gave rise to a unique collaborative clinical research activity in terms of patient recruitment and samples collected and analyzed. Significant progress has been made concerning data integration of each clinical trial (requiring standardization of assay protocols, sample management, clinical terminology, formats…) and data analysis (longitudinal analysis but also pooled analysis using data from several clinical studies).

Total number of samples tested by RISET tests and by clinical study



Results obtained from the clinical studies provided important clues

Results obtained from the clinical studies provided important clues on the

- clinical conditions for complete drug weaning in liver transplant recipients
- clinical conditions for minimisation of immunosuppression (monotherapy) in kidney transplant recipients
- identification of potentially relevant biomarkers to guide weaning or minimization
- establishment of a critical mass of data to set up future clinical studies

Protocol T16. Early immunosuppression withdrawal/mimimization after cadaver liver transplantation using T cell depleting therapy and rapamycin

Sixteen patients have been included. Ten of them remain evaluable.

Results indicate that, in tolerance-inducing strategies using peritransplant T-cell depletion, memory T-cells present at transplantation, resistant to depletion and further expanding after transplantation could represent a barrier for tolerance induction. In these conditions, the use of treatments able to specifically target or control memory cells could be mandatory to allow establishment of transplantation tolerance.

Protocol T20. Minimization of immunosuppression in renal allograft recipients

The minimization of immunosupression to double therapy consisting of prednisolone and everolimus is safe in contrast to minimization to prednisolon and mycophenolate sodium

Better renal function found in the prednisolon/everolimus arm.

Protocol T06. Prospective trial for recognizing patients suitable for minimization of immunosuppression based on CAMPATH-1H and anti–TNF antibodies and maintenance regimen using either tacrolimus or sirolimus

20 patients were enrolled and 2-yr follow-up almost completed. The novel combination of targeting T/B cell clonal size by Campath-1 mAb and recently activated effector T cells by anti-TNF mAb allows a safe CNI-based monotherapy already early post-Tx. The renal graft function and histology were excellent during the follow-up period, even in patients who expressed high pre-transplant allospecific memory T-cell level (ELISPOT) – a situation associated with poor graft function under conventional immunosuppression. By contrast, rapamycine monotherapy was not successful in 4/7 patients, as some developed proteinurea and histological signs of chronic rejection. Therefore, these patients were switched to standard therapy and organ function could be preserved. This arm of the study was canceled after recruitment of 7 patients. The CNI-based monotherapy following the novel induction protocol, however, is a promising protocol for a safe monoherapy in almost all patients that might be combined in the future with tolerogenic approaches, such as Treg therapy.

The study was accompanied by extensive biomarker analysis - altogether 850 samples were collected for analysis using RISET tests. Low long-lasting urinary IP-10 levels further support the power of this new protocol

Protocol T18. Search for immunological signature of operational tolerance in liver transplantation

In stable liver transplant recipients operational tolerance is much more prevalent than previously estimated at late time points after transplantation.

The magnitude of the differences in gene expression between tolerant and non-tolerant recipients at baseline is smaller than originally estimated. Despite this, peripheral blood expression patterns can be employed to predict the success of immunosuppression weaning strategies before drugs are discontinued.

These data provide the rationale and starting point for the development of a non-invasive diagnostic test of tolerance in liver transplantation, and for its use within large clinical studies aiming at the personalized use of immunosuppressive drugs.

No differences in alloantibody measurements at baseline have been noted between tolerant and non-tolerant liver recipients.

Markers of operational tolerance in liver recipients are different from those found in kidney transplantation (data published in JCI 2010 – see Annexe 5).

Biomarker tests to develop novel diagnostics to identify patients for whom immunosuppressive treatments could be safely minimized or withdrawn were developed, validated and demonstrated their feasibility for multicentre trials.

Inter-lab cross-validation demonstrated the feasibility and robustness of the RISET tests. Some tests are now ready for commercial development in collaboration with the industry. Such developments would allow broad access to the RISET tests by transplant centres throughout Europe as well as internationally thereby facilitating personalized medicine in clinical transplantation.

Biomarkers have been identified which enables the development of guidelines for drug minimization.

The key results obtained from the biomarker studies are summarized hereafter.

Delivery of a set of well standardized markers/tests to the RISET consortium.

Extensive cross-validation of cell-based assays (HLA-DR monocytes, ELISPOT, CTLp) and viral load tests resulting for the first time in tests which deliver comparable multicenter results.
The safety marker set of tests makes new trials in transplant patients with unpredictable "netto"-immunosuppression safer as an objective monitoring is easily possible.

Development of a bedside test (point-of-care test) for quantifying urinary IP-10

Demonstration of the association between cellular donor-hyporeactivity and good graft function in long-term transplant recipients

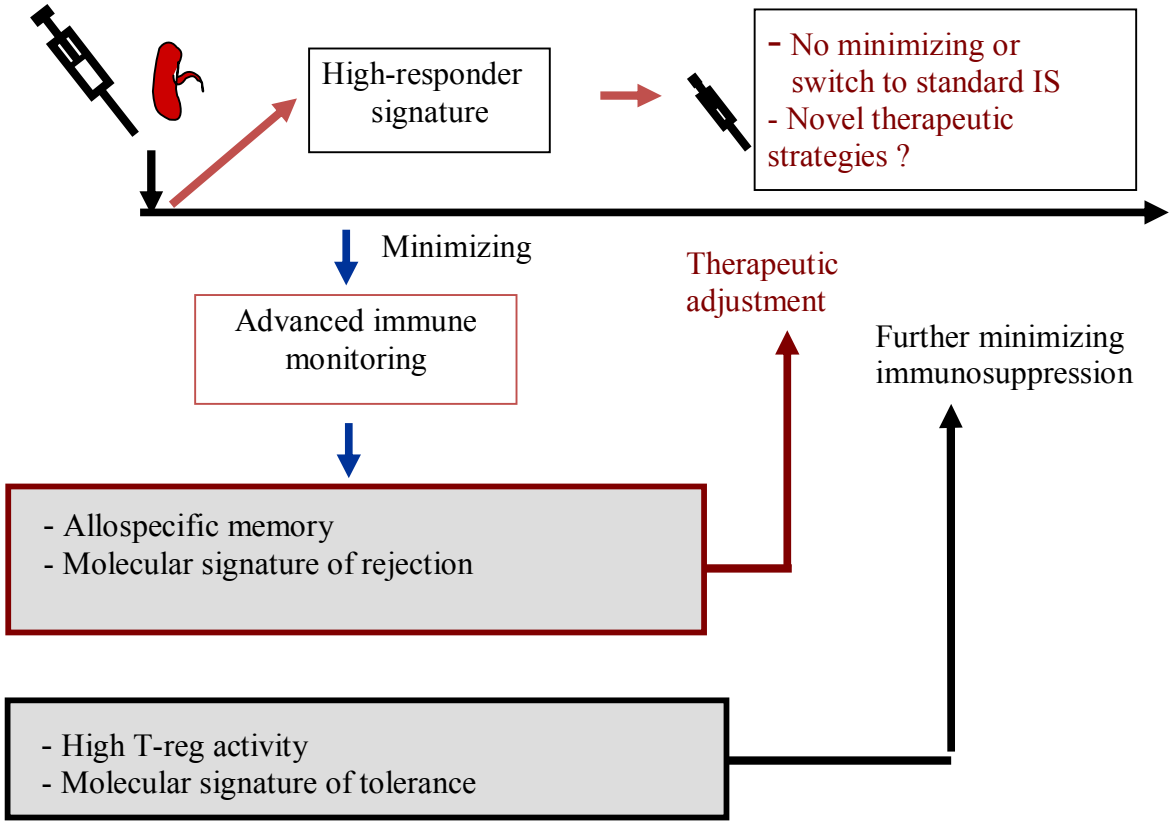
Discovering Toag-1 as a marker for quantifying the summative activation/silence balance of adaptive immunity in general, and better understanding the regulatory mechanisms behind
Insights into the mechanisms of novel therapies (e.g. cell-based therapies, targeting memory T cells by anti-TNF mAb)

Discovery of novel molecular markers/marker combinations for i) characterizing operational tolerance (patent submitted), ii) predicting failure/success of minimizing immunosuppression (validation still ongoing), iii) detecting high-responders less good suitable for minimization protocols already before transplantation

First biomarker-guided trial for minimizing immunosuppression in transplantation by perioperative ELISPOT analysis-based randomization of patients

The next critical step is the validation of the biomarkers identified by RISET in large multi-centre clinical studies to confirm cut-offs and personalize immunosuppression.

OUTLOOK – PERSONALIZED IMMUNOSUPPRESSION



In parallel, Miltenyi Biotec performed microarray experiments for the clinical studies. In total, 1,063 clinical samples and 77 experimental samples were processed. As a result, they have been involved in a series of publications with the academic and clinical RISET partners and took part in a patent application (Biomarkers of tolerance in kidney transplant recipients, see below).

Description of a cross-platform set of biomarkers of tolerance in kidney transplant recipients

As a follow up of the FP5 Indices of Tolerance project, RISET demonstrated that drug-free tolerant patients display an expansion of peripheral blood B and NK lymphocytes in the absence of donor-specific antibodies. Additionally, direct pathway donor-specific hyporesponsiveness by IFNγ ELISpot and a high ratio of FoxP3/α-1,2- mannosidase expression were observed in drug-free patients. Thanks to the customized Agilent RISET 2.0 microarray, which was designed with a focus on transplantation research related genes, we identified a series of them, whose expression on whole blood allowed the identification of 100% of the tolerant recipients of the training set and 93% in the test set.

When using cross-platform biomarkers on patients we were able to improve the diagnostic features on the test set. This validated 'tolerant fingerprint' can now be used to shape drug weaning protocols of kidney transplant patients. Data published JCI, 2010 (see Annexe 5).

This set of biomarkers needs now to be tested in patients undergoing immunosuppression.

From the experiences related to the development of novel biomarker assays, it appears that:

- FOX P3 promoter methylation analysis is feasible in transplant patients. To date, it requires additional standardisation markers at the the same platform (methylation PCR) like CD3, 4.
- B-cell ELISPOT looks promising but is not ready to go as some methodical variation has to be solved

Experimental studies led to the identification of new genes and molecules relevant for the induction of transplantation tolerance

RISET experimental studies have led to the identification of new genes and molecules relevant for the induction of transplantation tolerance. Diagnostic genes markers have been defined from a panel of RISET genes, including IL27p28, EB13, TGFβ and TORID, since each of these genes was found to be up-regulated in tolerant grafts and/or regulatory T cells. Valuable translational data have been obtained for allospecific regulatory T cells in humanized mice (published in Nature Medicine, 2010 – see Annexe 5) and Tolerogenic DCs (published in Transplantation, 2010 – see Annexe 5).

These studies contributed to demonstrate that the immune system's tolerance of the donated organ can be improved if so-called 'tolerogenic' cells are transplanted along with the organ. These tolerogenic cells effectively educate the host immune system to accept the new organ, thereby reducing the need for immunosuppressive medicines.

Dexamethasone was studied to promote tolerogeneic DCs and induction of expression of candidate genes has been studied by different partners.

EBI3 and TORID are genes that are up-regulated in tolerogenic DCs.

Studies demonstrated that donor drug-treated DCs although very efficient *in vitro* in expanding/inducing Tregs, *in vivo* are processed and presented in a stimulatory manner. A critical review about the use of "tolerogenic" DCs has been written by different partners and submitted to Transplantation.

Interesting data of the injection of immature syngeneic DCs in combination with anti-CD3 treatment on islet allograft survival have been obtained. The study of their therapeutical potential will be pursued in clinic by some partners.

The characterisation and expansion of allospecific regulatory T cells enabled to confirm their functional properties and therapeutical potential in different models. The study of their therapeutical potential will be pursued in clinic by some partners.

Alloantigens-specific Tregs have been generated by transduction and tested for solid allograft or GvHD following *in vivo* injection. In the human, polyclonal Tregs have been expanded in a GMP compliant manner and strategies to expand alloantigen-specific Tregs are now in place.

TORID mRNA expression was analyzed in PBMC samples from kidney transplant recipients and appears to be a possible marker of development of renal chronic rejection or degradation of function. This result was validated in different clinical studies and using the newly designed RISET 2.0 microarray.

Cell therapy in transplantation with the generation and characterization of tolerogeneic DCs and regulatory T cells will be tested in a phase I kidney allograft protocol in the context of the FP7 collaborative project "The ONE Study".

The "humanized" mouse models of xenoGvHD and human skin transplantation were demonstrated to be valuable for translational studies, particularly for testing therapeutic agents that are specific for human targets.

The first experiments involving injection of Treg lines were launched. Human CD25+CD127- T cells have been expanded in vitro and analyzed for their suppressive properties in vitro and in vivo (published in Nature Medicine, 2010 – see Annexe 5). CD8+ and CD4+ Treg have been characterized and their functional properties have been demonstrated in vivo by different partners.

For the humanized mice model of anti-CD3, the therapeutic potential of this antibody has been demonstrated. Indeed, transgenic huCD3-NOD mice has been validated as a reliable pre-clinical model for the study of the therapeutic effect of human CD3-specific Abs and interesting results on islet allograft survival have been obtained. Moreover, a collaboration between partners showed a synergistic effect of anti-CD3 Ab and recipient-type immature DC administration.

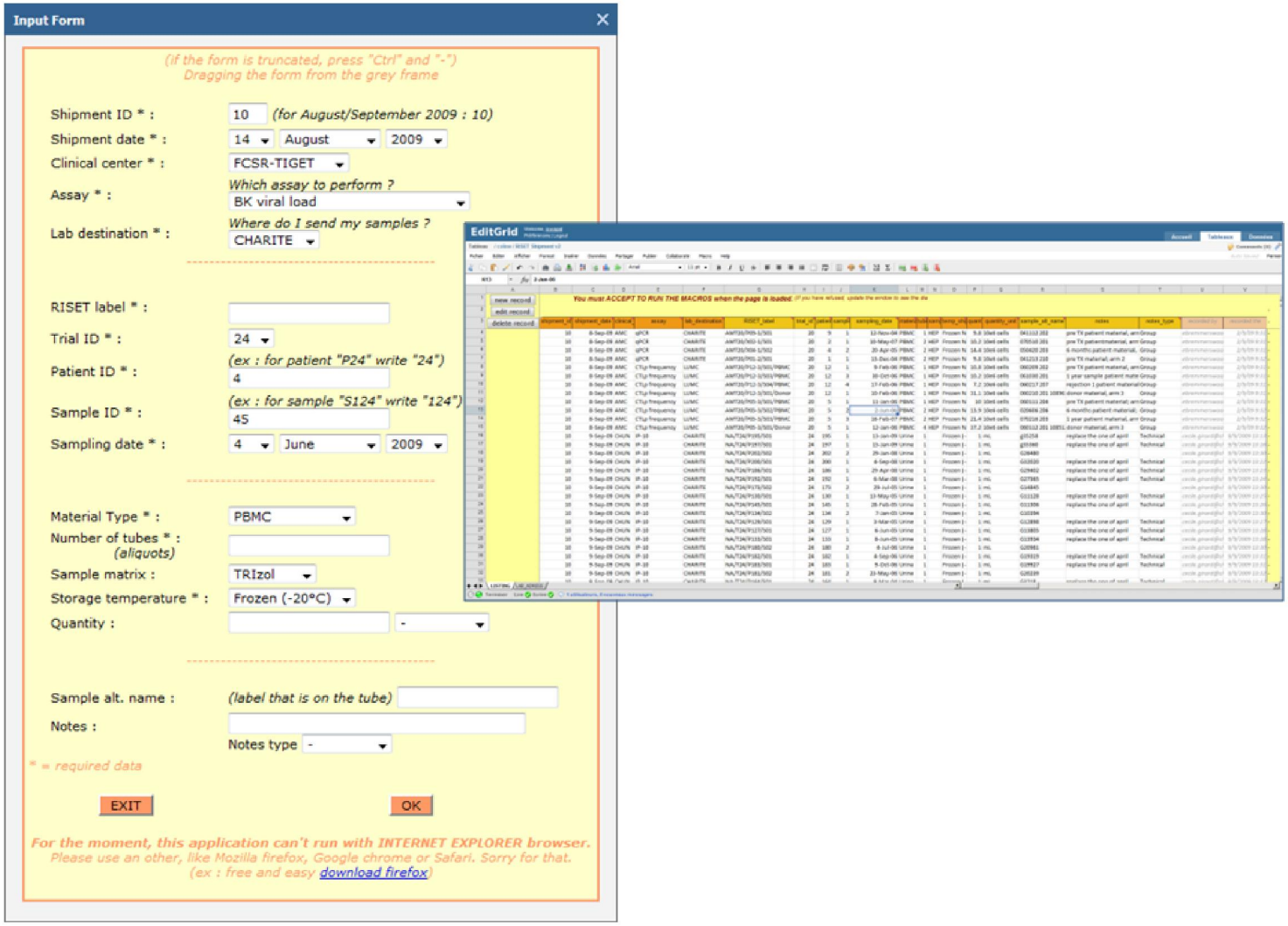
I.5 Tools

Several tools were developed
and are available for further studies

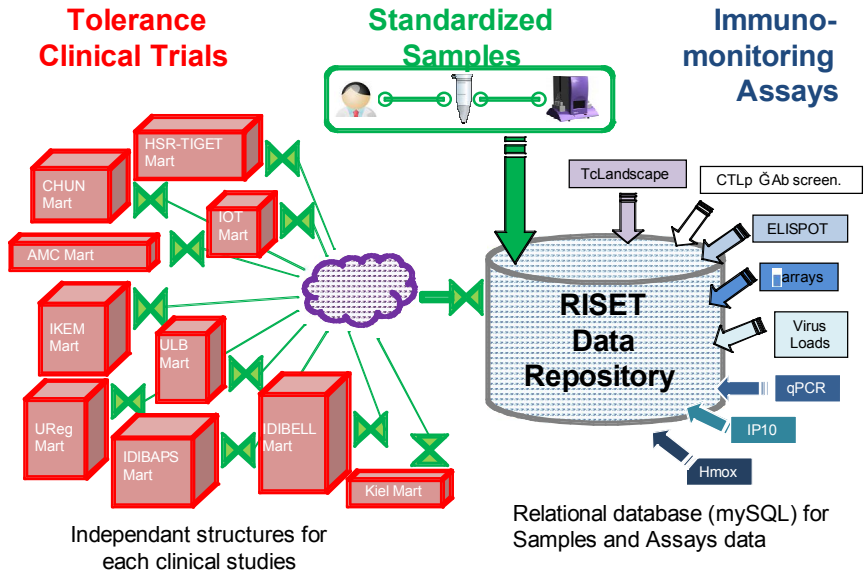
Standard Operating Procedures (SOPs) on sample preparation and the performance of the assays were designed and validated by RISET partners and made available to the scientific community.

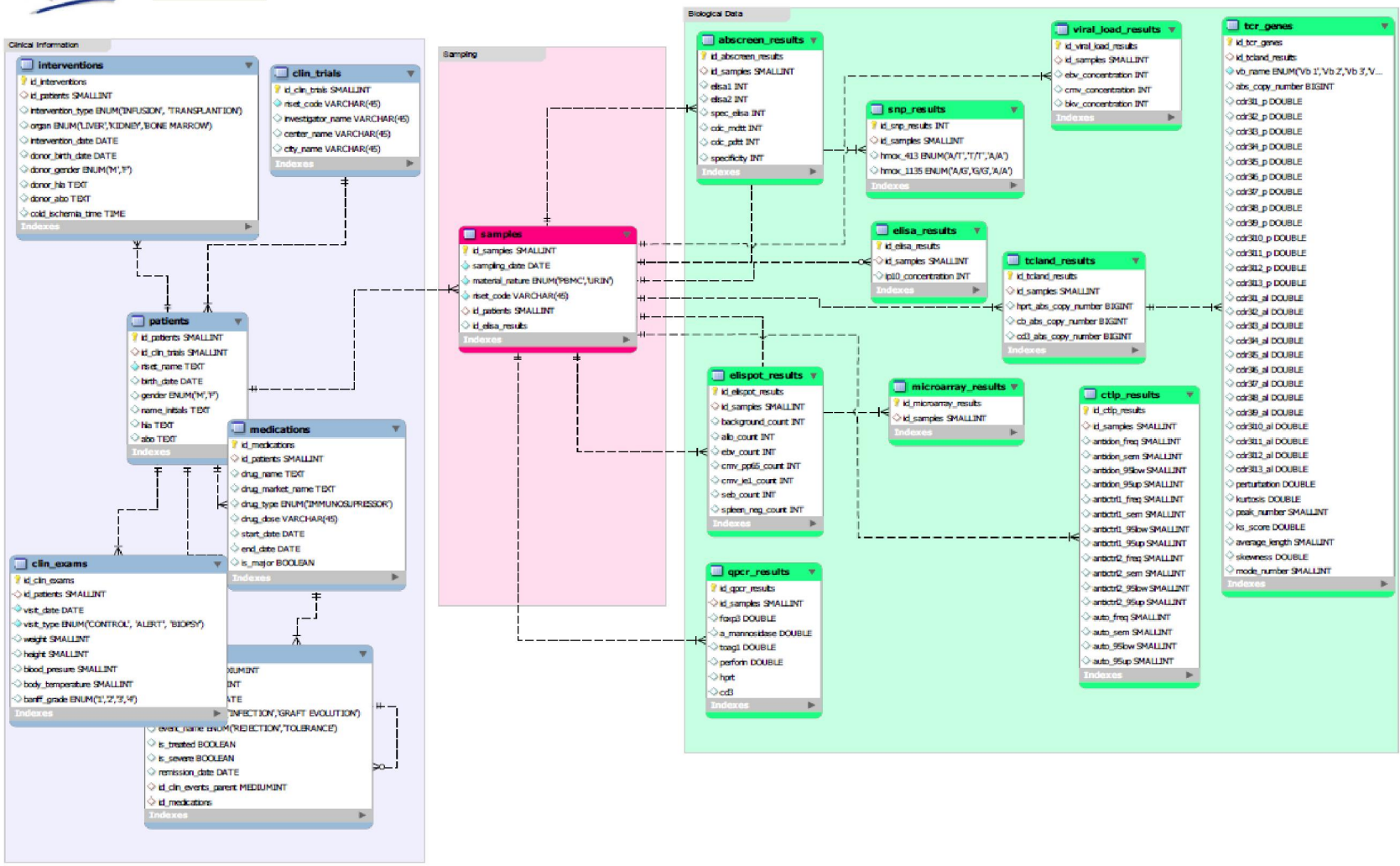
A tool enabling sample tracking on the RISET website has been developed and proved its usefulness for the management of multicentre clinical studies. This application was built on the EditGrid resource: a web-based online spreadsheet which allows creating and sharing spreadsheets documents and macros. For the last collective shipments, each clinical partner was invited to log on the private RISET page of the EditGrid website. This page contains a form dedicated to name and specify the characteristics of each sample. The assay that will be performed and the laboratory that will receive the sample are also mentioned. In order to avoid typos, the interface with as much listbox and button as possible was designed. Because partners share the same resource on the Web, the flow of samples between partners is easy to visualize and check.

Screenshot of the web-based tool dedicated to the sample exchange process



A Data Repository stores the clinical data and immunomonitoring results obtained with the tests. This Data Repository is a unique resource on the follow-up of patients in tolerance investigation studies. A longitudinal visualization tool, the LifeLines program allows representing both clinical and experimental data in a unified way and investigating the relationship between clinical events and test results in order to support the validation of biomarkers.




Version 0.2 (22/04/2009)
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donor_abo TEXT
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abo TEXT
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perforin DOUBLE
hprt
cd3
Indexes

To analyse a common set of tolerance genes in different animal models, partners involved in RISET experimental studies have set up different tools to study the function of these molecules

(1) Dkk3 specific antibodies were generated and administration of anti-Dkk3 antibody was shown to increase the rejection rate of tissue transplants in a model of transplantation tolerance mediated by CD8+ Treg cells.

(2) EBI3 and IL27p28 protein expression was analyzed in different rodent models of tolerance and in human renal allograft biopsies and 70 pre-transplant kidney biopsies.

(3) TORID antibodies were generated.

(4) For the characterization tolerogenic DC, models of generation of tolerogeneic DCs (by Dexamethasone, aspirin, C1q, TLRL treatments, low GMCSF culture) were developed.

(5) For the study of pre-clinical models in the mouse, humanized transgenic animals were generated to test specifically therapeutic properties of anti-CD52 and anti-CD3 engineered antibodies. However, for the humanized models of CD52 mice, homeostatic expansion was observed, so blockade of IL7 is under investigation to refine this model. Additional confirmatory experiments completed on mechanism of anti-IL7R blockade synergy with anti-CD52-mediated depletion for tolerance induction to allogeneic skin grafts. The therapeutic potential of a human CD3 antibody has been demonstrated.

Ethical recommendations providing guidelines on pilot clinical studies for tolerance induction were published in various places

Based on the analysis of the literature, internal debates within the consortium and discussions with regulatory authorities and local ethics committees, the RISET ethics team produced a set of ethical recommendations on pilot clinical studies for tolerance induction. These guidelines were published in series of Books and Journals. 9 posters were produced covering various aspects of the ethical debate and communicated in international meetings such as the EFI. In particular, the ethics team focused on 2 topics. First, on biomarker validation and qualification – a series of recommendations about biomarkers validation conditions, the use of biosamples for research activities, ethnic specific biomarkers, the benefit/risk balance, the avoidance of hype and the information to the patients and the general public were published. Second, on the inequality of access to transplantation and the novel accompanying therapies across Europe - recommendations were developed that insist on the necessity to develop the ethical dimension related to the translation of novel treatments accompanying transplantation, on the role of the national transplant centres to facilitate access to these innovative treatments, as well as on the need to further assess the economic impact of these treatments.

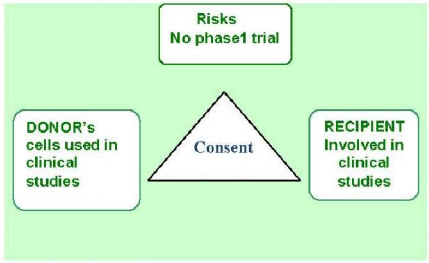
Recommendations proposed by an EU consortium regarding ethical aspects of pilot clinical assays for tolerance induction in transplantation

V. Commin, A. Cambon-Thomsen on behalf of the RISET FP6 EU integrated project
cambon@cict.fr, Epidemiology and public health analysis, Inserm U 558, Toulouse, France

Immunosuppression is needed to control rejection. It is efficient on the short term but has drawbacks for long term transplantation outcome and secondary effects. Tolerance induction could be a solution.

Clinical studies for tolerance induction in transplantation:
Performed within a **European research programme consortium: RISET (Reprogramming Immune System for the Establishment of Tolerance).**

Deal with tolerance induction protocols through donor cell infusion into the recipient, associated to the organ or haematopoietic stem cells transplantation from a living donor.



Ethical & legal framework:
- **European Medicine Agency** (EMEA) guideline on human cell-based medicinal products (2007)
- **Council of Europe:** the provisions of safe products meeting high quality standards (2004)
- **Council of Europe:** Recommendation for research using biological material of human origin (2006)
- **EU directives** :
  - ✓ Clinical trials (2001/20/EC),
  - ✓ Tissues,cells (2004/23/EC, 2006/17/EC, 2006/86/EC)

Specific dimensions are:

The issues below are not individually new, but their association is unique. On account of the overlap between the therapeutic act and the research protocol it may be difficult for the patient to distinguish between the two acts.

Extract of the handbook of 10 recommendations related to these specific dimensions:

➡ **clinical researchers, ethics committees, health authorities.**

Information and consent related to:

Risks

Assessment of risks/benefits balance is tricky;
- ✓ Loss of a transplant may happen in a short period whereas the benefits of diminishing immunosupression are long term.
- ✓ There is no phase I assay on healthy volunteers

How to deal with information about adverse events when assessing risks/benefits balance ?

Both recipient and donor involvement

These tolerance induction protocols involve both donor and recipient:
- ✓ Need of an adequate information for both of them **before beginning the trial**
- ✓ Right to withdraw of the donor versus the right to participate of the donor in case of iterative donor cell procurement

How to deal with an adequate information and consent?

Risks/ Benefits balance

Recommendation 1: That for tolerance pilot studies a **sufficient time period** is maintained between the enrolment of each patient in order that possible adverse events observed can be taken into account before the next patients are enrolled.
Discussion: how to define "sufficient time"? Criteria used may vary but should be explained when asking for ethical approval.

Recommendation 3: That **specific research for constructing tools** for **assessing risk/benefit balance** in such protocols should be set up at European level.

Adequate information and consent

Recommendation 4:
- a. That **independent informed consent** for the research should be given both by donor and recipient
- b. That both donor and recipient should be informed that the other, in addition to consenting to giving (receiving) the transplant, is also asked for consent to participate in the research part of the procedure including cell therapy
- c. That for such protocols an **extensive dialogue** with the donor and the recipient should be required with **specific information** provided about the study **that cannot be or is not included** in **general hospital information leaflet**
- d. That both donor and recipient should have the possibility of several days of reflection before the consent signature.

Recommendation 7:
- a. That donor and recipient should be informed of the **peculiarities relating to the right to withdraw** from a tolerance induction study, since it can have an impact on the follow up of the tolerance induction for the transplant recipient
- b. That, for the recipient, information concerning the right to withdraw from the trial at any time should be given, as usual
- c. **That while maintaining the principle of withdrawal, the donor, when relevant to the protocol, should be informed about his/her responsibility towards the recipient who would, as a result no longer be able to continue to participate in the clinical tolerance** study in case of donor withdrawal.

Other recommendations

Deal especially with information content, education of personnel, return of results, medical and psychological follow up.

This set of specific recommendations for ethics in tolerance induction clinical pilot studies has been validated within the RISET consortium (http://www.risetfp6.org/). It is currently being circulated for comments to various experts, committees, institutions and other stakeholders, prior to an open consultation and then its publication.

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Cambon-Thomsen, cambon@cict.fr

Riset website: www.risetfp6.org/

Riset Inserm

The RISET ethics team linked up with the regulatory authorities and was acknowledged as an interested party by the Committee for Advanced Cell Therapies

Regular updates were provided to the RISET consortium about EU and other international legal and ethical aspects relevant to the field of transplantation. These were presented and discussed with the consortium. RISET views were communicated to 16 public consultations issued by the EMA.

Understanding the way transplanted patients and the general public perceive tolerance induction

Understanding the way transplanted patients and the general public perceive tolerance induction revealed that patients are familiar with the main characteristics of immunosuppressive therapies, but their knowledge about the side effects of these therapies is rather limited. Similarly, their understanding of tolerance induction treatments is generally mistaken. Ten factors were identified to explain the motivations underlying their acceptance or their refusal to participate in clinical studies in this field. Results of these studies were communicated to the EFI Conference in 2010.

II. DISSEMINATION AND USE

II.1 Exploitable knowledge and its Use: description of results, perspectives and potential commercial applications

Understanding tolerance mechanisms

- ✓ *Analysis of the tolerogenic properties of DCs in vitro and in vivo*. The results obtained in this study are very important and highlight the risk of using "tolerogenic" allogeneic DCs for clinical therapy to induce tolerance. KCL has data in the rat, mouse and human to demonstrate that drug-treated DCs have a tolerogenic phenotype and function in vitro. However, once injected in vivo while in the rat they can induce tolerance to a kidney allograft, in the mouse they are very quickly reprocessed and represented in a stimulatory manner by endogeous APC. The prediction is that recipient DCs pulsed with alloantigens injected i.v. or locally can induce tolerance.

- ✓ *Targeting of DC subsets directly in vivo to induce tolerance* as an alternative strategy to negative vaccination with tolerogenic DCs. This strategy has been very successful. Importantly, one subtype of DC (33D1) that by targeting with alloantigens lead to deletion of alloreactive T cells has been identified.

- ✓ *Analysis of regulatory T cells as cell therapy* to induce transplantation tolerance. We have evidence that regulatory T cells can induce indefinite survival of an allograft in murine models of heart transplants. Murine Tregs with allospecificities are more efficient than polyclonal Tregs. In the human system we have addressed a series of questions in vitro related to Treg therapy using polyclonally activated Tregs. We have established the advantage of using Rapamycin to maintain Treg phenotype and function. We have now

started to expand in a more consistent manner Tregs with allospecificities to be tested both in vitro and in vivo in humanised mouse models. If this strategy is feasible clinically to induce tolerance, new constructs need to be made to target in vivo human DC subsets in transplanted patients.

- ✓ *Low molecular weight dextran sulfate facilitates long-term graft acceptance in an animal model with prolonged cold graft ischemia*. Ischemia / reperfusion injury, initiated by cold graft ischemia, seems to be a limiting factor for immunological tolerance induction. Its prevention by the use of low molecular weight dextran sulfate – or possibly also other substances which protect the graft endothelium during the early post-transplantation period – offers a clinical perspective to immunological tolerance induction strategies.

- ✓ *Low molecular weight dextran sulfate prevents TLR4-induced phenotypic and functional maturation of human dendritic cells*. These findings suggest that DXS prevents TLR-induced maturation of human DC and may therefore be a useful reagent to impede the link between innate and adaptive immunity. Patenting of the application of DXS to prevent maturation of dendritic cells was evaluated but not pursued since the substance itself (low molecular dextran sulfate) cannot be patented and a pure application patent was not seen as an option.

- ✓ *Expression and function of C1q in tolerogenic DC* provides new insight into the functions of C1q and other molecules of the innate immune system. Although initially associated with proinflammatory processes, it has now become clear that especially C1q also has an immune regulatory function.

- ✓ *The potential of Dexamethason to alter the function of professional antigen presenting cells* and to promote their tolerogenic function. Dexamethason is at present one of the well established pharmacological agents which is able to alter the function of dendritic cells. In our hands, the functional modulation is stronger and more stable than when VitD3 is used.

- ✓ *Anti-GITRL antibodies can promote tolerance* in autoimmune disease: the findings suggest that DXS prevents TLR-induced maturation of human DC and may therefore be a useful reagent to impede the link between innate and adaptive immunity.

- ✓ *Exploiting lymphocyte depletion by using anti-IL7R antibodies* to favour regulation is an important finding given the wide use of CAMPATH-1H (alemtuzumab) in transplantation.

- ✓ *Identification of a role for Treg within accepted tissue transplants* should open up research avenues to enhance that local benefit without a too great impact systemically.

- ✓ *Tm4 family members expressed in Treg* cells offer an explanation of how a low affinity TCR can still signal for regulation, and how Treg are responsive to low levels of antigen.
- ✓ *Treg interact with tissues to enable their protection*. A search for substances mediating these effects may lead to drugs which can enable their expression.

- ✓ *Establishment of a transgenic mouse autoimmune strain expressing the ε chain of the human CD3/TCR complex in an autoimmune genetic background*. The humanized mouse model described may be of use in a variety of situations to device strategies to induce immune tolerance not only in transplantation and autoimmunity but also in the context of regenerative medicine (i.e. cell therapy approaches).

- ✓ *Modalities of the induction of transplantation tolerance to fully mismatched islet allografts using CD3-specific antibodies*. This demonstrates that the tolerogenic ability of CD3-specific antibodies is totally independent from their intrinsic characteristics. It also confirms that pathophysiological mechanisms mediating autoimmune and alloimmune destruction of target tissues are similar and may ultimately be controlled by identical T cell-targeting modalities. These findings point to the importance of the therapeutic window that stresses the unique ability of CD3-specific antibodies to exert their effect in primed hosts harbouring. This also indicates that activation state of functionally distinct T cell subsets involved in the ongoing immune reaction is a key determining factor to promote the CD3 antibody-mediated immune tolerance.

- ✓ *Induction of transplantation tolerance to mismatched skin allografts using CD3-specific antibodies* (see above). In this model, the skin grafts are performed across a minor incompatibility mismatch that is H-Y which allows tracking alloreactive T cells. From a fundamental perspective, as compared to the islet transplant model, this will allow a more precise evaluation of the impact of CD3-specific antibody treatment on this key effector population.

- ✓ *Use of combination therapy using CD3-specific antibody and syngeneic bone marrow dendritic cells to induce transplant tolerance*.

- ✓ *Characterization of CD8 regulatory T cells in a mouse model of transplantation tolerance*: neonatally induced dominant CD8 T cell tolerance remain stable under lymphopenic conditions also in the presence of systemic inflammation induced by TLR ligands. However, when lymphopenic recipients are irradiated, the tolerant status is lost, as CD8 T cells acquire effector functions in an IL-15-dependent fashion.

Developing specific tools to measure tolerance *in vitro* and *in vivo*

- ✓ *Protocol for sorting and expanding human T regulatory cells with high suppressive capacity in vitro and in vivo*. This could be exploited as a proof of concept of the effectiveness of *ex vivo* expanded human Treg cells *in vivo*. The production of reagents, equipment for the isolation and expansion of T cells may be explored. The development of a strategy to sort and expand highly pure and highly suppressive human Treg would allow future application in the clinic as a cellular therapy. Using Treg cells in the clinic would allow minimizing immunosuppressive drug usage and thus avoiding side-effects of pharmacological immunosuppression.

- ✓ *Humanized mouse models of transplant rejection*. This model is useful for testing different population of human Treg before clinical implementation. It

maybe also used for testing various tolerising strategies. This would give an ethically approved way of assessing effectiveness and safety of human Treg in an in vivo context, thus leading to quicker and safer development of Treg-based clinical protocols. Collaborations with SME and larger pharmaceutical companies to test novel therapeutic agents in the humanised mouse model are being explored.

Identifying novel biomarkers of tolerance induction in DCs and in Tregs through gene analysis

- ✓ *CD103, TORID, TGFbeta and IL27* were found to be up-regulated in tolerant grafts and/or Treg cells. They are thus considered as possible markers of immune regulation. Mouse Treg cells over express a small group of genes that may be potential markers of immune regulation. If validated a gene expression profile associated with transplantation tolerance could be the subject of a patent and the test commercialised.

- ✓ *Identification of marker molecules for regulatory CD8 T cells*. Among the 31 differentially up-regulated gene products in regulatory CD8 T cells, 8 were represented (Iba1, LYL, CD39, beta Ig-H3, Mirf5, TGFβ1, Mirf7 and CD103) within the 12 most commonly found candidate genes of all RISET partners. These data were confirmed by Miltenyi Biotec when gene expression of these regulatory CD8 T cells was compared to the one of regulatory CD4 T cells.

- ✓ *Generation of tools for analyses of the selected candidate molecules*: since no antibodies against the selected candidate proteins EBI-3 and Dkk3 were available at the time of selection, various recombinant mouse and human proteins were generated and used to immunize the respective knock-out mice to establish monoclonal antibodies. In addition, Hes 1 containing recombinant retroviruses were established.

- ✓ *Functional evaluation of candidate gene products as possibly suitable biomarkers*. An involvement of EBI-3 and Hes1 in the induction and maintenance of tolerance could not be detected in the test systems used. However, Dkk3 as novel immune modulator, is essential for the observed tolerance mediated by the regulatory CD8 T cells. It does not interfere with T cell activation in the peripheral lymphoid compartments, limits the number of IFNg producing CD8 T cells locally in the brain during EAE, directly acts on CD4 and CD8 T cells (reversibly attenuating T cell responses), and is involved in MAP kinase inhibition in T cells.

- ✓ *B cell ELISPOT* is a new approach which may allow the detection of humoral sensitization with risk of humoral rejection at an early stage, preceding the emergence of serum HLA antibodies. Participation in this part of the project has helped ProImmue demonstrate that its technologies are not only relevant for measuring T cell immune responses, but that they can also play part in measuring other important adaptive immune responses that are relevant in transplantation. Results for the B cell ELISPOT are promising and the technology as such offers an understanding of B cell responses in transplantation that is at a significantly more detailed level compared to simply detecting serum antibodies in standard immune assays such as ELISA. ProImmune has recently pushed into the area of measuring B cell immune responses, by launching services for B cell epitope prediction and B cell epitope mapping. We anticipate that publication of the results from the B cell ELISPOT development will help promote our efforts in this area.

- ✓ *Extensive immunophenotyping with 12-colour facs*: immunophenotyping lead to interesting results to be combined with other biomarkers

- ✓ *CD107 expression as an alternative for CTLp*: CD107 expression is not a suitable alternative for the CTLp as the sensitivity is not comparable.

- ✓ *Foxp3 TSDR analysis of whole blood or PBMC samples* of patients enrolled into RISET clinical studies aiming on the active induction of tolerance or enrolled into drug weaning trials might be an additional test suitable to detect operational tolerant patients and thus enhance safety of such clinical trials. However, the efficacy to detect operational tolerant patients and discriminate them from patients with an ongoing pathogenic immune response has to be first tested in retrospective clinical trials. Furthermore, performing a TSDR analysis as a quality control step of Treg batches prior to in vivo transfer will ensure efficacy and safety of cell transfer approaches. TSDR analysis as such is protected by a patent of Prof. HAMANN (Charite) and Epiontis GmbH. It has been licensed to Charite for developing qPCR (quantitative TSDR analysis) in human samples.

- ✓ *Performance of Class II MHC Ultimers* for the indirect pathway lead to a better understanding of MHC multimer technology and to the validation of MHC Ultimers at least for staining of control responses. Having validation data for MHC Ultimers was important for ProImmune in terms of promoting this product line as a whole and therefore it has had a positive impact for ProImmune across many business areas.

- ✓ *Identification of new HLA binding peptides derived from HLA class I molecules and binding to HLA – DR molecules*. The new binding peptides identified could play a part in the indirect pathway response, subject to further validation in functional assays. This work has contributed for ProImmune to the validation of data for the REVEAL system. REVEAL is the only full service epitope discovery system for determining new relevant T cell responses available anywhere in the world.

- ✓ INF-γ ELISPOT to monitor anti-viral responses in transplanted patients. Influenza vaccination is not associated in renal allograft recipients with de novo production and/or increased titers of anti-HLA antibodies (HLA-Ab) and therefore can be performed safely in regular clinical practice. This study allowed U.DESCARTES to set up anti-influenza ELISPOT assay that are presently used to monitor allograft recipients.

Biomarkers validation and qualification

- ✓ *12 tests were validated methodologically* (reproducibility, coefficient of variation, sensitivity and specificity) and applied to the biosamples collected from the clinical studies: HLA-DR expression on monocytes, Urinary viral load (BKV/Polyoma), HLA alloantibody detection (ELISA, CDC, Flow cross match) , TCR landscaping, Gene expression profiling (PCR /cDNA-Array) , CTLp frequency, Multiplex cytokine assay, DNA polymorphism, IP-10 (CXCL-10) level in urine, ELISPOT - allospec. TH1, CMV/EBV spec. T-cells (ELISPOT), ELISPOT IFN-gamma. The allo-ELISPOT was first time used for guiding early CNI-avoidance protocols following kidney transplantation.

- ✓ *Cross-validation of the CTLp frequency assay to monitor anti-donor-specific alloreactive responses in transplanted patients*: CTLp assay is a reliable parameter for monitoring the immune response of recipients of living related renal allografts versus donor and third party antigens. This method appears promising as part of diagnostic monitoring tools applied to perform tailor-made drug minimization studies. This assay is now used in U.DESCARTES to direct drug minimization in individual patients presenting particular conditions (i.e. development of tumours or of severe infections) and in the context of prospective protocols.

- ✓ *Gene expression profiling by qPCR test*. It has been discovered that Toag-1 is a marker for quantifying the summative activation/silence balance of adaptive immunity in general, and better understanding the regulatory mechanisms behind. Toag-1 and aMann expression analysis is protected by a patent and licensed to Milteniy Biotec.

- ✓ *TcLandscape analysis of longitudinal samples from patients enrolled in several clinical trials*. It allows a better understanding of complex immune responses. The diversity of protocols and organ transplanted (bone marrow, kidney and liver) gave all its potential to the TcLandscape. It has also allowed developing longitudinal data analysis procedures. TcLand succeeded in applying their technology in different areas in transplantation research. TcLand will pursue the validation of the test and will propose it as a service of the company.

- ✓ *Description of a cross-platform set of biomarkers of tolerance in kidney transplant recipients*: drug-free tolerant patients display an expansion of peripheral blood B and NK lymphocytes in the absence of donor-specific antibodies. Additionally, direct pathway donor-specific hyporesponsiveness by IFNγ ELISpot and a high ratio of FoxP3/α-1,2- mannosidase expression were observed in drug-free patients. Using the customized Agilent RISET 2.0 microarray focused on immune-related genes, we identified a series of them whose expression on whole blood allowed the identification of 100% of the tolerant recipients of the training set and 93% in the test set. When using cross-platform biomarkers on patients we were able to improve the diagnostic features on the test set. This validated 'tolerant fingerprint' can now be used to shape drug weaning protocols of kidney transplant patients. This set of biomarkers needs now to be tested in patients still receiving immunosuppression.

Early immunosuppression withdrawal/mimimization after cadaver liver transplantation using T cell depleting therapy and rapamycin

- ✓ Resistance of memory T-cells to ATG-mediated T-cell depletion: these results confirmed previous data when using high doses ATG. They indicate the limits of this strategy for tolerance induction in clinical setting.
- ✓ Clinical tolerance to high doses ATG in the immediate period after liver transplantation. Memory T-cells as mediators of acute rejection in lymphopenic environment. Potential to use such regimen for further tolerance protocol in organ transplantation (i.e. in combination with donor cells infusion).
- ✓ Pre-transplant level of Toag-1 gene as a predictive marker for rejection after liver transplantation.

Use of unresponsive anergised interleukin (IL-) 10 driven T lymphocytes of donor origin in recipients of allogeneic bone marrow

- ✓ Cell therapy with IL-10 DLI after haploidentical HSCT is safe and feasible. IL-10 DLI provides immune reconstitution without severe GvHD after haploidentical HSCT. The proposed cell therapy is ready to be used in multicenter studies for the treatment of cancer patients and of patients with genetic hematologic diseases in the need of allogeneic HSCT, not only from haploidentical donor but also from matched unrelated donors. Importantly, the cell therapy protocol with IL-10 DLI can also be extended to prevent rejection after organ transplants.

- ✓ Signs of long-term tolerance induced by IL-10 producing Tr1 cells in IL-10 DLI treated and informative patients. The discovery of in vivo biomarkers that can be related to tolerance induced by IL-10 would represent a major step towards the application of new therapeutic intervention. Results need to be validated in the patient samples and cross-validated in different biological systems.

Using a combination of anti-CD3 and anti-CD7 immunotoxins to induce long-standing remission of severe high grade graft-versus-host disease

- ✓ Results confirmed that the immunotoxin combination, nor its components, induce cytokine release when incubated ex vivo with PBMC. Moreover, it has been confirmed that the immunotoxin combination appears to be safe in a monkey sub-acute toxicity study. Currently attempts are being made to find investors to restart the IT-combination project and to perform the Phase I/II study in GVHD.

Using monocyte-derived Transplant Acceptance Inducing cells (TAIC) of donor origin in recipients of allogeneic kidney

- ✓ The TAIC studies pioneered cell-based immunotherapy in transplantation, teaching us a great deal about the clinical practicalities of such procedures. We expanded our understanding of human M regs, an important suppressor macrophage population. Mreg infusion resulted in up-regulation of the immune "silence" marker Toag-1. Blasticon GmbH holds the intellectual property rights to the clinical application of human regulatory macrophages in transplantation and the treatment of autoimmune diseases.

Pilot parallel prospective monocentric trial to evaluate the efficacy of an immunosuppression regimen based on an induction therapy combining anti-CD52 (CAMPATH-1H) and anti-TNF monoclonal antibodies followed by a maintenance regimen based on either tacrolimus or sirolimus

- ✓ Alemtuzumab (Campath 1H) and infliximab induction followed by tacrolimus monotherapy is associated with normal histology and excellent graft function at 12 months. Results should be validated in larger prospective trials.
- ✓ HOX-1 gene polymorphism has no significant impact on the outcome of renal transplantation.
- ✓ Specific molecular phenotypes of acute rejection predict the outcome of kidney transplantation. More work planned in chronic antibody mediated rejection.
- ✓ These are potentially new biomarkers for results prediction. Yet they are not ready for commercial use.

Analysis of the T cell repertoire can be useful to understand graft outcome

- ✓ Data suggest that a classification based on the shape of the T cell repertoire could be a tool to score the severity of chronic humoral rejection. The shape of the T cell repertoire could constitute a valuable parameter to assess the graft outcome, to guide the medical management of patients with chronic rejection and to warrant long-term follow-up of stable patients with altered T cell repertoire.

Analysis of the involvement of molecules overexpressed in blood from operationally tolerant patients (SMILE or TMTC3, a 104 kD protein containing tetratricopeptide repeats whose function is still unknown).

- ✓ Findings suggest a role for SMILE in stress resistance, likely via enhanced capacity of SMILE overexpressing cells to degrade proteins.
- ✓ Increasing evidences suggest the implication of ER stress in mediating allograft injury. Learning more about this molecule may provide a novel framework for further strategies to discover new pathways of regulation of graft acceptance and to increase our understanding of tolerance.

Development of validated analyses of renal transplant biopsies to identify local markers of tolerance and to predict the development of fibrosis

- ✓ IL-17 protein in renal allograft upon acute rejection correlates with inflammation, fibrosis and poor renal outcome. To the best of our knowledge, this is the first report concerning IL-17 producing cells and renal allograft outcome. We found that IL-17+ infiltrates upon acute allograft rejection correlate with tubulitis, interstitial inflammation and interstitial fibrosis. However, no relationship between IL-17+ infiltrates and response to conventional anti-rejection therapy was observed. Interestingly, there was a correlation with poor renal outcome at 6 months after an episode of rejection and return to dialysis. A striking finding in our study is that only few intragraft TH17 cells, defined as double positive cells for IL-17 and CD3, can be found upon acute rejection. In contrast to the small amounts of TH17 cells, many IL-17+ cells are in close contact with CD3+ cells and show double staining for either neutrophils or mast cells.
- ✓ HMOX Intragraft tubular vimentin and CD44 expression correlate with late renal allograft function and interstitial fibrosis/tubular atrophy. Treatment with P/CsA or P/everolimus allows boosting of T-cell dependent and T-cell independent secondary humoral responses. P/everolimus treatment does not even affect the primary response.
- ✓ Although still not optimal, better tools for immunological monitoring of transplant patients can be developed that will facilitate our clinical decision making.

Minimization of immunosuppression in renal allograft recipients

- ✓ We found that recipients prone for acute rejection had a higher precursor frequency of alloreactive CD8+ T cells and a lower percentage of IL-7Rα expressing CD8+ T cells after allospecific stimulation than non-rejectors. These data point to quantitative and qualitative differences between T cells of patients who will experience acute cellular rejection episodes from those who will not.
- ✓ Treatment with P/MPA completely disturbs primary and secondary humoral responses. Treatment with P/CsA or P/everolimus allows boosting of T-cell dependent and T-cell independent secondary humoral responses. P/everolimus treatment does not even affect the primary response.

- ✓ Identification of clinical variables associated with the development of allograft tolerance in liver transplantation. IDIBAPS has determined that, among various criteria, the time elapsed since transplantation, the age of the recipient and the type of immunosuppression employed are associated with the feasibility of successfully withdrawing immunosuppressive therapy in liver transplantation, and that this phenomenon is much more prevalent than originally estimated at later time points after transplantation. The achievement of allograft tolerance allows for the discontinuation of immunosuppressive drugs, and can therefore substantially diminish the morbidity and mortality associated with the chronic use of these drugs.

- ✓ Identification of transcriptional biomarkers capable of predicting the outcome of immunosuppression withdrawal in stable liver transplant recipients. IDIBAPS has indentified a series of genes whose expression is associated with the success of immunosuppression withdrawal in liver transplantation, and that can be employed therefore as a diagnostic test of tolerance. The validation of a diagnostic test of tolerance will have a major impact in liver transplantation by allowing the discontinuation of immunosuppressive drugs from a sizeable population of liver recipients. IDIBAPS has applied for 2 patents to protect the intellectual property associated with our findings and has signed a licensing agreement with TcLand Expression SA for the commercial exploitation of the results.

- ✓ Identification of transcriptional biomarkers associated with rejection in stable liver transplant recipients undergoing immunosuppression drug withdrawal. We have analyzed liver biopsies and peripheral blood samples collected from tolerant and non-tolerant recipients at different time-points during the immunosuppression withdrawal procedure employing microarray gene expression profiling to investigate which pathways are associated with the rejection response. This has resulted in the identification of a number of genes whose expression is associated with rejection in blood and liver tissue. Interestingly, the changes in the blood expression levels of some of the genes associated with rejection occur weeks before liver function is disturbed, suggesting that these markers could have utility as non-invasive diagnostic biomarkers of liver graft rejection. This will contribute to a better understanding of the overall rejection response and could constitute the basis for a future patent application.
- ✓ Identification of the molecular pathways associated with tolerance in liver tissue. We have analyzed liver biopsies collected from tolerant and non-tolerant recipients before immunosuppressive drugs are discontinued to investigate which pathways are associated with the tolerance phenotype. This has resulted in the identification of iron metabolism genes as being closely related to tolerance in human liver transplantation. This finding opens a completely novel approach in the field. Understanding the pathways associated with tolerance in liver transplantation at the level of the graft is critical in order to be able to increase the proportion of recipients who can attain this state.

- ✓ IFN-gamma ELISPOT cross-validation. Using the same protocol, 3 different labs have been able to show similar results of different allostimulation experiments from healthy volunteer blood samples in blinded interlab cross-validation . Also, stimulation against CMV and EBV peptides has shown very similar responses among the 3 groups. However, it s important to note that shipment of fresh blood samples preserved in ice is not recommendable for routine use so far as the cells seem to easily die. Moreover, frozen samples from the same individual seem to lose some amount of response (<10%) as compared to a fresh sample. Noteworthy, negative controls (PBMC co-cultured with medium alone) show less background when they have been frozen than when dealing with fresh samples, probably because of recent mechanical manipulation of these cells. Fortunately, the cross-validation data show that the data are quite comparavle if tested at different centers meaning a core unit is not permanently necessary (only for reglar quality control) This is a potentially useful assay to be used in transplant recipients in order to assess their alloimmune risk of cellular-mediated rejection and the need of high-dose immunosuppression (or avoidance of weaning protocols). This assay needs further collaboration between labs in order to continue its validation for being used in the clinical practice among transplant patients – a commercialization is aimed.

- ✓ Development of clinical protocols for the generation of therapeutic human T reg preparations. These studies revealed that the lineage stability of Treg cell populations is in parts determined by the DNA methylation state of Treg-specific enhancer regions. DNA methylation at such sites results in loss of FOXP3 expression preferentially in antigen-experienced (CD45RO+) Treg cells (Hoffmann et al, Eur J Immunol. 2009 Apr;39(4):1088-97). In contrast, CD45RA+ Treg cells remain demethylated at those sites and thereby maintain a stable expression of FOXP3 even after extensive in vitro expansion (Schmidl et al, Genome Res. 2009 Jul;19(7):1165-74; Hansmann et al, Eur J Immunol. 2010 Mar 3. [Epub ahead of print]). Based on this detailed characterization of Treg cell subpopulations, the CD45RA+ Treg cell population seems the ideal starting population for the generation of homogeneous in vitro expanded Treg cell products.
- ✓ For the development of GMP-compatible Treg isolation and expansion protocols, extensive preclinical studies have been performed (funded from other sources). These include the comparative analyses of different T cell stimulation reagents, GMP-compatible media and serum sources, re-stimulation intervals and intensities and the development of quality control assays. Furthermore, multiple cell isolation strategies aiming at the purification of the rare CD45RA+ Treg subpopulation have been tested in collaboration with Miltenyi Biotec GmbH and BD Biosciences. These include magnetic and flow cytometric cell separation technologies.

- ✓ It is anticipated that approval for the production of human T regs for treatment of solid organ transplant patients will be given by Spring 2011. At this time, we will be ready to proceed with the proposed study of T regs as a tolerance-promoting therapy in liver transplant recipients. This work holds enormous promise for transplant recipients as it may, for the first time, allow for a reliable tolerance induction without the toxic conditioning regimens necessitated by alternative protocols.

II.2 Patents and licences

Discoveries	Inventors	IP Protection measures	Commercial / Industrial Exploitation
Optimization of the use of CAMPATH-1H therapy in the induction tolerance: exploiting lymphocyte depletion by using anti-IL7R antibodies to favour regulation.	H. WALDMANN, U. Oxford	IP protection request is ongoing	-
Method of determining an individual's transplantation tolerance by determining the level of a number of biomarkers. Designing a kit comprising reagents for detecting the levels of the biomarkers and to a sensor for detecting the expression levels of a plurality of genes to determine an individual's transplantation tolerance.	King's College London, Miltenyi Biotec GmbH and Charité - Universitätsmedizin Berlin	Patent application n° GB 1007454.0 filled on the 04/05/210 at the UK patent office	No licensees have currently been identified
Identification of transcriptional biomarkers capable of predicting the outcome of immunosuppression withdrawal in stable liver transplant recipients.	A. SANCHEZ FUEYO, IDIBAPS	Applications filed n° WO2008081039 and WO2010000848	1 licensing agreement has been settled with TcLAND Expression SA for the commercial exploitation of the result
TSDR analysis	Prof. Hamann (CHARITE) and Epiontis GmbH	TSDR analysis as such is protected by a patent of Prof. Hamann (CHARITE) and Epiontis GmbH. It has been licensed to CHARITE for developing qPCR (quantitative TSDR analysis) in human samples	
Gene expression profiling by qRT-PCR: Toag-1 is a marker for quantifying the summative activation/silence balance of adaptive immunity in general, and better understanding the regulatory mechanisms behind	B. SAWITZKI, H.-D. VOLK, Charité - Universitätsmedizin Berlin M. LEHMANN, K. WOOD, U. Oxford	Toag-1 and aMann expression analysis was already protected by a patent: "Immune marker fur die Diagnose und Therapie von transplantation". Patent application filed n° WO 2004/018504 A2, 04.03.2004-	The commercial exploitation of the result has been licensed to Miltenyi Biotec GmbH
Improved minimizing immunosuppression by targeting allospecific memory (campath/infliximab)	IKEM, Charité - Universitätsmedizin Berlin	IP protection request is ongoing.	
ELISPOT analysis for detecting cellular sensitized transplant patients	REINKE, VOLK, Charité - Universitätsmedizin GRINYO, IDIBELL		Collaboration initiated with Autoimmun Diagnostika GmbH (AID). ELISPOT assay will be commercialised by AID.
B-Cell ELISPOT for detecting alloreactive memory B-cells	Charité - Universitätsmedizin Leiden University Medical Centre	IP protection opportunity is currently under evaluation	
Bedside test for urinary IP-10	Charité - Universitätsmedizin		A collaboration with Miltenyi Biotec GmbH has been settled for the co-development of a commercial test

II.3 Dissemination of Knowledge

Collaborative publications are highlighted in red

Forthcoming publications

Chassang. G., Rial-Sebbag E., Cambon-Thomsen A. Les fondements de l'éthique de la recherche en droit communautaire (accepted in International Journal of bioethics)

Mahalatchimy, E. Rial-Sebbag, V. Tournay, A. Cambon-Thomsen, A-M. Duguet, F. Taboulet Access to advanced therapy medicinal products in the EU: where do we stand? To be submitted to the European Journal of Health Law

Cambon-Thomsen A., Mahalatchimy A., Rial-Sebbag E. Translating Proteomic Biomarkers Into Clinic: New Ethical Issues Or Different Perspectives On Classical Ethical Dilemma? Proteomics (submitted, August 2010, Special issue)

Mahalatchimy, A. Cambon-Thomsen "L'accès aux biotechnologies dans l'Union Européenne: mesures d'incitation et limites" European Summer School, 30 June- 8 July 2010, Toulouse: To be published in Les Etudes Hospitalières, Collection « Séminaire d'actualité de droit medical »

Van Kooten, C., G. Lombardi, K.A. Gelderman, P. Sagoo, M. Buckland, R. Lechler, and M-C. Cuturi. Dendritic cells as a tool to induce transplantation tolerance: obstacles and opportunities. 2010. Transplantation, provisionally accepted

Chantal Kuhn, Sylvaine You, Fabrice Valette, Geoff Hale, Peter van Endert, Jean-François Bach, Herman Waldmann and Lucienne Chatenoud. Human CD3 transgenic mice: a robust avenue for preclinical testing of antibodies promoting immune tolerance. (submitted)

Sylvaine You, Chantal Kuhn, Fabrice Valette, Virginia Sauvaget, Florence Porte, Nicolas Ducrot, Sabine Sarnacki, Jean-François Bach, Birgit Sawitzki, Hans-Dieter Volk and Lucienne Chatenoud Immune tolerance to fully mismatched allografts using CD3 antibodies requires delayed treatment at the time of T cell activation burst. (submitted)

S. Heidt, D.L. Roelen, Y.j.h. de Vaal, C. Eijsink, S. Thomas, H.D. Volk, M.G.D. Kester, F.H.J. Claas, A. Mulder. A novel ELISPOT assay to quantify HLA-specific B cells in HLA-Immunized individuals. Manuscript in preparation.

Sylvaine You and Lucienne Chatenoud. New Generation Cd3 Monoclonal Antibodies: Are We Ready To Have Them Back In Clinical Transplantation?
Current Opinion in Organ Transplantation (In Press)

Renaud Snanoudj, Sophie Candon and Christophe Legendre. Targeting B-Cells In Sensitized Kidney Transplant Patients: State Of The Art And Future Perspectives
Current Opinion in Organ Transplantation (In Press)

Ilias I.N. Doxiadis, Dave Roelen, Frans H.J. Claas. Mature Wines Are Better: Cdc As The Leading Method To Define Highly Sensitized Patients (In Press)

Maria-Carlota Londoño, Marta-Cecilia López, and Alberto Sánchez-Fueyo. Minimization Of Immunosuppression In Adult Liver Transplantation: New Strategies And Tools (In Press)

Golshayan D, Wyss J-C, Buckland M, Hernandez-Fuentes M and Lechler RI. Differential role of naïve and memory CD4+ T-cell subsets in primary alloresponses (2010) Amer J Transplantation (in press)

Van Kooten, C., and K.A. Gelderman. In vitro generated DC with tolerogenic functions: perspectives for in vivo cellular therapy. 2010. Meth Mol Biol. (In Press)

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Hutchinson et al. Macrophages driven to a novel state of activation have tolerogenic properties in kidney transplant recipients

Donckier et al. Acute liver transplant rejection upon immunosuppression withdrawl in a tolerance trial. Prediction by whole blood qPCR analysis

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Alcock S, Smyth LS, Sagoo P., Buckland M., Tanriver Y., Lechler^ R., and Lombardi^ G. Tolerogenic dendritic cells risk sensitisation *in vivo* due to processing and presentation by recipient antigen-presenting cells**.**

Afzali B, Mitchell P, Rani A, Khamri W, Kordasti S, Ray KP, Bamford KB, Grimbacher B, John S, Lechler RI, Lombardi G. Sustained activation of STAT3 and inactivation of SOCS3 by IL-1b leads to conversion of human regulatory T cells to Th17.

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Pre-transplant measurement of alloreactivity by multiparameter MLC-CFSE assay identifies patients at risk for acute rejection. (2010, submitted by AMC Ten Berge)

Rapid T cell repopulation after Anti-Thymocyte globulin (ATG) treatment is mainly driven by cytomegalovirus (in preparation)

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Mahalatchimy, E. Rial-Sebbag, V. Tournay, A. Cambon-Thomsen, A-M. Duguet, F. Taboulet Access to advanced therapy medicinal products in the EU: where do we stand? To be submitted to the European Journal of Health Law

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Search for the immunological signature of tolerance in liver transplantation (IDIBAPS)

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Intragraft molecular profiling of tolerant liver transplant recipients (IDIBAPS)

Ünsal Yapici[1], Joris J.T.H. Roelofs[1], Fréderike J. Bemelman[2], Jaap Groothoff3, Chris M. van der Loos[1], Karlijn van Donselaar-van der Pant[2], Mirza Idu4, Nike Claessen[1], Ineke J.M. ten Berge[2]*, Sandrine Florquin[1]* IL-17 protein in renal allograft upon acute rejection correlates with inflammation, fibrosis and poor renal outcome.

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