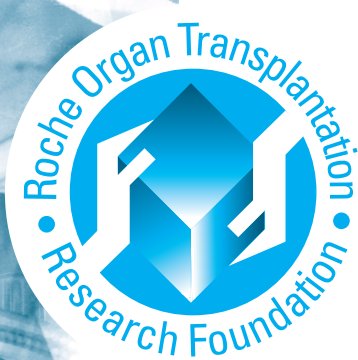




R O T R F

*Roche Organ Transplantation
Research Foundation*



BIANNUAL REPORT

October 2001



The Roche Organ Transplantation Research Foundation

The mission of the Roche Organ Transplantation Research Foundation (ROTRF) is to advance the science of solid organ transplantation in order to improve the care of the thousands of patients undergoing transplantation every year. The results of the funded research projects will contribute to an understanding of many aspects of the clinical and scientific adventure of transplantation, e.g. the mechanisms of long-term organ deterioration, the consequences of tissue injury, and the opportunities to intervene in these processes.

The Foundation is an independent medical research charity that provides operating funds to established academic staff in universities, transplant centres and research institutes. The Foundation supports research in solid organ transplantation, particularly where there is an unmet medical need.

The funding, a donation from F. Hoffmann-La Roche Ltd., provides the Foundation with 25 million Swiss francs over the first five years. The funds are disbursed as grants of up to 300,000 Swiss francs distributed over three years. The Foundation is legally independent from F. Hoffmann-La Roche Ltd. and is guided solely by the Board of Trustees according to its charter.





Table of Contents

1. Preface	7
2. Facts and Figures	8
3. ROTRF Grant Awards in Cycle VI	
<i>“Delayed Type Hypersensitivity (DTH) Status and MMF Monotherapy”</i> Dr. William J. Burlingham, Madison, USA	12
<i>“Protective Effect of A20 against Transplant-Associated Vasculopathy”</i> Dr. Christiane Ferran, Boston, USA	13
<i>“Paclitaxel and Rapamycin: A One-Two Punch Against Rejection and Cancer in Organ Transplantation”</i> Prof. Edward K. Geissler, Regensburg, Germany	14
<i>“The Immunologic Basis of Hepatic Progenitor Cell Transplantation”</i> Dr. David A. Gerber, Chapel Hill, USA	15
<i>“Dendritic Cells Transduced with the FasL Gene as a Tool to Regulate Allograft Immunity”</i> Prof. Michel Goldman, Bruxelles, Belgium	16
<i>“The Role of Plasmacytoid Dendritic Cells in the Development of Tolerance”</i> Dr. Michael D. Gunn, Durham, USA	17
<i>“Prevention of Skin Allograft Rejection by HLA-G-Modified Dendritic Cells”</i> Dr. Anatolij Horuzsko, Augusta, USA	18
<i>“VEGF: A Central Mediator in both Acute and Chronic Transplant Rejection”</i> Prof. Ian Hutchinson, Manchester, UK	19
<i>“Source of Intimal Smooth Muscle like Cells in Aortic Allograft Arteriopathy”</i> Dr. Koichi Shimizu, Boston, USA	20
<i>“Tolerance through Hematopoietic Cell Transplantation with Costimulation Blockade”</i> Dr. Thomas Wekerle, Vienna, Austria	21
4. Progress Reports of ROTRF Grantees	
<i>“Role of Costimulatory Molecules in Cardiac Allograft Rejection”</i> Dr. Marie-Luisa Alegre, Chicago, USA – Grantee in Cycle IV	23
<i>“Immunosuppression and Tolerance Induction by Genetically-Engineered Dendritic Cells Expressing Cell Surface Anti-CTLA-4 mAb”</i> Dr. Jeffrey A. Bluestone, San Francisco, USA – Grantee in Cycle I	29

<i>“Chemokine Antagonists to Block Alloimmune Responses”</i>	
Prof. Jonathan Bromberg, New York, USA – Grantee in Cycle II	34
<i>“Creating Artificial Immunoprivilege for Allograft Acceptance”</i>	
Dr. Francois Denis, Laval, Canada – Grantee in Cycle IV	37
<i>“Potentiation of Dendritic Cell Tolerogenicity with NF-κB Antagonist Phosphorothioate Oligonucleotide”</i>	
Dr. Lina Lu, Pittsburgh, USA – Grantee in Cycle II	46
<i>“Suppressing Clonotypic and Bulk Alloreactive CTL with Soluble Peptide-Loaded MHC Dimers”</i>	
Dr. Sean O’Herrin, Madison, USA – Grantee in Cycle II	49
<i>“Thymic Transplantation to Achieve Tolerance”</i>	
Dr. David H. Sachs, Boston, USA – Grantee in Cycle II	52
<i>“Phenotype and Function of Immunoregulatory T Cells Responsible for Tolerance to Alloantigens in vivo”</i>	
Prof. Kathryn Wood, Oxford, UK – Grantee in Cycle II	56
<i>“Use of Proteasome Inhibitors to Control Rejection and to Induce Long-Term Tolerance of Organ Grafts”</i>	
Dr. Jiangping Wu, Montreal, Canada – Grantee in Cycle II and IV	58
<i>“Induction of Transplant Tolerance by Antibody Against CD45RB”</i>	
Dr. Robert Zhong, London, Canada – Grantee in Cycle II	60

5. Final Reports of ROTRF Grantees

<i>“Ex vivo Induction of Regulatory Donor-Specific T cells for Immunosuppression in Transplantation”</i>	
Dr. Irma Joosten, Nijmegen, The Netherlands – Grantee in Cycle III	63
<i>“A Novel Approach for Effective Expansion of Transplanted Isolated Hepatocytes”</i>	
Dr. Ezio Laconi, Cagliari, Italy – Grantee in Cycle III	68

6. How to apply for an ROTRF grant

74

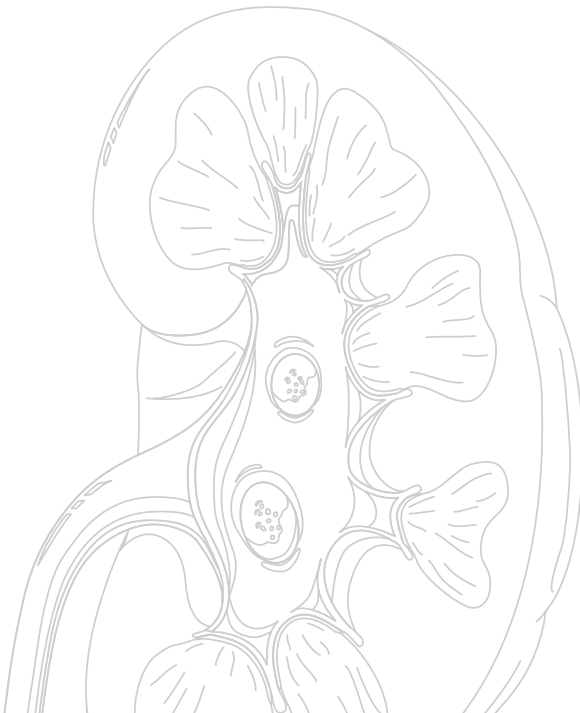


The Roche Announcement

F. Hoffmann-La Roche Ltd renews support for the ROTRF for a further three years

F. Hoffmann-La Roche Ltd announced on 6 December 2001 that it will extend the support for the Roche Organ Transplantation Research Foundation (ROTRF) for a further three years until 2006. The annual donation of 5 million Swiss francs to the independent research foundation was established in 1998 for an initial period of 5 years. This extension demonstrates a long-term commitment to research to improve the lives of thousands of patients undergoing organ transplantations every year.

William M. Burns, Head of Roche's Pharmaceuticals Division, said "Roche has been very impressed by the calibre of studies being supported by the ROTRF. The decision to extend the financial support of the Foundation reflects the commitment of Roche to the transplantation community. We are proud of our association with this worthy Foundation."





1. Preface

Summer 2001 has been a very important and successful period for the ROTRF.

The ROTRF was established by the Roche Group in 1998 as a charitable, autonomous and legally independent organisation. At that time, the Roche Group indicated its commitment to the international transplant community by providing funds of CHF 25 million for the first five years, with two funding cycles per year. The transplant community was grateful for this generous gift and responded vigorously to the opportunity.

The ROTRF has just passed the midpoint of the first five years with the completion of the sixth cycle of grant review. I am pleased to announce, on behalf of the Board of Trustees, that ten research grants with a total of 2 million Swiss francs (CHF) have been awarded to scientists around the world in this funding cycle.

The Trustees and the Scientific Advisory Committee are very pleased that the ROTRF continues to receive applications of generally high scientific quality and originality. Details of the increasing accomplishments of the researchers funded by ROTRF can be found in the lay abstracts and grant reports in this and previous ROTRF Biannual Reports. Many high-quality papers have already been published in respected international journals, and it is to be expected that the number of papers will grow considerably over the next few years.

The ROTRF is fulfilling its mission to advance the science of solid organ transplantation. It is a great and unique achievement – an international competition with an international review – and much of the credit for the success goes to the scientists sending their projects to the ROTRF and the excellent reviews of the Scientific Advisory Committee. Everybody involved can be proud of the Foundation. The new knowledge will contribute to the progress of our clinical mission.

Therefore, we, as the Trustees of the ROTRF, are very pleased that the Roche Group has decided to continue their strong commitment to the transplant community and to continue their support of the ROTRF with an additional CHF 15 million distributed over three years. On behalf of the international transplant community and the patients who have and need organ transplants, we want to express our deep gratitude to the Roche Group for this new gift and for their vote of confidence in us and in the field of organ transplantation.

On behalf of the Board of Trustees

Phil Halloran



2. Facts and Figures

Funding Cycle VI – Letter of Intent Submission in April 2001

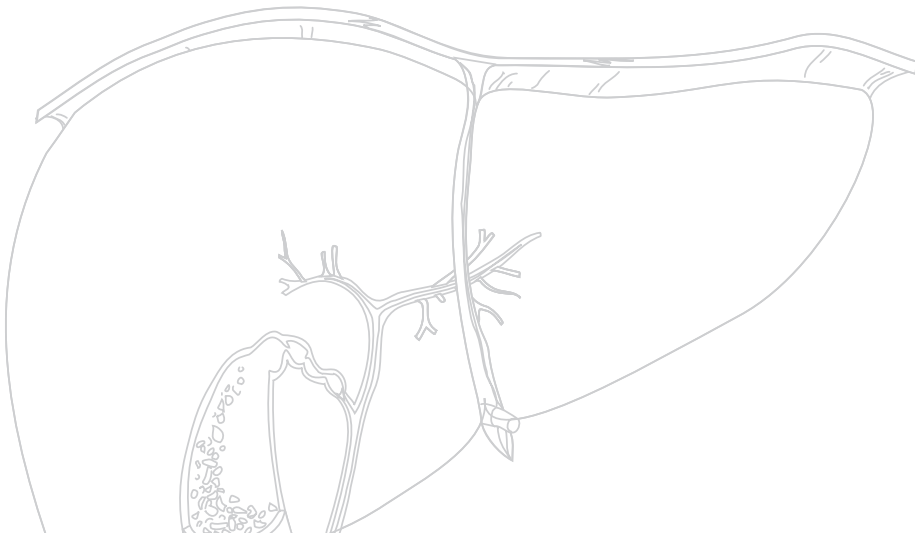
In the sixth ROTRF funding cycle, 103 letters of intent were received from scientists around the world. More than half of the applications came from the United States (51%), while 36% came from Europe, the major countries being the UK (12%) and Germany (8%). In total, North America accounted for 54% of the applicants, and Asia, Australia and South America accounted for the remaining 10% of the applicants.

The Scientific Advisory Committee of the ROTRF evaluated all the applications, which were submitted electronically via the ROTRF homepage (www.ROTRF.org), on the basis of originality and scientific excellence. The authors of the 21 most highly ranked applications were invited to submit full paper applications, and the 18 applications received subsequently underwent a thorough review by the Scientific Advisory Committee and the Board of Trustees.

ROTRF Grants were awarded to 10 applicants: six from the USA, and one each from Austria, Belgium, Germany, and the UK (see the world map on the following page).

The abstracts of the novel and promising research projects that received funding in cycle VI are presented on the following pages. Their research interests focus mainly on the following: improvement of long-term graft survival and prevention of chronic organ dysfunction; induction of tolerance; relevant immune recognition; regulation, and effector mechanisms; inflammation; and the development of new immunosuppressive agents.

In this sixth cycle of ROTRF Grant Awards, a total of two million Swiss francs was allocated.





Statistics on Applications to the ROTRF

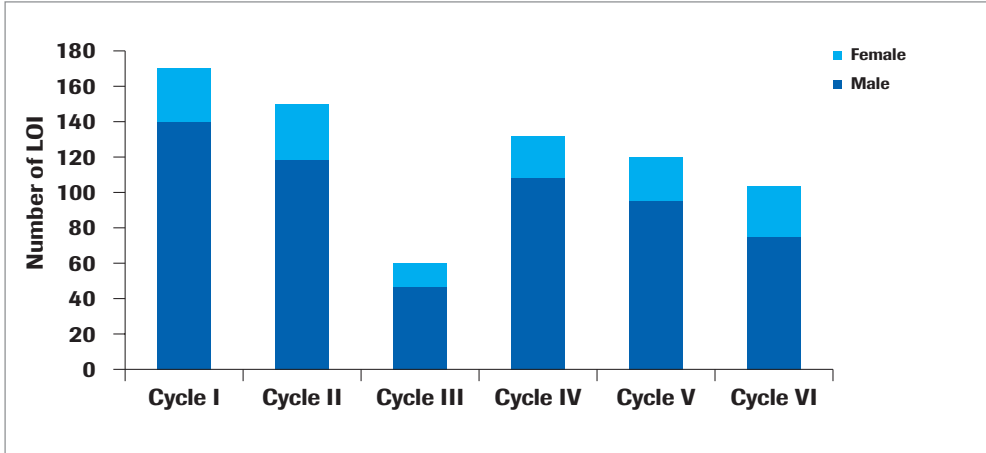


Figure 1. Number and gender of applicants who submitted letters of intent (LOI) to the ROTRF during the first six ROTRF funding cycles.

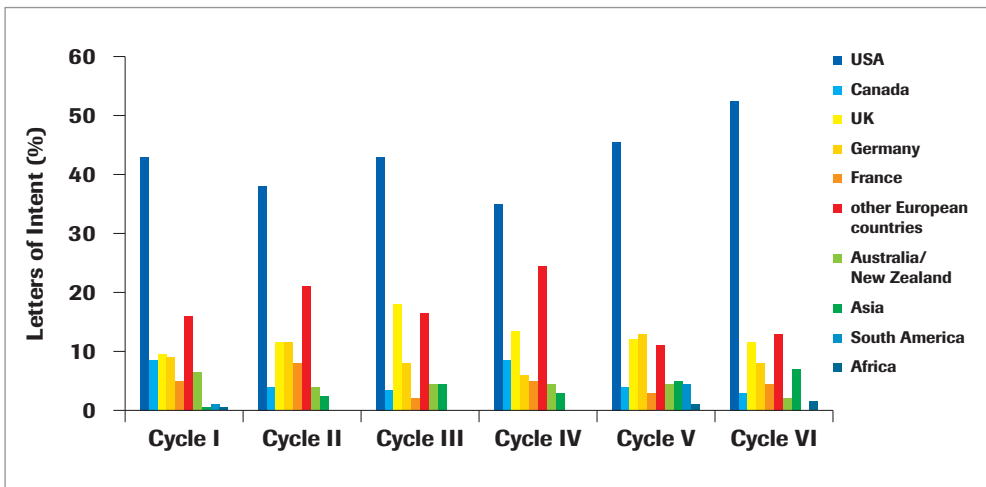
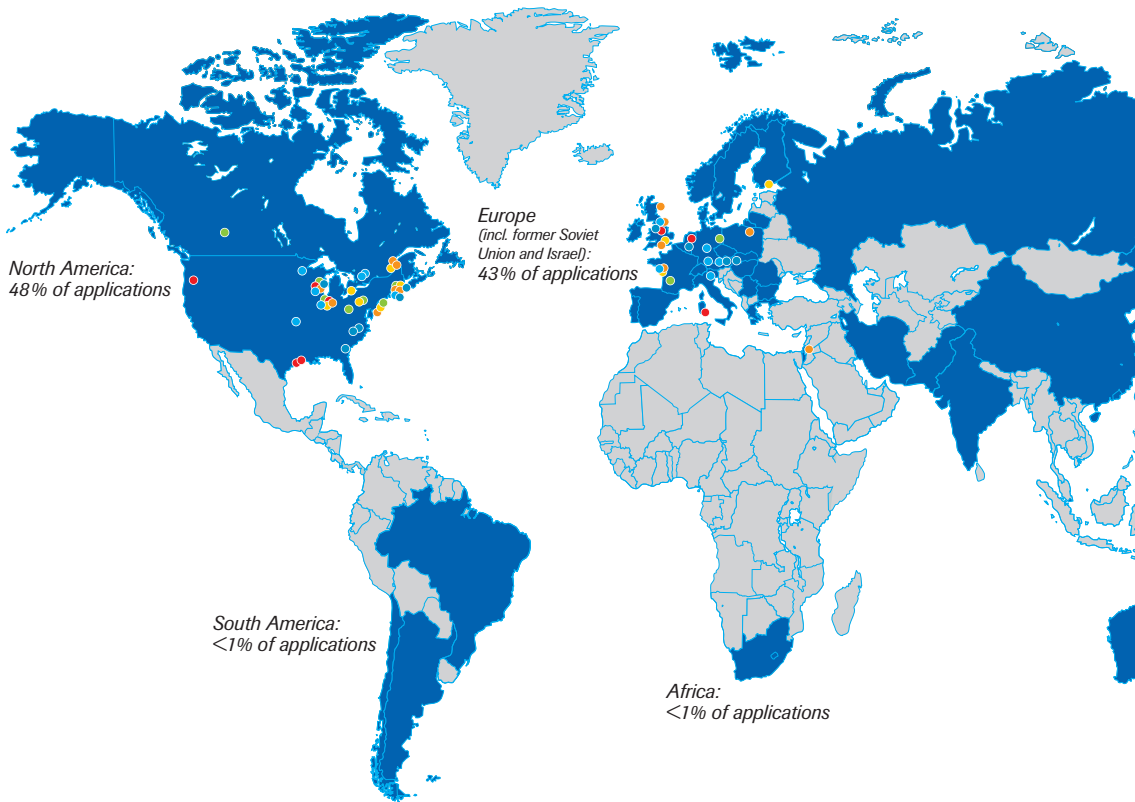


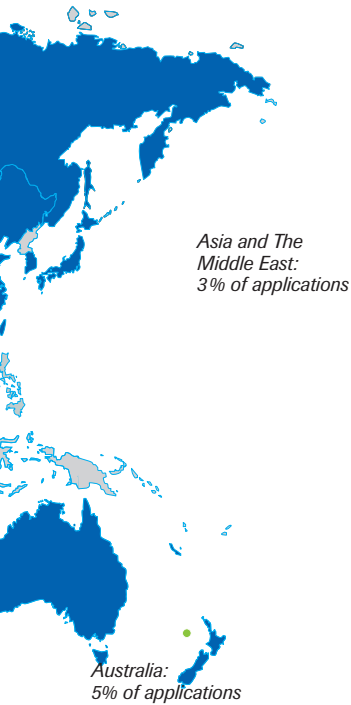
Figure 2. Geographical distribution of the applicants who submitted letters of intent (LOI) during the first six ROTRF funding cycles.



The Global View of Applications to the ROTRF

Distribution of ROTRF applications worldwide





■ at least one application ever received
 ■ no application received

Cycle I Grantees

<i>Berlin, Germany</i>	<i>Madison, USA</i>
<i>Bordeaux, France</i>	<i>Melbourne, Australia</i>
<i>Boston, USA</i>	<i>New Haven, USA</i>
<i>Cincinnati, USA</i>	<i>Pittsburgh, USA</i>
<i>Edmonton, Canada</i>	<i>San Francisco, USA</i>

Cycle II Grantees

<i>Boston, USA</i>	<i>Nantes, France</i>
<i>Chicago, USA</i>	<i>New York, USA</i>
<i>Helsinki, Finland</i>	<i>Oxford, UK</i>
<i>London, Canada</i>	<i>Pittsburgh, USA</i>
<i>Montreal, Canada</i>	

Cycle III Grantees

<i>Atlanta, USA</i>	<i>Madison, USA</i>
<i>Birmingham, UK</i>	<i>Nijmegen, The Netherlands</i>
<i>Cagliari, Italy</i>	<i>Portland, USA</i>
<i>Houston, USA</i>	<i>Winnipeg, Canada</i>
<i>Houston, USA</i>	

Cycle IV Grantees

<i>Boston, USA</i>	<i>Nantes, France</i>
<i>Boston, USA</i>	<i>Newcastle-upon-Tyne, UK</i>
<i>Chicago, USA</i>	<i>Oxford, UK</i>
<i>Dundee, UK</i>	<i>Philadelphia, USA</i>
<i>Laval, Canada</i>	<i>Rehovot, Israel</i>
<i>Madison, USA</i>	<i>Warsaw, Poland</i>
<i>Montreal, Canada</i>	

Cycle V Grantees

<i>Bergamo, Italy</i>	<i>Minneapolis, USA</i>
<i>Boston, USA</i>	<i>Munich, Germany</i>
<i>Chicago, USA</i>	<i>Nantes, France</i>
<i>Edgbaston, UK</i>	<i>Oklahoma City, USA</i>
<i>Hanover, Germany</i>	<i>Toronto, Canada</i>
<i>Heidelberg, Germany</i>	<i>Toronto, Canada</i>
<i>Madison, USA</i>	

Cycle VI Grantees

<i>Augusta, USA</i>	<i>Durham, USA</i>
<i>Boston, USA</i>	<i>Madison, USA</i>
<i>Boston, USA</i>	<i>Manchester, UK</i>
<i>Bruxelles, Belgium</i>	<i>Regensburg, Germany</i>
<i>Chapel Hill, USA</i>	<i>Vienna, Austria</i>



3. ROTRF Grant Awards in Cycle VI

Dr. William J. Burlingham, Principle Investigator

Dr. Hans Sollinger, Co-Applicant



University of Wisconsin Medical School, Madison, USA

Delayed Type Hypersensitivity (DTH) Status and MMF Monotherapy

The lifelong administration of medications that suppress the immune system of the organ transplant recipient can prevent or slow down the rejection process but have debilitating side effects. Most patients who stop taking these drugs entirely lose their transplant and must undergo re-transplantation. However, cancer, infections or toxicity to healthy organs sometimes make it desirable to discontinue one or more of the medications.

Research scientists studying tolerance, the state in which a patient can maintain transplant function for years without any immunosuppressive drug therapy, have found that such individuals make their own immunosuppressive chemicals in response to the donor organ. This phenomenon of self-suppression is revealed by a test called the delayed-type hypersensitivity (DTH) test. DTH suppression is found uniformly in patients and experimental animals successfully take off all immunosuppressive drugs, and in a proportion (25–40%) of patients still taking one or more immune suppressive drugs.

The significance of this study is that it will determine whether the latter group of patients may be reduced safely from 3 drugs to a single drug (MMF) without a substantially increased risk of rejection. If our hypothesis is incorrect, then the outcome of partial drug withdrawal will be entirely independent on a patient's DTH status at the time of drug reduction.

Dr. Christiane Ferran, Principle Investigator



Beth Israel Deaconess Medical Center, Boston, USA

Protective Effect of A20 against Transplant-Associated Vasculopathy

Effective immunosuppressive regimens used in transplantation have drastically reduced the failure of vascularized grafts due to acute rejection (<10%). Chronic rejection is the major obstacle to long-term transplant function. Chronic rejection is characterized by the development of an accelerated arteriosclerosis, termed “transplant-associated vasculopathy” (TAV). TAV is particularly dramatic in cardiac transplant recipients and has become the principle cause of late death and graft dysfunction. The precise mechanisms of chronic rejection and TAV are poorly understood and likely multifactorial, with both immunological and non-immunological causes.

Numerous approaches to treating TAV (including increased immunosuppression, anti-hypertensive and lipid lowering agents) have been largely unsuccessful and carry additional toxicity. We propose a novel anti-TAV approach aimed at shielding the blood vessel wall from immune and non-immune effectors of TAV. Our approach is based on our finding that the fate of the graft does not solely depend upon the host immune and non-immune effectors, but also on its ability to protect itself from injury. Our data suggest that this might be safely achieved by genetic engineering of the vessel wall with a single gene: A20. A20 is a physiologic protective response to injury in endothelial cells (EC) and smooth muscle cells (SMC), preventing TAV regardless of the precipitating offender. A20 is anti-inflammatory and anti-apoptotic in EC, and anti-inflammatory and anti-proliferative in SMC. Additionally, A20 sensitizes SMC to apoptosis. The conventional paradigm emphasizes the critical role of EC and SMC activation/proliferation in the progression of TAV lesions. Protection from TAV will best be achieved if we promote death of neointimal SMC while helping EC to survive. Our aim is to provide direct *in vivo* proof for the protective effect of A20 against TAV using A20 based gene therapy in rat aortic transplants.

Prof. Edward K. Geissler, Principle Investigator

Dr. Markus Guba, Co-Applicant

Dr. Markus Steinbauer, Research Associate

Dr. Christian Graeb, Research Associate



University of Regensburg, Regensburg, Germany

Paclitaxel and Rapamycin: A One-Two Punch against Rejection and Cancer in Organ Transplantation

The success of organ transplantation remains dependent on the use of drugs that suppress a recipient's immune system from destroying foreign transplanted tissue. Unfortunately, one of the major side effects of suppressing the general immune system is that cancer cells are not as likely to be destroyed by this protective system. Therefore, the occurrence of cancer is a frequent and dreaded side effect of immunosuppressive therapy. This creates a difficult situation, because removal of immunosuppression to reduce *de novo* neoplasm occurrence puts the organ transplant at risk for rejection. Paradoxically, organ transplants are sometimes done to cure isolated cancer of the liver or lung, but these cancers tend to recur in the transplanted organ, most likely due to the tumor-promoting effect of conventional immunosuppression on residual cancer cells.

In the present study we propose that two specific drugs, rapamycin and paclitaxel, could together have a unique potential to promote allograft survival, while attacking tumor cells. This hypothesis is based on our preliminary data, which shows that the well-known immunosuppressive drug rapamycin can also act to destroy tumors in mice. Additionally, we have also found that the powerful anticancer drug paclitaxel has potent immunosuppressive activity that promotes heart transplant survival in rats. Therefore, we propose to use this "one-two-punch" of rapamycin and paclitaxel to treat tumors in both mice and rats that subsequently receive a heart or liver transplant. In these experiments we will test the protective effect of the "one-two-punch" on the transplanted organ and its potential simultaneous destructive effect against cancer. The mechanisms by which these drugs may complement each other with regard to immunosuppression and tumor growth inhibition are critical, and will be explored. We believe results from this study could have a direct impact on the future successful maintenance of organ transplants in situations where organ replacement is a curative option for early cancer, or when *de novo* cancer occurs in transplantation patients.

Dr. David A. Gerber, Principle Investigator

Jian Wang, Research Associate

Lujun Song, Technician



University of North Carolina, Chapel Hill, USA

The Immunologic Basis of Hepatic Progenitor Cell Transplantation

Liver cell therapy has the potential to successfully address many problems associated with liver transplantation (e.g. the need for immunosuppression, morbidity of surgery and shortage of organs). A single liver could be a potentially therapeutic cellular source for multiple patients. Previous experimental attempts at liver cell therapies have unsuccessfully made use of unfractionated adult liver cells as a potential treatment for patients with inborn errors of metabolism or patients with fulminant hepatic failure that need a temporary bridge until their native liver regenerates. In addition, the past failures of mature hepatocyte transplantation have at least partly been related to issues of immunogenicity.

Progenitor cells (a cellular population that is defined as pluripotent, capable of extensive growth, and possibly able to replicate) should be able to expand extensively *in vivo* offering the greatest hope for liver reconstitution. The potential of hepatic progenitor cells in transplantation is dependent upon understanding their ability to mature to a functional cell and understanding their ability to initiate an alloimmune response or induce tolerance in the host. The goal of the following experiments is to develop a greater understanding of the cellular milieu of the liver and to develop and understand the immunogenicity of the hepatic progenitor cell population.

Further advances in the field of hepatic stem cell biology will potentially allow us to offer a cell-based therapeutic modality for patients with fulminant hepatic failure, end stage liver disease and/or inherited diseases of the liver that is as efficacious as solid organ transplantation, but does not require long-term immunosuppression.

Prof. Michel Goldman, Principle Investigator

Dr. Véronique Flamand, Research Associate



Université Libre de Bruxelles, Bruxelles, Belgium

**Dendritic Cells Transduced with the FasL Gene
as a Tool to Regulate Allograft Immunity**

There is growing evidence that the induction of tolerance to organ transplants will require the infusion of donor cells endowed with tolerogenic properties. Along this line, it has been suggested that donor-type dendritic cells expressing the death-inducing molecule Fas-ligand (FasL) might selectively eliminate the recipient's T cells responsible for transplant rejection. In preliminary experiments, we found that dendritic cells genetically engineered to overexpress FasL rapidly died unless they are deficient in Fas, the molecule which transmits the death signal inside the cell. When Fas-deficient dendritic cells overexpressing FasL were obtained, we observed that although these cells were endowed with killing properties they induce strong inflammation at the site of injection and promote efficient T cell responses. The purpose of this project is to delineate the conditions that allow dissociation of the inflammatory reaction elicited by dendritic cells overexpressing FasL from their capacity to kill T cells specific for alloantigens expressed at their membrane. The data that will be generated should provide a better understanding of the links between inflammation/innate immunity and adaptive immune responses leading to transplant rejection. Hopefully, they will also lead to new cell therapy approaches for the induction of transplantation tolerance.

Dr. Michael D. Gunn, Principle Investigator

Manabu Yanagita, Research Associate



Duke University Medical Center, Durham, USA

The Role of Plasmacytoid Dendritic Cells in the Development of Tolerance

It has long been thought that a population of antigen-presenting dendritic cells within the lymph nodes and spleen may act to inhibit immune responses by eliminating excessive or unwanted T lymphocytes. Such a function is required to eliminate self-reactive T lymphocytes and to terminate immune responses that have served their purpose. These cells may also be stimulated to eliminate T cells that mediate the rejection of organ transplants.

We have identified a mutation in mice that leads to excessive T cell responses, the decreased elimination of unwanted T cells, and a failure to develop transplant tolerance. This mutation involves genes that control cell migration and our studies suggest that this defect is due to the failure of a specific dendritic cell population to migrate into lymph nodes. In pursuing this defect, we have identified a novel dendritic cell type that may be involved in this process.

In these studies, we will use a model in which T cells are eliminated in normal mice but not in our mutant mice to identify the specific dendritic cell type that mediates the elimination of unwanted T cells. We will also examine the requirement for specific molecules that may be involved in this process and will examine the requirement for specific dendritic cell types in the development of transplantation tolerance.

This work should lead to a greater understanding of the mechanisms by which tolerance is initiated. In addition, the identification of a cell type that promotes the development of tolerance may have clinical applications.

Dr. Anatolij Horuzsko, Principle Investigator

Prof. Andrew Mellor, Co-Applicant



Medical College of Georgia, Augusta, USA

**Prevention of Skin Allograft Rejection
by HLA-G-Modified Dendritic Cells**

A more complete understanding of natural immune mechanisms is needed to design better therapeutic approaches to achieve acceptance of transplanted tissues with minimal intervention by immunosuppressive drugs.

In vivo models of immune suppression are the most useful models for studies in transplantation because of their relevance to clinical transplantation in humans. The newly developed HLA-G transgenic mouse is an excellent model system for such studies. HLA-G is a human MHC class Ib molecule that is thought to regulate immune responses during pregnancy. We have generated HLA-G transgenic mice that express HLA-G in all major tissue. We found that the rejection of allogeneic skin grafts (donor skin from a different strain of mouse) is significantly delayed. In these mice dendritic cells were deficient in spontaneous maturation and induced T cell hyporesponsiveness. We found that this is due to the presence of HLA-G molecules. The HLA-G tetrameric complexes specifically bind a subset of myelomonocytic cells and inhibit maturation of dendritic cells *in vitro*. We propose that this binding occurs on murine cells via the receptor, a close homologue of the human inhibitory receptor, which is known to bind HLA-G. Determining the molecular mechanism and cellular process by which murine cells expressing HLA-G interfere with maturation of dendritic cells is the main focus of this study.

The proposed studies will help to establish a new approach to the processes that induce immune suppression. The results have the potential of providing new strategies for the management of transplants, allergy, autoimmune disease, resistance to infection and tumors, and immunodeficiency.

Prof. Ian Hutchinson, Principle Investigator

Dr. Vera Pravica, Co-Applicant



University of Manchester, Manchester, UK

VEGF: A Central Mediator in both Acute and Chronic Transplant Rejection

Transplantation is now a successful treatment, but patients still lose their grafts to acute and chronic rejection. These damaging processes appear to be linked, so that someone who suffers acute graft rejection is more prone to develop chronic rejection. We have identified a molecule called VEGF that appears to play a role in both the acute and chronic rejection processes.

The amount of VEGF a person produces is genetically determined. One version of the gene produces higher amounts of VEGF while the other version produces lower amounts. Those people born to be higher producers of VEGF seem to suffer more acute and chronic transplant rejection. This is important because a simple genetic test may help us to identify, in advance of transplantation, recipients who need more drugs, or different drugs, to control their tendency to reject the transplant.

In this project we continue with our studies of VEGF in transplant patients. We will study what naturally turns the production of VEGF on and off, and how this is influenced by inheritance of higher or lower producer versions of the VEGF gene. Because VEGF appears to be a central player in both acute and chronic rejection, we also want to know what influence the immunosuppressive drugs that are given to transplant recipients have on the production of VEGF. Similarly, we want to know if immunosuppressive agents have the ability to block the damaging activities of VEGF once it has been formed. This will help us to choose the most appropriate immunosuppressive agents for our patients.

Dr. Koichi Shimizu, Principle Investigator

Dr. Richard Mitchell, Consultant

Dr. Peter Libby, Consultant



Brigham & Women's Hospital, Boston, USA

**Source of Intimal Smooth Muscle like Cells
in Aortic Allograft Arteriopathy**

Because of excellent therapeutic strategies to prevent acute allograft rejection, graft arterial disease (GAD) is now the most important long-term obstacle to successful solid organ transplantation. The arteries of allografted organs typically develop severe, diffuse intimal hyperplastic lesions leading eventually to luminal stenoses and to ischemic graft failure. These lesions consist primarily of smooth muscle cells (SMCs) and associated extracellular matrix, admixed with infiltrating mononuclear leukocytes. The intimal lesions result from an initial alloimmune response, although the exact mechanisms remain to be elucidated. The SMCs forming the intimal hyperplastic GAD are thought to originate from medial SMCs.

Although bone marrow (BM) stem cells can develop into multiple mesenchymal lineages, only recently has it been demonstrated that BM-derived circulating cells also have the specific ability to differentiate into the intimal SM-like cells in vascular grafts. To show this we performed murine aortic transplantation into irradiated recipients of β -galactosidase transgenic (ROSA26) BM cells. The aortic graft intima contained numerous SM-like cells, which also showed β -galactosidase activity. We hypothesize that GAD SM-like cells derive primarily from such circulating BM stem cells. Such a finding is a paradigm shift from the current view that these cells derive from the underlying media. Moreover, this has profound implications regarding the therapy for GAD, as well as for more typical atherosclerotic lesions.

Our objective is the identification and characterization of the cells forming the intimal hyperplastic lesions of allograft arteriopathy, and in particular their recruitment and activation requirement.

The demonstration of BM-derived intimal SM-like cells and identification of SM-like cell specific recruitment and activation will lay the groundwork for future experiments where GAD-preventive therapeutic genes or drugs may be targeted not to donor medial SMC, but rather to recipient SM-stem cells.

Dr. Thomas Wekerle, Principle Investigator

Prof. Megan Sykes, Research Associate



Vienna General Hospital, Vienna, Austria

Tolerance through Hematopoietic Cell Transplantation with Costimulation Blockade

The lifelong non-specific immunosuppressive drug therapy that all transplant recipients currently have to take allows good short-term results. Long-term graft survival, however, is severely limited by episodes of drug-resistant acute and chronic rejection and drug-related side effects. The induction of donor-specific immunological tolerance is therefore a long-sought goal in order to re-educate a patient's immune system so that it accepts the "foreign" organ like own tissue without the use of chronic immunosuppressive drugs. It has been known for some time that the transplantation of donor bone marrow together with the organ graft allows the induction of robust tolerance. The clinical application of this concept has been inhibited, however, in large part by the toxicity of the recipient conditioning required for successful conventional allogeneic bone marrow transplantation (BMT).

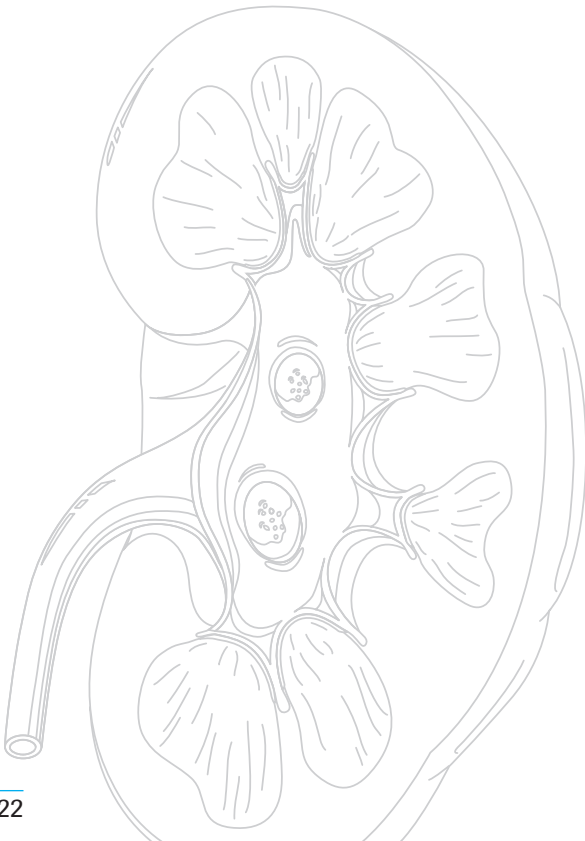
Recently, a mouse model of allogeneic BMT using very high numbers of bone marrow cells and two new costimulation-blocking antibodies has been developed which, for the first time, avoided all cytotoxic (i.e. cell-destructive) recipient conditioning. This very mild protocol holds great promise for clinical tolerance induction in the future, but currently two main factors limit its clinical applicability:

- 1) the amount of bone marrow cells required cannot be obtained from one human donor, and
- 2) it is successful only in approximately half the treated mice. Mobilized peripheral blood stem cells (mPBSC) are routinely used for transplantation for hematological indications and offer the advantage that very high numbers of hematopoietic cells can be harvested from a living donor. mPBSC differ, however, in several important respects from bone marrow cells.

The proposed project, therefore, aims to achieve the following:

- 1) to induce tolerance through the transplantation of clinically obtainable amounts of mPBSC with costimulation blockade;
- 2) to improve reliability by transiently using immunosuppressive drugs; and
- 3) to identify and use the donor cell population which is responsible for one of the main tolerance mechanisms in this protocol, namely the peripheral deletion of donor-reactive T cells.

The goal of these studies is to develop a clinically applicable protocol for tolerance induction in living- (un)related kidney transplantation. Such a protocol would be expected to substantially improve graft survival and morbidity after organ transplantation.





4. Progress Reports of ROTRF Grantees

Dr. Maria-Luisa Alegre, Principal Investigator

Dr. Richard J. Thistlethwaite, Resource Person

Dr. Ping Zhou, Research Assistant



University of Chicago, Chicago, USA

Role of Costimulatory Molecules in Cardiac Allograft Rejection

Antigen-specific immune responses are controlled by signals that potentiate and signals that down-regulate T cell activation. Ligation of CD28 by B7 is a major requirement for T cell activation, and blockade of this pathway has provided a promising tool to prevent donor-specific T cell activation in transplantation models. In contrast, interaction of CTLA-4 with B7 reduces the magnitude of an immune response and prevention of this binding can lead to more vigorous T cell responses. A careful understanding of the role of each of these interactions in concert with TCR stimulation may aid in the design of therapeutic regimens to promote tolerance.

Specific Aim. To determine the mechanism of rejection operating in CD28-deficient mice *in vivo*. Although CD28-deficient T cells have impaired IL-2 production and proliferation *in vitro*, these animals are capable of rejecting cardiac allografts *in vivo*. This suggests either a co-stimulation-independent mechanism of rejection, or involvement of alternative co-stimulatory molecules. Preliminary data suggest that CD8⁺ T cells are required for cardiac allograft rejection in CD28-deficient mice. This model will allow us to study alternative co-stimulatory molecules specifically involved in CD8 activation during allorecognition. This is especially important as preliminary results show that CD8⁺ T cells appear to play a major role in rejection of cardiac allografts in wild-type mice when CD4⁺ T cell responses are impaired, a situation likely to occur in clinical settings.

RESULTS

1. CTLA4-Ig treatment prolongs cardiac allograft survival in wild-type but not CD28-deficient mice. CTLA4-Ig and anti-B7 mAbs have been used extensively to prevent ligation of the co-stimulatory molecule CD28 to B7 on antigen producing cells (APCs) in an attempt to induce antigenic tolerance. Previous results had indicated that B7 blockade prevents cardiac allograft rejection in normal mice whereas CD28-deficient mice that also lack CD28-mediated signals are capable of rejecting heart transplants. To investigate the existence of co-stimulatory receptors on T cells other than CD28, capable of binding B7 ligands, CD28-deficient B6 recipient mice were transplanted with BALB/c hearts and treated with CTLA4-Ig. As previously published and shown in Figure 1, CTLA4-Ig treatment in wild-type mice prolonged cardiac allograft survival beyond 100 days post transplantation, whereas untreated animals

rejected their grafts rapidly (MST 8 ± 1 d). Rejection of the heart allografts was slightly delayed in CD28-deficient recipients (MST 17 ± 2 d; $p < 0.001$). However, in contrast to wild-type animals, CTLA4-Ig treatment did not prolong graft survival in CD28-deficient mice. Similar results were obtained following treatment with anti-B7-1 plus anti-B7-2 mAbs (data not shown). These results suggested that rejection of heart allografts in CD28-deficient mice was not due to cryptic CD28-like T cell co-stimulation.

2. Both wild-type and CD28-deficient recipients mediate graft rejections via a CD40L-dependent pathway. Several possibilities were considered to explain why CD28-deficient mice rejected cardiac allografts. Effective cardiac allograft rejection in CD28-deficient hosts could have indicated that T cells developing in the absence of CD28 become co-stimulation-independent or rely on alternative co-stimulatory pathways to bypass CD28 requirements. To investigate whether CD28-deficient mice rejected cardiac allografts in a complete co-stimulation-independent manner, wild-type and CD28-deficient B6 recipients transplanted with BALB/c hearts were treated with repeated doses of anti-CD40L mAb. Administration of anti-CD40L mAb resulted in prolongation of cardiac allograft survival in both wild-type and CD28-deficient recipients (Figure 2). The prolongation observed in the CD28-deficient mice was significantly greater than that observed in wild type mice (MST 105 ± 7 d versus MST 39 ± 7 d, respectively). These results indicate that CD28-deficient T cells are not co-stimulation-independent and that, like wild-type T cells, they depend on CD40L-mediated signals. Moreover, the data confirm that CD40L/CD40 mediated T cell co-stimulation is not solely due to its regulation of B7 expression on APCs as previously suggested.

rejected their grafts rapidly (MST 8 ± 1 d). Rejection of the heart allografts was slightly delayed in CD28-deficient recipients (MST 17 ± 2 d; $p < 0.001$). However, in contrast to wild-type animals, CTLA4-Ig treatment did not prolong graft survival in CD28-deficient mice. Similar results were obtained following treatment with anti-B7-1 plus anti-B7-2 mAbs (data not shown). These results suggested that rejection of heart allografts in CD28-deficient mice was not due to cryptic CD28-like T cell co-stimulation.

3. T cells from CD28-deficient mice are less potent at rejecting cardiac allografts than wild-type T cells. The slightly slower rejection observed in the untreated CD28-deficient animals when compared with wild-type recipients, together with the enhanced prolongation of graft survival by anti-CD40L treatment in these animals suggested that the CD28KO T cells might

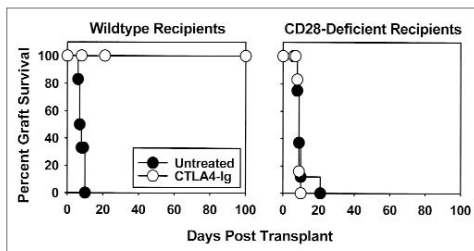


Figure 1. CD28-deficient mice effectively reject cardiac allografts.

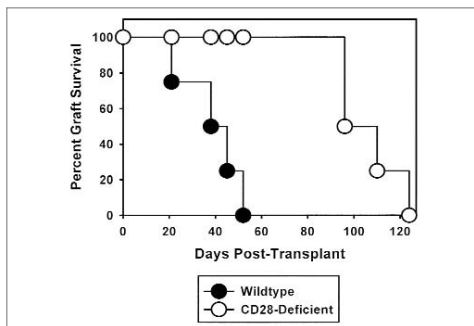


Figure 2. Anti-CD40L mAb prolongs cardiac allograft survival in CD28-deficient mice.

in fact be impaired *in vivo*. Alternatively, it remained possible that the alloreactive T cells from CD28-deficient animals were indeed more potent on a per cell basis and that the delayed rejection time was due to impaired APC capacity. To directly compare wild-type and CD28-deficient T cells, different numbers of purified T cells from wild-type or CD28-deficient mice were adoptively transferred into B6 nude recipients previously transplanted with BALB/c hearts. As shown in Figure 3, an order of magnitude more CD28-deficient than wild-type T cells was necessary for rejection to occur, as 50% of the grafts were rejected in 30 days following transfer of 20×10^6 CD28-deficient T cells or of 1.75×10^6 wild-type T cells. This was probably not due to increased death of CD28-deficient T cells following the adoptive transfer, as similar numbers of wild-type and CD28-deficient CD4⁺ and CD8⁺ T cells were found in the spleen of transplanted animals 90 days following the adoptive transfer of T cells (data not shown). Rather, this result suggests that CD28-deficient T cells are less potent at triggering cardiac allograft rejection than wild-type T cells.

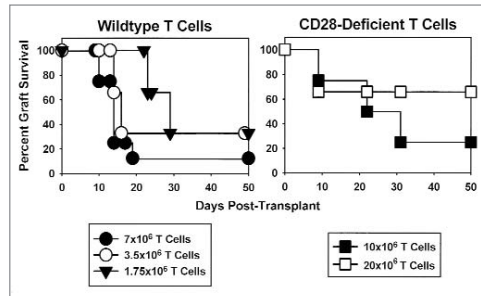


Figure 3. Increased numbers of CD28-deficient T cells required to reject cardiac allografts.

4. Increased numbers of infiltrating T cells in grafts from CD28-deficient recipients. Thus, it appeared that the T cells from CD28-deficient mice remained co-stimulation-dependent and were far less effective on a per cell basis. These results suggested that an alternative mechanism of allograft rejection might be operative in the CD28-deficient mice for rejection to occur relatively promptly. To investigate this possibility, T cell subsets were analyzed qualitatively in grafts from wild-type and CD28-deficient mice. Tissue sections from heart transplants harvested at the time

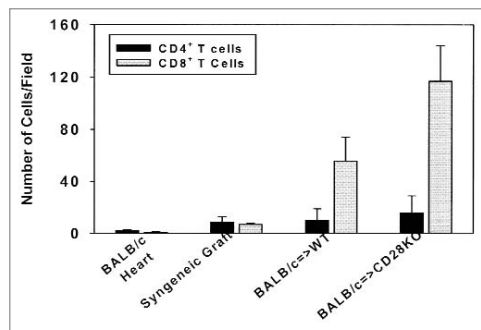


Figure 4. Increased T cell infiltrate in grafts from CD28-deficient recipients.

of rejection were stained by immunohistochemistry with anti-CD4, anti-CD8, or control mAbs. The majority of the infiltrating cells were CD8⁺ in both wild-type and CD28-deficient mice. There was approximately 3 to 10 fold fewer CD4⁺ than CD8⁺ T cells observed in the sections (Figure 4), and no staining was detectable following use of the control mAbs (data not shown). More importantly, there were significantly larger numbers of infiltrating CD8⁺ T cells in grafts from CD28-deficient animals as compared with grafts from wild-type mice ($p < 0.001$). These data suggested that, unlike wild-type animals, there might be a crucial role for CD8⁺ T cells in

graft rejection mediated by CD28-deficient mice.

It is important to note that B6 mice are thought to have increased numbers and function of CD8⁺ T cells as compared with other strains of mice. To exclude the possibility that the massive CD8⁺ T cell infiltration observed in rejected cardiac transplants was strain-dependent, the infiltrate in C3H cardiac allografts rejected by BALB/c recipients was also analyzed. In this strain combination, even more T cells were of the CD8⁺ phenotype than in B6 wild-type recipients (CD4⁺ T cells: 47±15; CD8⁺ T cells: 142±54), suggesting that a high ratio of CD8⁺/CD4⁺ T cells is a normal occurrence during cardiac allograft rejection in mice.

5. Differential requirement for CD8⁺ T cells for cardiac allograft rejection by wild-type and CD28-deficient recipient mice.

We next tested the possibility that the rejection observed in CD28-deficient mice was CD8⁺ T cell dependent. Wild-type and CD28-deficient mice were injected with depleting anti-CD4 or anti-CD8 mAbs. As shown in Figure 5, depletion of CD4⁺ T cells resulted in long-term acceptance of most cardiac allografts in

both wild-type and CD28-deficient mice. In contrast, depletion of CD8⁺ T cells did not prevent allograft rejection in wild-type mice (MST 16±2d), but resulted in long-term cardiac allograft survival (MST greater than 100d) in CD28-deficient animals. This suggests that cardiac allograft rejection in CD28-deficient mice was also dependent on CD8⁺ T cells whereas CD4⁺ T cells were sufficient to mediate graft rejection in wild-type animals.

6. Defects in CD4⁺ T cells in CD28-deficient mice may explain CD8⁺ T cell dependency in this model.

Taken together, the previous results supported the notion that CD4⁺ T cells from the CD28-deficient mice were compromised and thus required CD8⁺ T cells to facilitate graft rejection. Thus, we investigated the relative function of CD4⁺ T cells in wild-type versus CD28-deficient recipients. Mice transplanted with BALB/c hearts were treated with depleting anti-CD4 mAb two days before or four days after transplantation.

Depletion of CD4⁺ T cells before transplantation prevented rapid graft rejection in wild-type mice (see Figure 5). In contrast, depletion of CD4⁺ T cells on day four post-transplant did not prolong graft acceptance (MST 11±1 d). These results indicated that the presence of CD4⁺

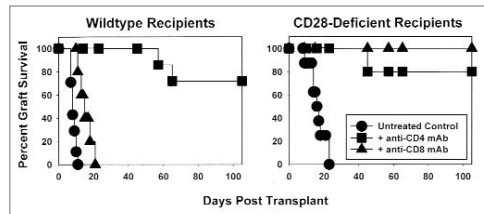


Figure 5. CD8⁺ T cells are required for cardiac allograft rejection in CD28-deficient but not wild-type mice.

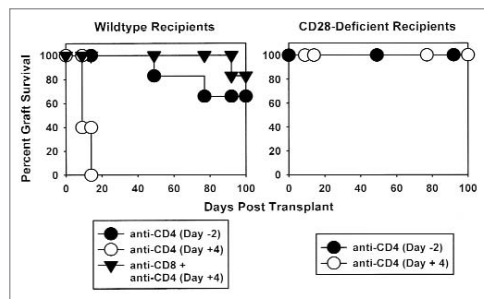


Figure 6. CD4⁺ T cells are required past day 4 for cardiac allograft rejection in CD28-deficient mice.

T cells after day four was not critical for graft rejection to occur in wild-type mice (Figure 6). In contrast, depletion of CD4⁺ T cells on day four in CD28-deficient mice induced long-term graft acceptance. Interestingly, rejection of allografts in the setting of aborted CD4⁺ T cell response became CD8⁺ T cell-dependent in wild-type mice, as anti-CD8 therapy prevented cardiac allograft rejection in most animals (MST 99±1d, $p < 0.01$). These data may explain findings that CD8⁺ T cells accounted for the majority of the graft infiltrate yet could not be shown to be critical for cardiac allograft rejection in wild-type animals. Moreover, the results support the conclusion that in normal situations, CD4⁺ T cells are only necessary at the initiation of the alloimmune response in wild-type mice whereas rejection mediated by CD28-deficient mice requires the prolonged presence of CD4⁺ T cells.

7. Cardiac allografts rejection in anti-CD40L mAb-treated mice is CD8-dependent. These results suggested that cardiac allograft rejection may become absolutely dependent on CD8⁺ T cells in cases of impaired CD4⁺ T cell responses such as CD28 deficiency or aborted CD4⁺ T cell responses due to late subset depletion. To further test this hypothesis, wild-type mice were treated with anti-CD40L mAb, as this treatment has previously been shown to be more effective at impairing CD4⁺ than CD8⁺ T cell responses. The animals also received depleting anti-CD8 mAbs. Treatment with anti-CD8 mAbs prevented cardiac allograft survival in the majority of anti-CD40L-treated mice (not shown). Therefore, treatment with anti-CD40L mAb prolonged allograft survival but did not prevent rejection, and rejection in these animals was CD8⁺ T cell-dependent.

SUMMARY AND FURTHER STUDIES

In conclusion, we have found that the cellular mechanisms for acute cardiac allograft rejection are different in CD28-deficient and wild-type animals. This may explain the different outcome in CD28-deficient mice and wild-type mice treated with CTLA-4-Ig or anti-B7 mAbs. Because anti-CD40L mAb treatment was shown to prolong cardiac allograft survival in CD28-deficient mice, we have become interested in the alternative co-stimulatory molecules that may support T cell activation in CD28-deficient mice. Preliminary data *in vitro* has pointed to CD30 as an alternative co-stimulatory molecule with interesting signaling properties. However, the role of CD30 in immune responses *in vivo* is not known. To investigate whether CD30 can play a co-stimulatory role for cardiac allograft rejection in CD28-deficient mice, we have obtained CD30L-deficient mice from Dr. Podack (University of Miami, FL). These mice are in the process of being intercrossed with CD28-deficient mice to generate CD30L/CD28-deficient animals. Graft survival will be examined in these animals.

Publications related to this project

- Szot GL, Zhou P, Rulifson I, Wang J, Guo Z, Kim O, Newell KA, Thistlethwaite JR, Bluestone JA, Alegre ML. Different mechanisms of cardiac allograft rejection in wild-type and CD28-deficient mice. *Am J Transplantation* 2001; 1: 38-46.

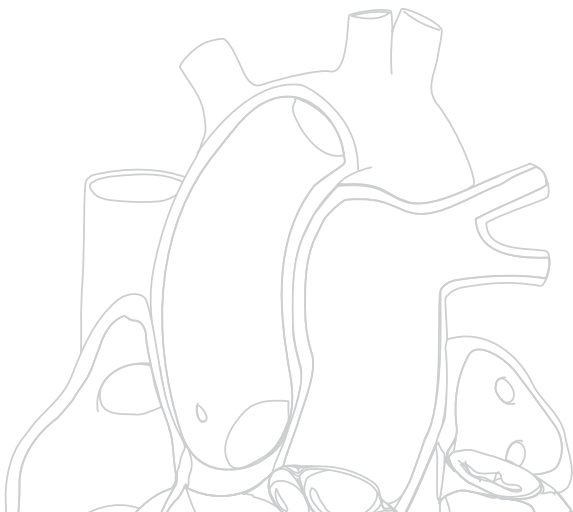
2000-2001 Publications related to transplantation research

- Zhou P, Szot GL, Guo Z, Kim O, He G, Wang J, Grusby MJ, Newell KA, Thistlethwaite JR, Bluestone JA, Alegre ML. Role of STAT4 and STAT6 signaling in allograft rejection and CTLA4-Ig-mediated tolerance. *J Immunol* 2000; 165: 5580-5587.
- Szot GL, Zhou P, Rulifson I, Wang J, Guo Z, Kim O, Newell KA, Thistlethwaite JR, Bluestone JA, Alegre ML. Different mechanisms of cardiac allograft rejection in wild-type and CD28-deficient mice. *Am J Transplantation* 2001; 1: 38-46.
- Zhong G, Meng L, Kim O, Wang J, Hart J, He G, Alegre ML, Thistlethwaite JR, Newell KA. CD8 T cell mediated rejection of intestinal allografts is resistant to inhibition of the CD40/CD154 costimulation pathway. *Transplantation* 2001; 71: 1351-1354.
- Zhou P, Szot GL, Guo Z, Kim O, He G, Wang J, Grusby MJ, Newell KA, Thistlethwaite JR, Bluestone JA, Alegre ML. STAT6 signaling may be necessary for tolerance induction by CTLA4-Ig. *Transplant Proc* 2001; 33: 214-216.
- Alegre ML, Fallarino F, Zhou P, Frauwirth KA, Thistlethwaite JR, Newell KA, Gajewski TF, Bluestone JA. Transplantation and the CD28/CTLA4/B7 pathway. *Transplant Proc* 2001; 33: 209-211.
- Zhong G, Wang J, Meng L, Wu Q, Kim O, Hart J, He G, Thistlethwaite JR, Alegre ML, Fu YX, Newell KA. Disruption of the membrane lymphotoxin/lymphotoxin receptor pathway inhibits intestinal allograft rejection. *Cutting Edge J Immunol* 2001, in press.
- Chin R, Zhou P, Alegre ML, Fu YX. Letter in response to « Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue ». *Nature Medicine* 2001, in press.

Presentations directly related to this report

The work summarized in this progress report was reported at:

- The American Transplant Society, Chicago, IL, May 2000
- The American Association of Immunologists FASEB meeting, Orlando, FL, March-April 2001.



Dr. Jeffrey A. Bluestone, Principle Investigator

Dr. Matthew D. Griffin, Co-Applicant



UCSF Diabetes Center, San Francisco, USA

Immunosuppression and Tolerance Induction by Genetically-Engineered Dendritic Cells Expressing Cell Surface Anti-CTLA-4 mAb

Introduction and Project Aims

Much evidence has accumulated in recent years that the maintenance of immune tolerance to self, as well as to non-threatening foreign antigens, is an active and ongoing process throughout life. A key element of this process is the recruitment of negative regulatory pathways during antigen encounter by T cells. The influence of such negative regulatory mechanisms is vital to the appropriate balance between T cell proliferation/effector function/memory – the hallmarks of an active cellular immune response and T cell apoptosis/anergy/suppression, the features of cellular immune tolerance. Indeed animal models in which negative regulatory pathways are genetically deficient, typically result in destructive autoimmune disease. The discovery of the important physiological role of negative regulatory pathways has generated the compelling possibility that augmentation of such pathways during presentation of alloantigen will favor specific tolerance to these same antigens, achieving the clinical goal of lasting immune tolerance to organ and tissue allograft.

Among the best characterized negative regulatory pathways is that mediated by the interaction of CTLA-4 (CD152) on T cells with a pair of ligands (B7-1 (CD80) and B7-2 (CD86)) expressed by professional antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells. A large body of literature now exists to show that CTLA-4 plays a central role in the generation of immune tolerance to peripherally expressed antigens. The strategy of recruiting additional CTLA-4/B7 interactions during alloantigen presentation is complicated, however, by the fact that CTLA-4 shares specificity for its natural ligands with a homologous T cell receptor (CD28) that transmits a potent activation signal when it is engaged along with the T cell receptor (TCR). We have developed and characterized an artificial surface-bound ligand for CTLA-4 that has no ability to bind to CD28. We initially hypothesized that this engineered ligand, derived from a single-chain anti-CTLA-4 antibody, would deliver a negative signal when expressed on the same cell surface as a TCR ligand (anti-CD3 or peptide / MHC complex). *In vitro* studies confirmed the predicted function for this cell-surface anti-CTLA-4 protein (1) and led us to develop the hypothesis and specific aims upon which the current project is based. **The overall hypothesis for the project is that, expression of a CTLA-4-specific surface ligand on professional APCs presenting alloantigen will**

promote subsequent immune tolerance to allografted organs and tissues expressing the same alloantigens.

We chose to focus our attention initially on the antigen presenting cells (APCs) of the immune system because of the growing appreciation that APCs, including dendritic cells (DC), B cells and macrophages, occupy central roles in the activation of alloreactive T cells *in vivo* while also having the potential to mediate immune tolerance to specific self and foreign antigens. We also elected to focus on murine systems of antigen-specific T cell activation and allotransplantation in order to employ transgenic technology and a potentially broad range of *in vivo* models. The specific aims of the project are as follows:

- Specific Aim #1:** To generate transgenic mice expressing a surface-linked CTLA-4-specific ligand on DCs.
- Specific Aim #2:** To utilize a retrovirus-based gene transfer system to engineer anti-CTLA-4-expressing APCs for tolerance induction.
- Specific Aim #3:** To compare the effects of control and anti-CTLA-4-expressing DCs in allotransplantation.

Significant Results During the Second Year

Specific Aim 1: The anti-CTLA-4 mAb used for the studies had been shown to effectively suppress T cell responses. However, the affinity of the antibody was sub-optimal. Thus, for the *in vivo* experiments, attempts were made to increase affinity. Yeast display mutagenesis was used to overcome the limitations decreased stability or antigen-affinity of mAbs. As shown in Figure 1, the selected mutations occurred in framework regions of the VL sequence rather than in the CDR loops. These mutants were shown to bind the ligand, CTLA-4, more effectively (2).

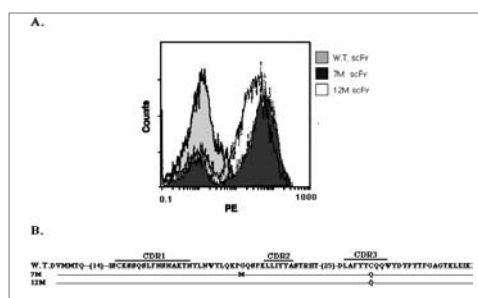


Figure 1A. Flow cytometric analysis of two mutant anti-CD152 scFvs with improved yeast surface display expression compared with the parent (W.T.) construct. Yeast surface-displayed anti-CD152 scFv was detected with biotinylated rabbit anti-mouse IgG_{2a} followed by streptavidin-PE. The yeast clones bearing selected mutant anti-CD152scFv (7M and 12M) demonstrated significantly enhanced surface binding of CTLA-4lg.

The amplification product from an error prone PCR of the anti-CD152 scFv gene was cloned into the yeast display vector pCT302 (1) and used to transform *E. coli* resulting in $\sim 2 \times 10^8$ colonies. Plasmid isolated from the *E. coli* library was used to transform the *Saccharomyces cerevisiae* EBY100 (3) to generate $\sim 10^8$ yeast transformants. Mutant anti-CD152 scFvs were isolated from the yeast library after four flow cytometric sorts with successively decreasing concentrations of the CTLA4lgG_{2a} fusion protein: 160 μ g/ml, 40 μ g/ml, 0.8 μ g/ml, 0.08 μ g/ml.

B. Amino acid sequence alignment of the two selected anti-CD152 scFv mutants. Individual yeast clones from the fourth sort were analyzed for improved binding to the CTLA4lgG_{2a} ligand and the plasmid rescued and sequenced. The complementarity determining regions of the variable light gene is overlined. The framework mutations are 7M:L43M, L89Q; 12M:L89Q.

Initial attempts to develop a transgenic mouse strain expressing the anti-CTLA-4 scFv on dendritic cells was unsuccessful. We utilized a vector from T. Broker (3). Unfortunately, none of the founder mice (n=4) expressed the single chain on the surface of dendritic cells. During the past year, we changed vectors to optimize expression. The new vector utilizes the I μ promoter and enhancer to drive constitutive expression on mature B cells (4). As seen in Figure 2, we have identified a founder mouse strain using this construct. One of these mice (7685) was observed to express the anti-CTLA-4 mem scFv on about 50% of B220⁺ B cells consistent with Ig expression. The CTLA-4Ig-FITC binding was specific as no staining was observed on the control or tg-mice.

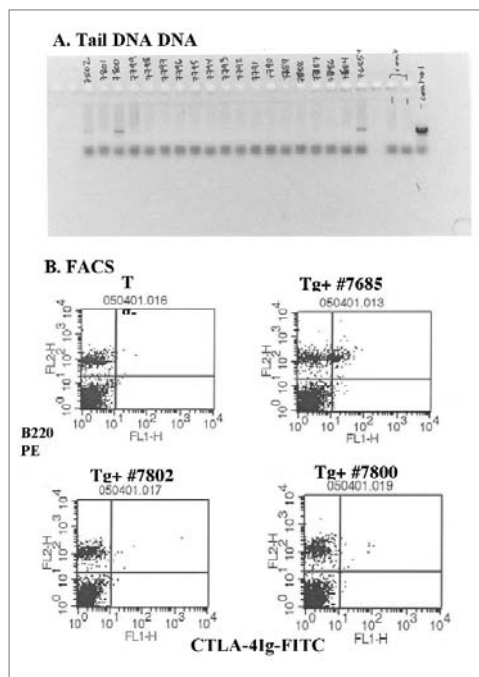


Figure 2A. PCR analysis of tail DNA from founder and offspring mice expressing anti CD152 scFvs. **B.** Flow cytometric analysis of offspring mice selected for expression of mutant anti-CD152scFv (7M). Significant surface binding of CTLA-4Ig-FITC was observed on a subset of B220⁺ cells.

Specific Aim 2: Although retroviral transfection vectors expressing surface-linked anti-CTLA-4 have been successfully generated and result in high level expression of the construct in a variety of murine cell lines it has not been possible to achieve high-efficiency transfection of cultured murine DCs using these vectors. For this reason, experiments have focussed on alternative techniques for achieving this aim. In addition modifications to the single-chain construct have been carried out with the goal of improving surface stability and allowing detection of the surface-expressed protein on B7-expressing APCs such as DCs. These goals have been met as follows:

- (a) The mutated anti-CTLA-4 construct developed by yeast, display technology in collaboration with Dr. Kranz (University of Illinois, Urbana, IL, USA). The construct has been further characterized and modified to incorporate a detectable peptide (c-myc) tag within the spacer region (2). This construct exhibits enhanced surface stability compared with the parent construct and is readily detected by a monoclonal antibody specific for the peptide (CT-14, anti-c-myc).
- (b) Using this modified anti-CTLA-4 construct, an adenoviral vector has been generated and shown to transduce a variety of murine cell lines and primary cells resulting in high level

expression of the protein. Furthermore, a protocol for high efficiency adenoviral transfection of murine cultured DCs has been developed. Adenovirally-mediated gene expression in cultured murine DCs has been shown to persist for at least one week following transduction allowing an ample duration of expression for functionally meaningful interactions with T cells to occur *in vitro* and *in vivo*.

(c) A system for the generation of significant numbers of anti-CTLA-4-expressing DCs for use in *in vitro* and *in vivo* experiments has, therefore, been developed.

Specific Aim 3: We have continued to pursue the use of anti-CTLA-4 membrane bound to suppress allogeneic responses. Using the newly developed mutant form of the anti-CTLA-4 mAb we now see robust inhibition of T cell responses. As seen in Figure 3, RAG-/- mice transplanted with P815 cells transfected with the 7M grow equally to normal P815 T cells. However, when 10^4 2C T cells (that recognize H-2L^d) are co-transferred, control vector transfected P815, but not tumors expressing membrane-bound anti-CTLA-4, are rejected (Figure 3). The difference is also seen in the survival (or lack thereof) of mice bearing the vector versus anti-CTLA-4 transfected tumor cells (Figure 4).

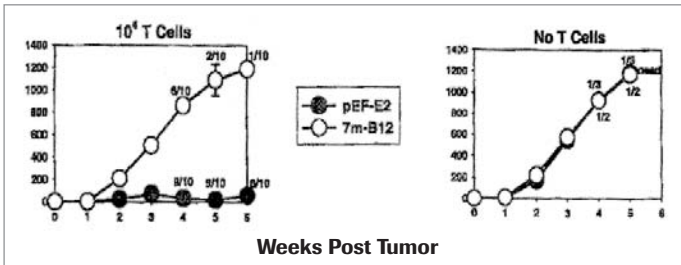


Figure 3. Increased tumor size in mice transplanted with anti-CTLA-4 expressing P815 tumor cells.

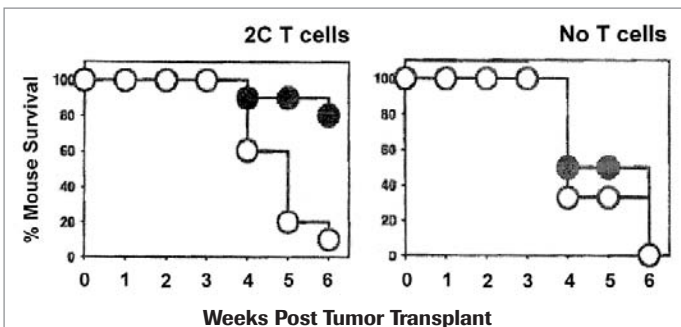


Figure 4. Decreased survival in mice transplanted with anti-CTLA-4 expressing P815 tumor cells.

Summary and Plans for Third Year: Experiments during the third year of the project will draw strongly from significant progress during the second year in the following areas:

(a) Continued analysis of transgenic murine lines expressing anti-CTLA-4 surface ligand on specific cell populations.

- (b) Overcoming the challenge of developing high-level expression systems for the anti-CTLA-4 surface-linked construct through the generation of an adenoviral vector.

Experiments will be carried out both in Dr. Bluestone's laboratory at the University of California, San Francisco and in Dr. Griffin's laboratory at Mayo Clinic, Rochester, MN. The immune phenotype of mice expressing anti-CTLA-4 on B cells will be characterized. Anti-CTLA-4-expressing B cells from these transgenic animals will also be used, isolated and compared with wild-type B cells for their ability to induce primary or secondary T cell activation or tolerance *in vitro* and *in vivo* using allogeneic and TCR transgenic T cell model systems. Additional transgenic mice transgenic for anti-CTLA-4 on all tissues will be generated using a class I MHC promoter-containing construct. The immune phenotype of these animals will be characterized. In addition, skin and islets from these transgenic animals will be used as donor tissue in allotransplantation experiments as a means to determine the outcome of direct CTLA-4 engagement by graft cells. Finally, bone marrow from these transgenic animals will also be used to generate DCs *in vitro* that constitutively express the construct for comparison with adenovirally-transduced DCs.

Adenoviral transfection of *in vitro*-derived DCs with control adenoviral vector or with adeno-anti-CTLA-4 will be used to determine the immune modulating functions of the anti-CTLA-4 construct *in vitro* and *in vivo*. Fully and partially mismatched allogeneic transplants and adoptively transferred TCR transgenic T cells will be used as model responder systems. In addition, adenoviral transfection of murine pancreatic islets will be used to test the immune modulating function of the anti-CTLA-4 construct when directly expressed on an allogeneic graft.

Publications related to this project

Griffin MD, Holman PO, Tang Q, Ashourian N, Korthauer U, Kranz DM, Bluestone JA. Development and application of surface-linked single chain antibodies against T-cell antigens. *J Immunol Meth* 2001; 248: 77-90.

References

1. Griffin MD, Ashourian N, Hong DK, Whitters M, Collins M, Segal D, and Bluestone JA. Surface-linked single chain antibodies deliver T cell receptor and co-stimulatory signals to resting T lymphocytes *in vitro* and *in vivo*. *J Immunol* 2000; 164: 4433-4442.
2. Griffin MD, Holman PO, Tang Q, Ashourian N, Korthauer U, Kranz DM, Bluestone JA. Development and application of surface-linked single chain antibodies against T-cell antigens. *J Immunol Meth* 2001; 248: 77-90.
3. Brocker T, Riedinger M, Karjalainen K. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes *in vivo*. *J Exp Med* 1997; 185: 541-550.
4. Nussenzweig MC, Shaw AC, Sinn E, Danner DB, Holmes KL, Morse HC, Leder P. Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin mu. *Science* 1987; 236: 816-819.
5. Boder ET, and Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotech* 1997; 15: 553.

Prof. Jonathan S. Bromberg, Principle Investigator



Mount Sinai School of Medicine, New York, USA

Chemokine Antagonists to Block Alloimmune Responses

Understanding and modifying the immune response in order to prolong allograft survival without acute or chronic rejection, and without the need for long-term immunosuppression, remain the goals of allogeneic transplantation. Most approaches to immunosuppression have relied on the systemic administration of antigen non-specific reagents that suppress multiple cells and arms of the immune response in order to prevent allograft rejection and try to achieve tolerance. In this regard then, local immunosuppression with either antigen specific, or even non-antigen specific modalities, may result in immunosuppressive management which has fewer side effects and deleterious problems, while resulting in equally effective immunosuppression. Indeed, the application of an immunologically non-specific reagent in an anatomically restricted fashion within the allograft, or restricted only to the site alloantigen presentation, may have the functionally equivalent effect of providing alloantigen specific immunosuppression. In this regard then, our laboratory has focussed on gene transfer and gene therapy approaches to immunosuppression for transplantation. The rationale is that the local administration of a gene transfer vector encoding an immunosuppressive molecule, which is subsequently expressed within the allograft, may suppress the local immune response, resulting in prolonged and perhaps indefinite allograft survival, without the effects of systemic immunosuppression and its complications. The current ROTRF project is based upon original preliminary data showing that plasmid mediated gene transfer of the genes vMIP-II and MC148, which are virally derived genes that block multiple CC and CXC chemokine receptors, could suppress the local immune response and prolong allograft survival. Thus, the specific aims were as follows:

- 1) Demonstrate that vMIP-II and MC148 gene transfer inhibit alloimmunity and evaluate their effects on T cells, B cells, and antigen presenting cell (APC) components of the alloresponse.
- 2) Co-transfer vMIP-II or MC148 with other immunosuppressive cytokine genes, such as IL-10, or with conventional immunosuppressive agents to determine if there is a synergistic effect on prolongation of graft survival and inhibition of alloimmunity.
- 3) vMIP-II and MC148 constructs will be humanized by replacing the beta sheet scaffolding of the carboxyl regions for the molecule with hIL-8 beta sheet scaffolding to produce clinically relevant chimeric molecules.
- 4) vMIP-II and MC148 constructs will be used to create adenoviral vectors to be evaluated in the allograft models.

The goal of aim one has been accomplished in one model and a manuscript published, as noted in last year's progress report. Additional optimization protocols for gene transfer were developed and published this year. During the course of studies to characterize T cell, B cell, and APC responses to gene transfer; it has become clear that there are significant innate immune responses, induced by alloantigen, organ transplantation, and also by vector gene delivery. Analysis of these innate immune responses is currently ongoing and our initial findings were published this year in a manuscript entitled, "TNF α and INF γ induced by innate anti-adenoviral immune responses inhibit adenovirus mediated transgene expression". It has become clear that the operative procedure, ischemia-reperfusion, alloantigen, and viral vectors all induce both innate and adaptive immune responses that amplify each other. Thus, there are deleterious effects on graft survival, gene transfer and gene expression, all of which impede the utility of the gene transfer vectors. Current experiments are aimed at further delineating the innate and adaptive immune responses induced by these various stimuli, how these different immune responses interact, and ultimately to devise techniques to prevent innate immune induction to prolong gene expression and efficacy of the gene transfer vector.

The second specific aim has been refocused because of the realization of the significant interaction of gene transfer vectors with innate and adaptive immunity. Thus, co-transfer of vectors encoding different immunosuppressive molecules, such as vMIP-II + IL-10 or MC148 + IL-10, did not result in any further enhancement of gene expression or allograft survival. Indeed, it is clear that innate and adaptive immune responses are limiting the efficacy of gene expression and gene transfer. Current experiments are utilizing other immunosuppressive modalities, such as brief pulses of systemic immunosuppressants, to inhibit initial innate and adaptive immune responses to improve the efficacy of gene transfer and gene expression.

Specific aim three will be initiated during the third year of the grant. Our results to date show that the adaptive immune response to viral components is not the major limiting factor to gene transfer and gene expression. Rather it is innate immune responses to viral and plasmid vector incursions that are the greatest limitation to the efficacy of gene transfer and gene expression.

Specific aim four has been mostly accomplished to date. Adenoviral vectors expressing vMIP-II or MC148 under the control of the elongation factor 1 α (EF1 α) promoter have been constructed. They produce transcripts and protein *in vitro* and are able to prolong allograft survival *in vivo*. Nonetheless, the same issues for these gene transfer vectors are, as noted above, related to the rapid induction of innate immune responses that impair gene expression and therefore gene transfer efficacy. Again, ongoing experiments are investigating the nature of innate immune responses that are induced, the cell types most responsible for those innate responses, and specific techniques for inhibiting those responses at the time of gene transfer.

The field of gene therapy, gene transfer, and gene medicine is now increasingly focussed on methods for improving and regulating gene expression. Increasing attention has been given to the importance of non-specific, innate immune mechanisms that limit vector efficiency and persistence. These innate immune mechanisms include vector clearance by resident macrophages, and the release of cytokines and chemokines, which induce a profound inflammatory response of macrophages, neutrophils, and natural killer cells. This inflammatory response is not dependent on viral gene expression, and viral antigen specific immune recognition is not required. This suggests that viral transduction of both parenchymal cells and leukocytes induces an innate, stereotyped response that inhibits viral function and gene expression. Results to date confirm these ideas. As can be seen from the accomplished and preliminary data noted above, our experimental efforts are being re-directed to understanding the innate immune responses that are induced by gene transfer vectors, defining the cellular and molecular aspects of these innate immune responses and their mechanisms, and using this knowledge to devise strategies to briefly and specifically inhibit the responses in order to significantly improve gene transfer, gene expression, and thus the efficacy of the gene transfer vectors in transplantation.

Publications for the second year of the ROTRF grant

1. Wang Y, Boros P, Liu J, Qin L, Bai Y, Bielinska AU, Kukowska-Latallo JF, Baker JR, Bromberg JS. DNA/dendrimer complexes mediate gene transfer into murine cardiac transplants *ex vivo*. *Molecular Therapy* 2000; 2: 602-608.
2. Qin L, Ding Y, Tahara H, Bromberg JS. Viral IL-10 induced immunosuppression requires TH2 cytokines and impairs APC function within the allograft. *J Immunol* 2001; 166: 2385-2393.
3. Sung RS, Qin L, Bromberg JS. TNF α and IFN γ induced by innate anti-adenoviral immune responses inhibit adenovirus mediated transgene expression. *Molecular Therapy* 2001; 3: 757-767.
4. Wang Y, Bai Y, Price C, Boros P, Qin L, Bielinska AU, Kukowska-Latallo JF, Baker JF, Bromberg JS. Combination of electroporation and DNA/dendrimer complexes enhances gene transfer into murine cardiac transplants. *Am J Transplant* 2001, in press.
5. Bromberg JS, Boros P, Ding Y, Fu S, Ku T, Qin L, Sung R. Gene Transfer Methods for Transplantation. *Methods in Enzymology* 2001, in press.



Dr. Francois Denis, Principle Investigator

Dr. Claude Daniel, Co-Applicant

Dr. Denis Girard, Co-Applicant



INRS-Institut Armand-Frappier, Laval, Canada

Creating Artificial Immunoprivilege for Allograft Acceptance

Excerpt from the original grant application

RATIONALE

Organ transplantation has allowed survival of individuals suffering from organ failure, yet immune recognition of non-self ultimately leads to organ rejection. Immunoprivileged sites are protected from the immune system mainly because Fas ligand (FasL) expression induces infiltrating T cell apoptosis. Researchers have tried to exploit immunoprivilege to improve graft acceptance, but this has revealed that FasL was a chemoattractant for neutrophils, causing infiltration and organ rejection. This novel property of FasL requires characterisation before immunoprivilege can be used to improve graft survival. This research project aims at defining the molecular determinants of FasL involved in chemotaxis and apoptosis to design non-chemotactic, pro-apoptotic forms of FasL. These novel FasL molecules will be engineered to specifically target allografts and their potency will be tested *in vivo* in a murine model of allograft rejection. It is expected that these reagents will create artificial immunoprivilege and induce allograft tolerance.

SPECIFIC AIM ONE: Delineation of FasL determinants involved in apoptosis/chemotaxis

FasL covalent trimers and forced monomers will be created. Membrane-bound and soluble FasL mutants (recombinant and derived from protease cleavage) will be compared *in vitro* for both neutrophil chemotaxis and lymphocyte apoptosis. Biochemical characterisation will be performed and the protease(s) involved in releasing FasL will be identified.

SPECIFIC AIM TWO: Creation of high affinity allograft-targeting molecules

Engineered FasL will be targeted to allografts using donor-specific MHC class-I molecules. Since high affinity is required to avoid non-specific toxicity, antibodies recognising donor MHC will be produced as ScFvs by phage-display and subjected to affinity maturation. Chimeric molecules between mutated FasL molecules and ScFvs will be constructed and tested *in vitro*.

SPECIFIC AIM THREE: Evaluation of the efficacy of artificial immunoprivilege *in vivo*

The efficacy of the constructs will be tested *in vivo* in a model of allograft rejection that allows evaluation of direct and indirect pathways of rejection in skin grafts and heterotopic heart

transplants. Adoptive transfer will be performed to mimic a realistic *in vivo* alloreactive precursor frequency and tolerance induction will be evaluated.

The ROTRF granting committee felt that completion of the first specific aim was essential to the success of the second and third specific aims and therefore provided a two-year grant to prove that the concept that FasL chemotactic and apoptotic properties could be dissociated. Preliminary results obtained in less than a year of funding (the grant was given in October 2000, but funds were not allocated until January 2001) indicate that the working hypothesis was valid. The granting committee did recognise that using single-chain antibodies as a means of targeting allografts was novel and attractive. Although it was suggested that the first specific aim be completed before pursuing the other aims, the encouraging preliminary results have prompted us to undertake the generation of allograft targeting constructs. It will be difficult to attempt to explore the third aim in this short two year funding period.

INTERIM PROGRESS REPORT

INTRODUCTION

While considerable success in organ transplantation has been attained, indefinite graft acceptance remains elusive. Organ rejection ultimately occurs because of recognition of non-self by T lymphocytes. T cells use two mechanisms to induce allograft apoptosis: cytotoxic CD8 T cell degranulation with release of perforin and granzymes, or engagement of Fas by its ligand, FasL, which is expressed by both all T cells. The Fas death pathway also plays an essential role in controlling immune responses. Following clonal expansion and clearance of the offender, expanded T cells destroy other T cells through FasL-Fas interaction. Neutrophils, monocytes/macrophages, activated T and NK cells and immunoprivileged tissues such as the eye, testis and placenta express FasL. FasL is a type II membrane protein, whereas Fas is type I membrane proteins. FasL can be membrane-bound or become soluble following cleavage by metalloproteinases. The biological significance of FasL cleavage remains controversial since soluble FasL (sFasL) can block or induce apoptosis. Membrane-bound and soluble FasL has been shown to exist as monomers, dimers and trimers, the latter being associated with maximal apoptotic activity. Fas multimerisation appears to be required for apoptosis since multivalent antibodies induce cell death, whereas divalent antibodies do not. While it is believed that sFasL is less active than membrane FasL, numerous reports have shown that sFasL is pro-apoptotic. The proteases involved in shedding and precise processing sites remain unknown.

Given the failure of immunosuppressive drugs to afford prolonged allograft survival, researchers have attempted to induce artificial immunoprivilege using FasL. Grafting FasL expressing Sertoli cells together with pancreatic islet allografts afforded graft acceptance in the absence of immunosuppression. A mixture of islet allograft and myoblasts engineered to

express FasL were accepted indefinitely. Transgenic expression of FasL in the thyroid successfully conferred immunoprivilege, although this was expression-level dependent. Xenografted neuroblastoma tumours expressing high levels of FasL were accepted and this was correlated with intratumoral T cell apoptosis. Implantation of FasL-expressing dendritic cells improved allograft acceptance. Other attempts at inducing immunoprivilege proved unsuccessful. Transgenic expression of FasL in pancreatic islets caused accelerated graft destruction due to granulocyte infiltration. Adenoviral-mediated gene transfer of FasL in pancreatic islets lead to rapid graft destruction through neutrophilic infiltration. Xenografted tumour cells engineered to express FasL were rapidly destroyed following neutrophil recruitment. Transgenic expression of FasL in heart allografts led to neutrophil-mediated graft rejection. All failed attempts at inducing artificial immunoprivilege were correlated with PMN infiltration, revealing a novel property for FasL, neutrophil chemotaxis.

Neutrophils play a key role in innate immunity and can be recruited to inflamed sites by several chemokines. While examples of forced FasL expression have led to neutrophil infiltration, it remains unclear whether these effects are direct or indirect as a complex network of cytokines and chemokines might be involved in this phenomenon. As *in vivo* mechanisms of inflammation are complex, micro-environmental differences could be responsible for the contradictory results obtained using FasL for induction of artificial immunoprivilege. The direct chemotactic activity of recombinant sFasL has been demonstrated *in vitro* using purified PMNs. The activity of sFasL depends on Fas since neutrophils from Fas⁻ mice are not chemoattracted. Furthermore, while multivalent antibodies are required to induce apoptosis, both multivalent and divalent anti-Fas antibodies elicit chemotaxis. The *lpr^o* mice carry a point mutation in the death domain of Fas and therefore do not undergo FasL-mediated apoptosis. Since neutrophils from *lpr^o* mice can still respond *in vitro* to sFasL, this shows that signalling events in chemotaxis differ from those in apoptosis.

While it is generally accepted that FasL trimers are required for apoptosis induction, the chemotactic form has yet to be characterised. Structural differences in FasL (soluble vs. membrane-bound; monomeric vs. multimeric) might be involved in distinguishing its chemotactic and apoptotic properties. Given that Fas trimerisation appears to be a prerequisite for apoptosis, it is likely that monomers or dimers could behave differently in chemotaxis. It seems reasonable that chemotaxis might require long range effects that soluble FasL could exert. These hypotheses will be tested by preventing dimer and trimer formation and creating covalent trimers by site-directed mutagenesis. The chemotactic and apoptotic potential of these constructs will be tested *in vitro*. Given that alternative metalloproteinase processing might modulate the biological activity of FasL, the proteases involved will be identified and the influence of processing will be correlated to biological activity. This will allow the design of mutants resistant to inactivating MMP cleavage. To avoid unwanted FasL toxicity, allografts need to be targeted with high specificity. Phage-displayed single-chain antibodies (ScFvs) recognising donor specific class-I molecules will be subjected to affinity maturation. These ScFvs will be genetically fused to FasL mutants having the desired properties.

RESULTS OBTAINED DURING THE FIRST YEAR OF FUNDING

FasL Mutagenesis

Murine FasL was cloned by RT-PCR and sequenced on an automated sequencer available in the laboratory. A FasL trimer was constructed by molecular modelling and mutagenesis strategies were designed using INSIGHT II. Forced monomers were generated by introducing a charged residue at the inner core of the trimer (Y244R) that should prevent trimerisation through charge repulsion. Inspection of trimeric FasL revealed that the L230 and A240 side-chains are located at distances and orientations conducive to interchain disulphide bound formation. Cysteines have been introduced (L230C, A240C) to create covalent trimers. As FasL and TRAIL possess an unpaired cysteine at the same position and this residue has been shown to participate in TRAIL dimer and trimer formation, it was mutated in FasL. A mutant predicted to be unable to form dimers was created by changing cysteine 231 to a serine (C231S). Finally, a FasL mutant unable to interact with Fas (Y218R), has been constructed as a negative control. These mutants have been cloned in the expression vector SR α puroMCS1 and introduced in COS-7 cells using Superfect and Neuro-2a cells by electroporation with a Gene-Pulser.

Functional Analysis of FasL Mutants

Populations of COS-7 transfectants were analysed by RT-PCR and showed similar levels of FasL mRNA expression. FACS analysis of these transfectants revealed virtually non-existent cell surface expression that was significantly enhanced upon treatment with the metallo-proteinase inhibitor O-phenanthroline (OPA) (Fig. 1), showing that COS-7 cells can process and release FasL. Figure 1 also shows that the transfectants express similar levels of FasL as detected with the conformational MFL3 anti-FasL antibody. The mutations introduced have apparently not altered FasL structural integrity as all mutants also showed positive staining

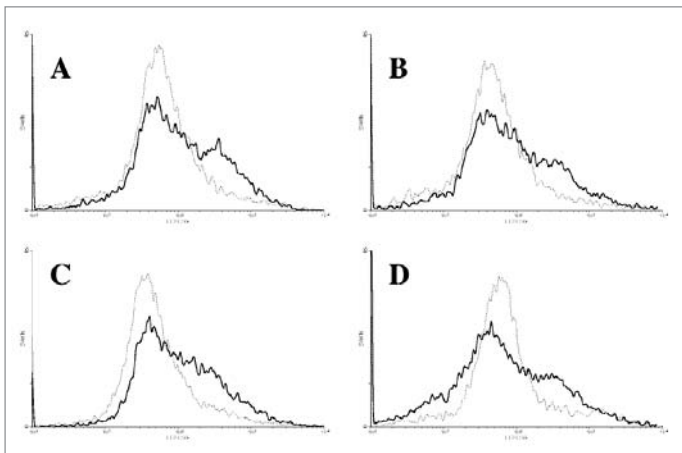


Figure 1. FasL expression in COS-7 transfectants treated with O-phenanthroline.

A. Wild-Type FasL.
B. Mutant L230C, A240C.
C. Mutant C231S.
D. Mutant Y244R.

- - - + OPA 15 min
——— + OPA 6 hrs

with the conformational Kay10 antibody, that recognises an epitope distinct from MFL3. Apoptosis assays were performed by co-culturing transfectants with Jurkat E6.1 cells for 20 hours in the absence of OPA. Even with the low FasL cell surface expression in the absence of OPA, the L230C, A240C trimeric mutant was as efficient as the EOS9.1 anti-Fas IgM antibody and superior to wild-type FasL at apoptosis induction (Fig. 2). Other mutants showed levels of apoptosis similar to untransfected cells. The superior efficacy of the trimeric mutant argues that molecular predictions were accurate. The transfectant populations are only about 20% FasL positive following OPA treatment (Fig. 1) and are currently being cloned by limiting dilution to repeat the assays above. Since COS-7 transfectants have negligible FasL cell-surface expression without OPA treatment, this clearly shows that culture supernatants contain sFasL. Culture supernatants were concentrated 10-fold by Centricon-10 centrifugation and preliminary chemotaxis experiments were performed using 5 mm pore transwells. HL-60 cells were differentiated into PMNs by a five day treatment with 1.25% DMSO and differentiation efficiency was estimated at 85% using the NBT dye reduction assay. HL-60 cells were labelled with CFDA and Figure 3 shows that the monomeric Y244R mutant was as potent as the chemoattractants IL-8 and fMLP, while all other mutants and wild-type FasL were ineffective. These preliminary data indicate that the mutants constructed appear to have distinct functional properties. The monomeric or multimeric nature of FasL molecules (soluble and membrane-bound) is currently being evaluated by western blot analysis using reducing or non-reducing conditions.

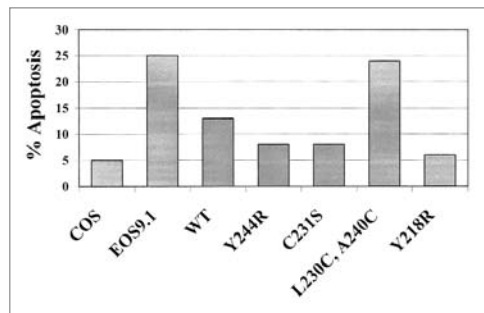


Figure 2. Apoptosis of Jurkat cells induced by FasL transfectants.

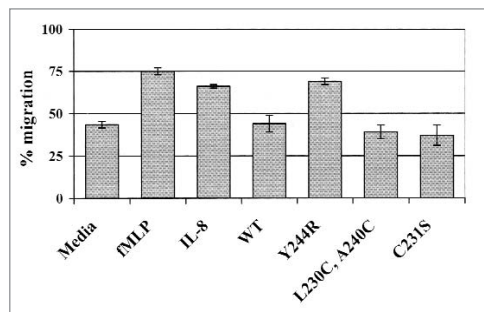


Figure 3. Migration of differentiated HL-60 cells in response to soluble FasL.

Allograft Targeting

Most attempts at inducing FasL-based immunoprivilege in murine models have used transgenics, which are clearly impractical in human subjects. Therefore, alternate targeting means are needed and MHC class I molecules have been chosen as molecular beacons to “coat” allografts with tolerance-inducing constructs. The relatively high affinity of FasL towards Fas (Kd: 7×10^{-8}), requires the targeting part to have superior affinity to avoid unwanted toxicity. Since transplanted organs are to be treated *ex vivo*, not only can systemic treatment be

avoided but apoptosis inhibitors can also be used to minimise potential FasL-mediated graft injury. In the murine model of rejection, allografts express either the p (direct) or b (indirect) MHC class I haplotypes, while the recipient expresses the k haplotype. The B8-24-3 antibody specifically recognises the p and b haplotypes, but not other class I haplotypes. Indeed, the purified antibody stains splenocytes from B10.P (p) and C57BL/6 (b) mice, but not those from B6.AKR (k) mice (Fig. 4). Because the sequence of the antibody was known, degenerated

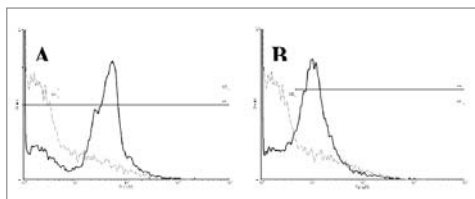


Figure 4. FACS analysis of haplotype specificity of the B8-24-3 antibody.

A. Splenocytes from B10.P mice (H2p).
B. Splenocytes from B6-Eµ/mHEL-Hb mice (H2b).

----- B6.AKR (H2k)
 ————— H2p or H2b splenocytes

oligonucleotides that can amplify all murine heavy and light chains were designed (Table 1). The individual heavy and light chains were individually amplified and cloned into pCR4Blunt-TOPO for sequencing. Ten independent clones from two independent PCRs were sequenced to ensure that PCR errors did not occur. The ScFv was assembled using the 5' Heavy and 3' κ oligonucleotides (Table 1), cloned in the pCANTAB5E phage-display vector and sequenced (Fig. 5). In order to perform panning following ScFv mutagenesis, class I molecules of donors and recipient were cloned by RT-PCR using oligonucleotides listed in Table 3. The β2-microglobulin chain was cloned in SRαneoMCS1 and stable COS-7 transfectants expressed 20-fold more murine β2 than endogenous β2, as assessed by RT-PCR. The individual class I heavy chains (D^p, K^p, D^b, K^b, D^k and K^k) were amplified by RT-PCR, sequenced, cloned in SRαpuroMCS1 and transfected in COS-7 cells expressing murine β2.

Table 1. Oligonucleotides used for heavy and light Ig chain and class I molecule amplification

5' Heavy	Sfi I Q V Q/K L Q Q/E S G TAT GCG GCC CAG CCG GCC CAG GTS MAA CTG CAG SAG TCW GG
3' Heavy	S G G G S G G G G G S S V T V T T G AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC
5' _1	G G G G S G G G S <u>D I V/L/L/M T Q S/T P</u> GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG <u>GAY ATY KTG MTG ACN CAR WCT CCA</u>
5' _2	G G G G S G G G S <u>Q/D I V L T Q S P</u> GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG <u>SA A ATT GTK CTV ACC CAG TCT CCA</u>
3' _	Not I R K IML E L K T G TTT TGC GGC CGC NCG TTT SAK YTC CAG CTT NGT NCC
5' _2	Xho I M A R C GGTCG CTCGAG TCGTCAGC ATG GCT CGC TCG G
3' _2	Bam HI * M D R D TC AGGATCC TTGA TCA CAT GTC TCG ATC CC
5' H2	Eco RI M G A M A P R T L L L GG GAATC ATG GGG GCG ATG GCT CCG CGC ACG CTG CTC CTG
3' H2	Bam HI * A K C D TT GGATCC AGGCAGCTGTCT TCA CGC TTT ACA ATC T
3'H2-Kp	Bam HI * A L S H TT GGATCC CGGCAGCTGTCT TCA TGC TAG AGA ATG T

```

A A Q P A Q V K L Q E S G A E F V R P G A S V K L
GCGGCCAGCCGGCCCAGGTGAAGCTGCAGGAGTCTGGGGCTGAGTTTGTGAGGCCAGGGCCCTCAGTCAAGTTG
S C T A S G F N I K D D Y M F W V K Q R P E Q G L
TCCTGCACAGCTTCTGGCTTAAACATTAAGACGACTATATGTTTTGGGTGAACACAGAGGCCTGAGCAGGGCCTG
E W I G W I A P D N G D T E Y A S K F Q G K A T I
GAGTGGATTGGATGGATTGCTCCTGACAATGGTGATACTGAATATGCCTCGAAGTTCAGGGCAAGGCCACTATA
T A D T S S N T A Y V Q L S S L T S E D T A V Y Y
ACAGCAGACACATCTCCAACACAGCCTACGTGCAGCTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTAC
C T T W G Y Y S Y V N Y W G Q G T T V T V S S G G
TG TACTACATGGGGTACTATAGTTACGTAACTACTGGGGCCAAGGGACCACGGTCCAGCTCTCCTCAGGTGGA
G G S G G G S G G G S D I Q M T Q S P A S L S
GGCGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCCAGATGACTCAGTCTCCAGCCTCCCTATCT
A S V G E T V T I T C R A S G N I H N Y L A W Y Q
GCATCTGTGGGAGAACTGTCAACATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCAG
Q K Q G K S P Q L L V Y N A K T L A N D V P S R F
CAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATAATGCAAAAACCTTAGCAAATGATGTGCCATCAAGGTTT
S G S G S G T Q Y S L K I N S L Q P E D F G S Y Y
AGTGGCAGTGGATCAGGAACACAATTCTCTCAAGATCAACAGCCTACAACCTGAAGATTTGGGAGTTATTAC
C Q H F W S T P W T F G G G T K L E I K R A A A
TG TCAACATTTTTGGAGTACTCCGTGGACGTTCCGGTGGAGGCCACCAAGCTGGAATAAAACGGGCGGCCGCA

```

Figure 5. Sequence of the assembled ScFv derived from the B8-24-3 antibody. The 5' Sfi I and 3' Not I sites are underlined, the middle flexible linker is shown in bold type and the CDR1, CDR2 and CDR3 amino acid sequences of the heavy and light chains are underlined.

EXPERIMENTS TO BE PERFORMED DURING THE LAST YEAR OF FUNDING

Identification of FasL Processing Site and Proteases Involved

The exact cleavage site(s) and metalloproteinase(s) involved in FasL shedding remain ill characterised and it appears likely that alternative processing may yield molecules with different biological properties. Since it is anticipated that selective MMP cleavage might lead to inactivation of FasL biological activity, it is essential to generate FasL molecules that would be resistant to proteolytic inactivation for the successful application of tolerance-inducing constructs *in vivo*. The extracellular domain of murine and human FasL has been cloned in pRSETc for expression as 6xHis-tagged proteins. The site of processing will be identified by producing soluble FasL *in vitro* and translation products will be purified by Nickel affinity chromatography. *In vitro* cleavage will be performed with commercially available MMPs (13 of the 20 known MMPs), namely MMP-1, 2, 3, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17. The processing sites will be identified by mass spectrometry following tryptic digestion. While these *in vitro* experiments will allow identification of the processing site(s) and the protease(s) involved, not all recombinant MMPs are commercially available. Furthermore, substrate accessibility might differ in the context of membrane insertion. To confirm and extend the

in vitro results, FasL will be co-transfected with individual metalloproteinases and processing sites will be identified by mass spectrometry. An expression survey of 13 of the 20 known MMPs has shown that MCF-7 cells only express MMP-15 and 16. In order to verify whether other MMPs are expressed, RT-PCR analysis is currently being performed using oligonucleotides specific to all known MMPs. Individual MMP cDNAs cloned in SR α hygroMCS1 will be transfected in FasL positive cells. Loss of cell-surface expression will be monitored by FACS analysis. Culture supernatants from the large-scale cultures will be harvested to identify the processing sites by mass spectrometry and to perform chemotaxis and apoptosis experiments.

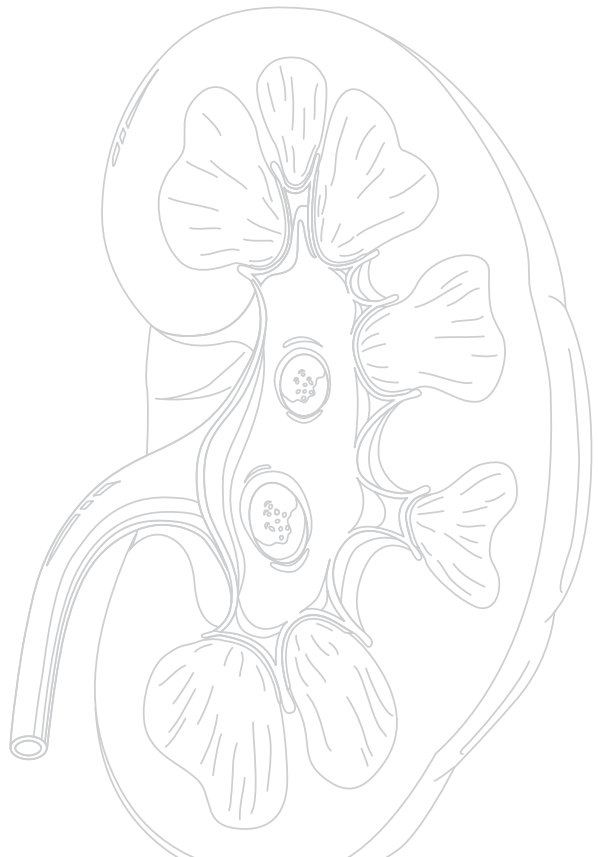
***In vitro* Apoptosis and Chemotaxis**

The engineered FasL molecules, expressed either as membrane-bound forms or produced as soluble forms, will be tested for apoptosis by flow cytometry using AnnexinV-PE and 7-AAD staining. The apoptotic potential of FasL mutants will be evaluated using the following sensitive targets: the Jurkat E6.1 human T cell lymphoma and the 2.102 hybridoma used to derive the TCR transgenic mice used in the *in vivo* model of allograft rejection. Co-cultures of transfectants with target cells will be performed at different cell ratios. Positive controls used for apoptosis will be the 145-2C11 anti-CD3 ϵ antibody for 2.102 and the EOS9.1 anti-Fas antibody for Jurkat. In parallel, cell surface release of FasL will be blocked using metalloproteinase inhibitors O-phenantroline shown to block FasL release. For *in vitro* chemotaxis assays, HL-60 cells have been chosen as these cells respond to soluble FasL when differentiated towards the neutrophil phenotype. Cells will be differentiated into neutrophils using 1.25% DMSO, and differentiation efficiency will be evaluated by cytology. To evaluate the contribution of Fas in neutrophil chemotaxis, murine neutrophils will be isolated from MRL/MpJ (Fas positive), MRL/MpJ^{lpr} (Fas negative) and MRL/MpJ^{lpr-cg} (Fas positive with a mutated Death Domain) mice and the chemotactic activity of FasL will be evaluated using a 96-well MBA96 chamber. Using CFDA-labelled neutrophils, spectrofluorometric quantification of emigrated cells will be performed.

Allograft Targeting

The complementary determining regions of light and heavy chains will be mutated by the PCR overlap extension using oligonucleotides with degenerated bases in the first position of the anticodons. Since mutations located outside of the CDR regions can also modulate the overall antibody affinity, the framework regions will be subjected to PCR mutagenesis with the nucleotide analogues dPTP and 8-oxoGTP that can induce a very high frequency of transitions or transversions. Pools of mutated ScFvs will be screened by panning against cells individually transfected with the D^p, K^p, D^b, K^b molecules (donors) and negative panning against cells transfected with the D^k and K^k molecules (recipient). While the antibody used does not recognise the k haplotype (Fig. 4), negative panning will ensure that the mutants obtained do not show increased reactivity towards recipient molecules. Panning and mutagenesis will be

repeated three times and the mutants obtained through independent mutagenesis showing the highest affinity will be combined together for final affinity maturation. ScFvs showing highest affinity towards the p and b haplotypes will be fused to FasL by a flexible (Gly(Ser)₄)₃ linker.



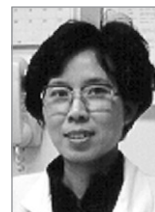
Dr. Lina Lu, Principle Investigator

Dr. Andrew Bonham, Co-Applicant

Dr. Nick Giannoukakis, Research Associate

Dr. Angus Thomson, Consultant

Dr. Paul Robbins, Consultant



University of Pittsburgh, Pittsburgh, USA

Potentiation of Dendritic Cell Tolerogenicity with NF- κ B Antagonist Phosphorothioate Oligonucleotide

PROGRESS REPORT

The proposal was funded for two years as a proof-of-concept study (Aim I and Aim II) by the ROTRF in October, 1999. During the past one and a half years, we have made significant progress towards achieving the goals proposed in Aim I and Aim II.

Aim I. To devise an effective strategy to deliver NF- κ B ODN to dendritic cells (DC) to arrest DC maturation and activation. The ability of dendritic cells (DC) to induce immunity or tolerance is related to their state of functional maturation. We proposed that treatment with short double-stranded oligodeoxyribonucleotides (ODN) with consensus NF- κ B binding sites might maintain DC in an immature state, enhancing their tolerogenicity.

In Aim I, the therapeutic window of NF- κ B ODN, i.e. the most appropriate dose that yields the maximal effect with minimal toxicity, and the duration of action of NF- κ B ODN on DC were determined. DC propagated from bone marrow (BM) with GM-CSF+IL-4 (IL-4 DC) efficiently took up ODN, with FITC-ODN detectable in DC as early as two hours after exposure, and persisted within the cells for more than 14 days without apparent toxicity.

NF- κ B ODN specifically block NF- κ B DNA binding activity:

- 1) Gel shift assays of nuclear/cytosolic protein fractions of cultured DC revealed that NF- κ B ODN bind specifically to cytosolic NF- κ B, blocking nuclear translocation of NF- κ B and subsequent DNA binding. Mutant ODN controls had no such effect.
- 2) Transient transfection with a luciferase transgene expressed under the influence of a NF- κ B promoter element revealed that transcription and transgene expression in response to NF- κ B inducing stimuli were inhibited by NF- κ B ODN, but not by mutant control ODN.
- 3) The production of nitric oxide (NO) in response to LPS, dependent on NF- κ B activation, was completely inhibited by NF- κ B ODN.
- 4) Expression of co-stimulatory molecules (CM) (CD80, CD86, and CD40) was significantly reduced in NF- κ B ODN DC. This was resistant to maturation stimuli, such as IL-4 or interaction with allogeneic T cells. However, costimulatory molecule inhibition required addition of ODN at the initial culture. NF- κ B ODN do not alter CM expression once DC become mature. Compared with mutant ODN DC or untreated DC, the proliferation of allo-T cells stimulated by NF- κ B ODN DC was reduced 80% in MLR, and the levels of

pro-inflammatory cytokines, such as $\text{INF-}\gamma$ and $\text{TNF-}\alpha$ were significantly reduced in the supernatants of MLR as determined by ELISA.

In summary, double-stranded NF- κ B decoy ODN are efficiently taken up by DC under defined conditions, inhibit NF- κ B binding in the nucleus, and arrest DC maturation without toxicity.

Aim II. To assess the influence of NF- κ B ODN DC on immune responses *in vivo* in allogeneic mice. The immunomodulatory effect of donor-derived DC treated with NF- κ B ODN administered to unmodified recipients was compared with that of IL-4 DC (immunostimulatory DC) and TGF- β DC (transiently tolerogenic DC, propagated with GM-CSF+TGF- β).

The fate of NF- κ B ODN DC after *in vivo* administration was determined by immunohistochemical staining with mAbs to anti-donor MHC class II and CD86. NF- κ B ODN did not alter the migration pattern of DC in allogeneic recipients. Increased numbers of NF- κ B ODN DC with low CM expression on their surface were seen in T dependent areas of host secondary lymphoid tissues.

Administration of donor-derived NF- κ B ODN DC before heart transplantation significantly prolonged survival of cardiac allografts in a donor-specific manner, and was associated with dramatically reduced $\text{INF-}\gamma$ production in heart grafts and draining lymph nodes, as determined by RNase protection assay and ELISA. Anti-donor CTL responses were inhibited in recipient spleen T cells.

Cytokine released by NF- κ B ODN DC at rest and after activation were analyzed by RNase protection assay and ELISA. IL-12 production of NF- κ B ODN DC was totally inhibited. In contrast, the expression of IL-12 mRNA in IL4-DC (mature DC) was high, and inducible in TGF- β DC (immature DC) by allostimulation or LPS.

In summary, our results demonstrated that NF- κ B ODN decoys inhibit CM expression and IL-12 production by DC during DC-T cell interaction in allogeneic recipients, resulting in inhibition of alloimmune responses. Further studies on T cell responses induced by DC, in particular focusing on NF- κ B signal transduction pathways, will aid us in designing a molecular therapy for prevention of allograft rejection and for inhibition of autoimmune disease.

Publications and Presentations supported by the ROTRF

1. Giannoukakis N, Bonham CA, Qian S, Chen Z, Peng L, Harnaha J, Li W, Thomson AW, Fung JJ, Robbins PD, Lu L. Prolongation of cardiac allograft survival using dendritic cells treated with NF- κ B decoy oligodeoxyribonucleotides. *Molecular Therapy* 2000; 1: 430-437.
2. Lu L and Thomson AW. Dendritic cell tolerogenicity and prospects for dendritic cell-based therapy of allograft rejection and autoimmunity. In: *Dendritic Cells Biology and Clinical Applications* (2nd edition) (Lotze M T and Thomson AW, eds), Academic Press Ltd, London, 2001.

3. Lu L, Thomson. Manipulation of dendritic cells for tolerance induction in transplantation and autoimmune disease. *Transplantation* 2001 (special issue for Beaune's seminar in transplant research), in press.
4. Bonham CA, Giannoukakis N, Qian S, Chen Z, Li W, Fung JJ, Robbins PD, Lu L. Maturation Arrest of Dendritic Cells by Blockade of NF- κ B with Decoy Oligodeoxyribonucleotides: Potentiation of Dendritic Cell Tolerogenicity. *Transplantation* 2000; 69: S412.
5. Bonham CA, Peng L, Chen Z, Du L, Robbins PD, Qian S, Lu L. Immunomodulation by dendritic cells transfected with CTLA4Ig is enhanced by NF- κ B decoy oligodeoxyribonucleotides. *Am J Transplantation* 2001; 1: 395.
6. Wang L, Liang X, Bonham AC, Qian S, Fung J and Lu L. Cytokine expression by subpopulations of dendritic cells derives differentiation of various T cell subsets. *Am J Transplantation* 2001; 1: 139.
7. Thomson AW and Lu L. Strategies to promote tolerance induction using dendritic cells. In *Therapeutic Immunosuppression* (AW Thomson, Ed.) Kluwer Academic 2001, in press.

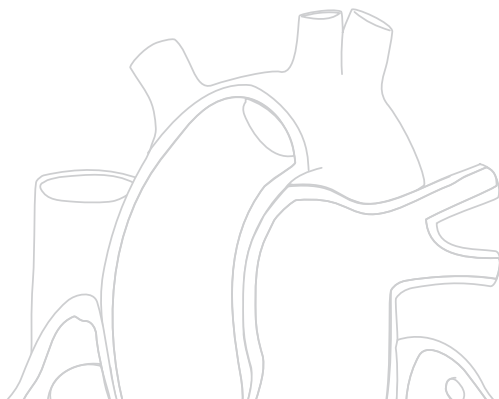
Presentations

The related abstracts were selected as oral presentation at

- 1) the First Joint Annual Meeting of the American Society of Transplant Surgeons and the American Society of Transplantation, May 13-17, 2000, Chicago, IL,
- 2) the XVIII International Congress of the Transplantation Society, Rome, Italy, August 27-September 1, 2000,
- 3) the V International Dendritic Conference, Austria, May 2000, and
- 4) the Joint American Transplant Meeting 2001, Chicago, May 11-16, 2001.

Invited talks:

1. Lu L. The balance between transplantation tolerance and immunity: Prospects of dendritic cell based vaccine. The 11th Walter Brendel Symposium on Applied Immunology and Microcirculation, Maials, Italy, February 5-10, 2000.
2. Lu L. Subsets of dendritic cells in the liver: Implications of the role of dendritic cells in liver transplant tolerance and autoimmune disease. The 4th International Liver Disease Conference, Shanghai, China, September 23-26, 2000.
3. Lu L. Manipulation of dendritic cells for tolerance induction: An overview. Beaune's Seminar, France, June 7-9, 2001.



Dr. Sean O'Herrin, Principle Investigator

Dr. Kenneth A. Newell, Collaborator

Dr. Jeffrey A. Bluestone, Collaborator



University of Wisconsin, Madison, USA

**Suppressing Clonotypic and Bulk Alloreactive CTL
with Soluble Peptide-Loaded MHC Dimers**

Alloreactive T cells play an important role in graft survival. Therefore, the elimination or suppression of their activity is an important goal in transplant therapy. Current methods of suppression and elimination are non-specific, and thus have the side effect of suppressing immune responses in general. This makes the graft recipient vulnerable to infectious diseases and cancer, which would otherwise be responded to by a robust immune system. Therefore, specific suppression, i.e. suppression of only those T cells reactive to the graft, is a major pursuit in transplant immunology. The basis of this research has been to exploit the inherent specificity of the T cell receptor (TCR) for the major histocompatibility complex (MHC), in order to mediate specific suppression of alloreactive T cells. Exploiting this specific interaction has been made possible by previous discoveries that the MHC can be made soluble in dimeric form. The principle investigator of this grant, and others, have demonstrated that this dimeric form is able to interact and stimulate the TCR, whereas the monomeric form is not. We have decided to study whether or not interactions between the dimeric MHC and the TCR of alloreactive T cells *in vitro* and *in vivo* would suppress the T cells in a specific manner, resulting in prolongation of graft life. Furthermore, we are studying whether increased efficacy of suppression can be achieved by linking the dimer to a molecule that will deliver a negative signal.

During the second year of ROTRF funding, the principle investigator relocated from the University of Chicago to the University of Wisconsin. The previous collaborator in the intestinal allograft model, Ken Newell, also relocated during this time, to Emory Medical School. Consequently, a significant amount of time was spent establishing the new laboratories, personnel, and collaborations.

Nonetheless, in the past year, we have demonstrated the efficacy of pepMHC-Ig dimers in prolonging the survival of allograft tissue (O'Herrin et al, *J Immunol* 2001). The data go beyond the efficacy of the dimers and begin to examine their mechanism of action. This was done by demonstrating, at the cellular and molecular level, that pepMHC-Ig dimers activate the alloreactive T cells. This suggests that either aberrant migration away from the graft or activation-induced cell death is the mechanism for the observed prolongation of graft survival.

The character of the signal, as determined by phosphorylation patterns of key molecules, does not suggest that the dimers mediate an inactivation signal.

In order to increase the strength of a negative signal to auto-reactive T cells, it was proposed in Specific Aim 3 to link anti-CTLA-4 to the MHC dimers. This would allow co-engagement of the negatively regulating molecule, CTLA-4, simultaneously with the TCR. As a proof of this principle, we have made a similar construct using a dimer-linked stimulatory molecule for CD28 co-ligation. This proof of principle was done with a co-stimulatory molecule rather than an inhibitory molecule because the interpretation of increased proliferation mediated by a new construct is more straightforward than that of decreased proliferation. The heterodimeric MHC/co-stimulatory construct stimulated a five- to eight-fold increase in proliferation over the homodimeric MHC-Ig of splenocytes from a wild-type mouse. Use of the two unlinked molecules in soluble form resulted in eight-fold less stimulation of proliferation. Meanwhile, the anti-CTLA-4 linked dimer has been made and will soon be tested *in vitro* in a similar fashion. It will then be tested *in vivo* in the model currently being tested, as discussed.

A new collaboration with Dr. Debra Hullett and a Surgery Resident Research Fellow, Dr. Jacquelyn Aschenbrenner, has allowed the testing of Specific Aim 2 wherein the broad applicability of pepMHC-Ig dimers in suppressing a naturally derived alloresponse can be tested. This model involves the transplantation of neonatal heart tissue into the ears of adult recipients. In this model, the hearts begin to beat after about seven days, which can be observed with the aid of a dissecting microscope.

We are testing the grafting of C57BL/6 neonatal hearts into B6bm1 mice, a commonly used allotransplant model. In a test we have just completed, hearts were transplanted into 10 mice with no technical errors. Five mice were treated with an allodimer that is specific to the donor (K^b), and five were treated with an allodimer of no specific relevance to the donor (L^d). This treatment was done on days -1, 2, and 5. In the work described above, such a regimen suppressed the effector function of adoptively transferred primed cells such that graft survival time increased. In this case, this regimen increased effector function of naive cells such that graft survival time was decreased. The mean survival time (MST) of the graft in animals treated with allodimer was eight days, while that of animals treated with the dimer of no specific relevance was 18 days. These data are summarized in Table 1. Beyond the scope of our previous work, these data indicate that dimers loaded with undefined peptide can activate an allo-specific response, in this case, to the demise of the graft. It is likely that longer

Treatment	Mean survival time (days)
Relevant dimer, K ^b (donor MHC)	8
Non-specifically relevant dimer, L ^d	17.8

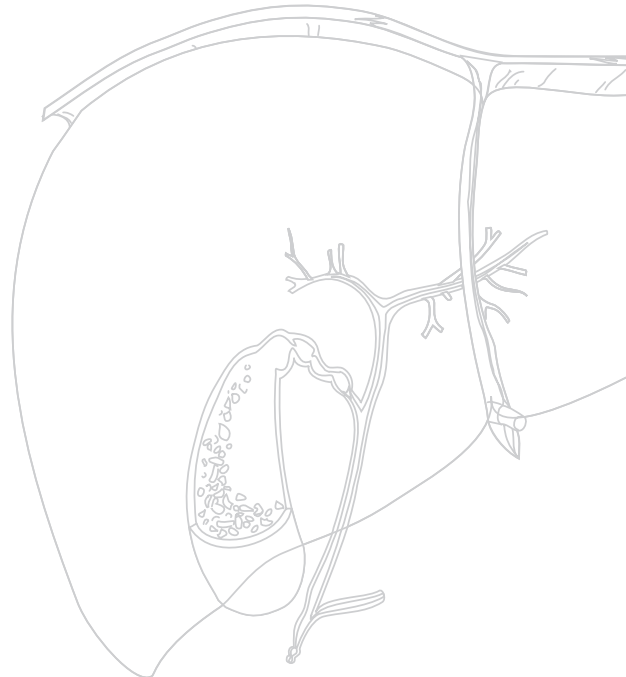
Table 1.

pretreatment, to mediate clonal exhaustion, and/or the use of the anti-CTLA-4/dimer conjugate (Specific Aim 3) will result in suppression of the response.

For the final year of the study, we want to continue use of the ear-heart model as a model of a vascularized graft. Our final aims will include: influence of the nature of the peptide loaded in the dimer on graft prolongation and repertoire selection; timing and quantity of dose for optimal graft survival (including survival time and extent of infiltrates studied histopathologically); efficacy of inclusion of negative signal moiety to dimer. It is expected that these experiments will yield further data.

Publication

O'Herrin SM, Slansky JE, Tang Q, Markiewicz MA, Gajewski TF, Pardoll DM, Schneck JP, Bluestone JA. *J Immunol* 2001; 167: 2555-2560.



Dr. David H. Sachs, Principle Investigator

Dr. Kazuhiko Yamada, Co-Applicant



Massachusetts General Hospital, Boston, USA

Thymic Transplantation to Achieve Tolerance

Introduction

We have made significant progress in year one, in our efforts to demonstrate the ability of vascularized thymic grafts, in the form of composite thymokidneys, to not only reconstitute depleted T cell populations, but also induce tolerance across allogeneic barriers in miniature swine. In year two, we mainly addressed Aim Three of our proposal. The specific accomplishments made in the year 2000-2001 are detailed in the following report.

Specific Aim Three: Test the effect of porcine thymus transplantation on the induction of xenograft tolerance. We have performed a total of 14 thymokidney xenotransplantations using transgenic hDAF swine as donors, and baboons as recipients. Fourteen animals had additional thymic tissue implanted into the omentum at the time of transplantation. The treatment protocol was either a cyclophosphamide and ATG based regimen (1) (with modifications of thymectomy, seven animals), or a T cell depletion regimen using an anti-CD3 immunotoxin conjugated antibody (FN18-CRM9, five animals) (Table 1).

Table 1. Experimental protocols and clinical courses

Expt	Thymectomy	Irradiation	FN18/ATG	Thymokidney Graftectomy	Death	Cause of Death
1	No	700 TI	ATG	11	12	DIC
2	Yes	150 WBI	FN18		7	Intussusception Peritonitis
3	No	700 TI	ATG		13	CV collapse
4	Yes	0	FN18		27	CMV
5	No	700 TI	ATG	18	>200	Euthanised viable thymic structure for 107 days
6	Yes	0	ATG		27	CV collapse Sepsis
7	No	700 TI	ATG	20	24	Peritonitis
8	Yes	0	FN18		12	Sepsis & Peritonitis
9	Yes	0	FN18		11	Myocardial Infarct
10	Yes	0	ATG		2	CV collapse Sepsis
11	Yes	0	ATG	15		Alive POD 77
12	Yes	0	ATG	16	16	Resp. Arrest
13	No	700 TI	ATG	30	32	Sepsis
14	No	700 TI	ATG		12	Rupture of Ao wall

Unfortunately several recipients died, due to either sepsis or cardiovascular collapse, without histologic evidence of rejection. Three animals (Baboons #4, #6 and #13) maintained thymokidney xenografts longer than 27 days. While the kidney xenografts demonstrated variable degrees of humoral rejection, both subcapsular and omental thymic grafts demonstrated the persistence of viable thymic epithelium and Hassal's corpuscles (Fig. 1). One of these three animals with long term xenografts had all immunosuppression stopped and showed reconstitution of lymphocyte counts to pre-transplant levels. MLR analysis (two of these three animals were tested) revealed the return of alloresponses, but with xeno (pig)-specific unresponsiveness *in vitro* (Fig. 2 a, b).

Figure 1. Histologic findings of thymic grafts in the thymokidney (POD 27).

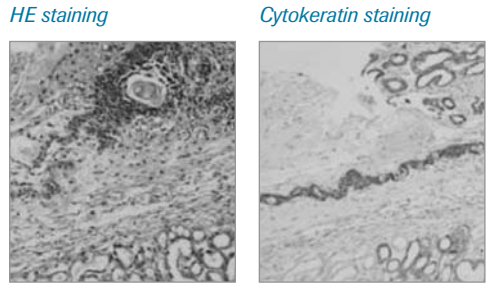


Figure 2. Mixed lymphocyte responses in recipients of thymokidney.

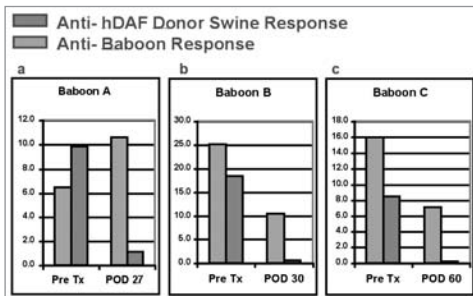
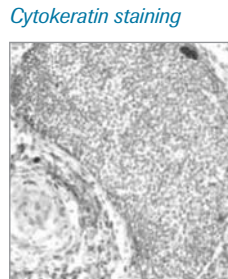


Figure 3. Histologic finding of omentum thymic graft (POD 107).



In addition, in one animal whose thymokidney xenograft was explanted on post-operative day 18, following early evidence of thrombotic microangiopathy (TMA) and systemic coagulation disorder, the omental porcine thymic tissue demonstrated the persistence of viable thymic structure for 107 days [histologic (see Fig. 3) and PCR analysis]. This animal also demonstrated normal alloresponses and a donor pig-specific unresponsiveness persisting for over two months based on MLR assays (Fig. 2c). This *in vitro* unresponsiveness could not be broken with exogenous IL2 stimulation, suggesting that anergy may not be the sole mechanism of this cellular hyporesponsiveness. This animal also demonstrated stable anti-Gal antibody levels, suggesting non-sensitization to xenoantigens.

While we have had problems of loss of recipients due to sepsis or CV collapse, as well as composite kidney graft loss secondary to humoral rejection, we have demonstrated evidence of thymic grafts playing a role in the development of donor unresponsiveness at the cellular

level (1). Two animals have developed donor unresponsiveness at the cellular level, with one animal maintaining donor-specific unresponsiveness for seventy days and maintaining stable anti-Gal antibody levels, suggesting non-sensitization at the humoral level. The omental thymic implants survived up to POD 107 in this same animal (2), with evidence of viable thymic stroma and the development of thymocytes. The data also suggested an importance of the thymokidney xenograft in protecting these omental thymic xenografts during the first weeks post-transplant. This form of shielding permitted these non-vascularized grafts to develop a host-derived vasculature free from xeno-antigen and alpha-Gal epitopes, which are common targets of humoral rejection.

Although we believe that these results made significant progress in this study, the treatment regimen of thymectomy, and nearly complete T cell depletion, has a high associated morbidity. Thus, the immunosuppressive regimen needs to be further optimized to minimize the risk of morbidity (infection, cardiac toxicity, and hepatic toxicity) and to address the humoral mechanisms of thymokidney graft rejection.

Summary

The data in year two have demonstrated the following: (1) thymokidney and thymic tissue xenografts can facilitate the development of donor-specific unresponsiveness and normal alloresponses, with the reconstitution of lymphocyte populations; (2) thymic xenografts do not sensitize humoral responses; and finally, (3) additional strategies to minimize humoral rejection could permit thymokidney plus omental thymic tissue transplantation to succeed in the development of cellular tolerance and long-term xenograft survival.

Plan

In year three, in order to prevent recipient loss due to factors such as infection or drug side effects, we will further optimize our immunosuppressive regimen. We hope that this will result in the prolongation of thymokidney graft survival, and the long-term survival of omental thymic xenografts, with the subsequent development of a donor-unresponsiveness at the cellular level.

We will also extend these studies to vascularized thymic lobe transplantation. Our thymokidney protocol has been limited solely to renal transplantation, and as such, the full potential of the vascularized thymic transplantation technique will only be realized if the scope of its applicability can be broadened. The creation of other thymic-composite organs has proven technically difficult; however, the ability to perform thymic transplantation as a vascularized thymic lobe allograft may permit thymic-facilitated tolerance to occur with any solid organ or tissue transplanted simultaneously.

The technical challenges of vascularized thymic lobe transplantation have limited its widespread application. Vascularized thymic lobe transplantation was first performed in the rat model and was later extended to a mouse model (2); however, no report has been published in any large animal models. With numerous experiences with porcine thymectomies

in our laboratory, we recently established the procedure of vascularized thymic lobe transplant in miniature swine. Our data have demonstrated that (1) vascularized thymic lobe transplantation is technically feasible in a large animal model; (2) vascularized thymic allografts are resistant to ischemic and structural damage following transplantation; (3) vascularized thymic allografts support thymopoiesis of recipient-type cells; and (4) CD4 and CD8 single-positive thymocyte development occurs simultaneously in the allograft (LaMattina et al. Manuscript accepted). Vascularized thymic transplantation has the potential for broad applications for many different organ and tissue transplantations. A model permitting transplantation of vascularized thymic tissue with any solid organ would clearly expand the scope of this protocol. We therefore, plan to transplant porcine vascularized thymic lobe grafts into baboons, using the same regimen used for recipients of the thymokidneys in this study.

References

1. Buhler L, Yamada K, Kitamura H, et al. Pig kidney transplantation in baboons: anti-Gal IgM is associated with acute vascular rejection and disseminated intravascular coagulation. *Transplantation* 2001; in press.
2. Kampinga J, Schuurman HJ, Pol GH, Bartels H, Broekhuizen R, Vaessen LM, Tielen FJ, Rozing J, Blaauw EH, Roser B. Vascular thymus transplantation in rats. Technique, morphology, and function. *Transplantation* 1990; 50: 669.



Prof. Kathryn Wood, Principle Investigator



University of Oxford, Oxford, UK

Phenotype and Function of Immunoregulatory T Cells Responsible for Tolerance to Alloantigens *in vivo*

The objective of this study is to determine the phenotype, function and specificity of immunoregulatory CD4⁺ T cells responsible for the maintenance of tolerance to alloantigens *in vivo* in a mouse model.

Specific Aim I: To determine if by CD45RB^{low} CD4⁺ cells are also responsible for immune regulation in mice following pre-treatment with anti-CD154 and donor alloantigen.

We have shown previously that mice with long term surviving cardiac allografts (Median Survival Time (MST) greater than 100 days) induced by pre-treatment with donor alloantigen and anti-CD4 therapy are operationally tolerant to donor alloantigens *in vivo* as demonstrated by their ability to accept a second donor type but not a third graft.

In the first part of the work for this project, we have examined whether CBA.Ca (H2^k) mice treated with anti-CD154 also develop operational tolerance to C57BL/10 (H2^b), donor alloantigens. When mice treated with anti-CD154 are transplanted with a second heart graft at day 100, only hearts of BL/10 survive; third party, BALB/c (H2^d) hearts are rejected within 20 days.

These data suggest that both anti-CD4 and anti-CD154 therapy can facilitate the development of operational tolerance to donor alloantigens *in vivo*.

Next, we investigated the phenotype and function of CD4⁺ T cells present in the peripheral lymphoid organs of CBA.Ca mice with long term surviving C57BL/10 heart allografts. We were able to show that CD4⁺ T cells present in these mice are heterogenous at the level of expression of one of the isoforms of the leukocyte common antigen (CD45RB) and expression of CD25, the α chain of the IL-2 receptor.

Purification of the different populations of cells by high speed cell sorting enabled us to investigate the functional properties of each of the subpopulations. CD4⁺ cells expressing high levels of CD45RB (CD45RB^{hi}) were able to promote rejection of an allogeneic skin graft when they were transferred to a T cell deficient host. These findings show that even in mice that are operationally tolerant to donor alloantigens, lymphocytes with the potential to reject a graft are present. In contrast, CD4⁺ cells expressing low levels of CD45RB (CD45RB^{low}) were unable to trigger rejection when transferred, but more importantly they were able to prevent the ability of CD45RB^{hi}CD4⁺ cells from either naïve or tolerant mice to trigger rejection of a BL10 skin

graft. CD45RB^{low}CD4⁺ T cells present in mice with long term surviving BL10 cardiac allografts were unable to prevent the rejection of BALB/c skin grafts.

These data demonstrate that CD45RB^{low}CD4⁺ T cells present in mice with long term surviving cardiac allografts have immunoregulatory activity that is specific for donor alloantigens.

Specific Aim II: To determine the role of TGF- β and CTLA-4 in immune regulation by CD45RB^{low} CD4⁺ cells present in the maintenance phase of tolerance

These experiments are in progress.

Publication of the ROTRF funded project

Hara M, Kingsley C, Niimi M, Read S, Turvey S, Bushell A, Morris P, Powrie F, Wood K. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. *Journal of Immunology* 2001; 166: 3789-3796.



Dr. Jiangping Wu, Principal Investigator



Notre-Dame Hospital, Montreal, Canada

Use of Proteasome Inhibitors to Control Rejection and to Induce Long-Term Tolerance of Organ Grafts

The proteasome is an important piece of machinery for protein degradation in a cell. This laboratory was amongst the first to discover that the proteasome has a pivotal role in proliferation and apoptosis of lymphocytes. The objective of this study is to use proteasome inhibitors to prevent graft rejection and to create long-term tolerance by clonal deletion. We will also try to elucidate mechanisms of action of proteasome inhibitors in induction of apoptosis.

With the support of the ROTRF grant, we have studied how proteasome inhibitors induce apoptosis of activated T cells, but leave resting T cells undamaged. This has established a base for further study in using proteasome inhibitors to eliminate antigen-specific activated T cells. The results have been published in the *Journal of Immunology* (Marshansky et al. JI 2001), and the summary of the results is as follows.

The mechanism underlying apoptosis induced by proteasome inhibition in leukemic Jurkat and Namalwa cells was investigated in this study. The proteasome inhibitor lactacystin (LAC) differentially regulated the protein levels of pro-apoptotic Bcl-2 family members and Bik was accumulated at the mitochondria. Bik over-expression was sufficient to induce apoptosis in these cells. Detailed examination along the respiration chain showed that LAC compromised a step after Complex III, and exogenous cytochrome c could overcome this compromise. Probably as a result, the succinate-stimulated generation of mitochondrial membrane potential (Dy_m) was significantly diminished. Bcl-xL interacted with Bik in the cells, and Bcl-xL overexpression prevented cytochrome c leakage out of the mitochondria, corrected the Dy_m defect and protected the cells from apoptosis. These results show that proteasomes can modulate apoptosis of lymphocytes by affecting the half-life of Bcl-2 family members, Bik being one of them.

Our findings imply that if the proteasome inhibitors preferentially induce apoptosis of activated T cells and prolong the graft survival when the drug is administrated *in vivo* after T cell activation, the specific T cells will be deleted, resulting in clonal deletion. Regardless of whether there is a complete or partial clonal deletion, the inhibitors will be especially useful in controlling clinical rejection episodes, which are normally diagnosed when the T cells are already activated, and are less responsive to conventional immunosuppressants.

In the next 12 months, we will further study roles of proteasomes in T cell activation, and

efficacy and side effects of a new proteasome inhibitor dipeptide bononic acid (DPBA) in rat and mouse organ transplantation. Moreover, we are trying to synthesize additional proteasome inhibitors of DPBA derivatives in order to screen for proteasome inhibitors with superior pharmacological features to DPBA and lactacystin.

Publication related to this ROTRF funded project

Marshansky V, Wang X, Bertrand R, Luo H, Duguid W, Chinnadurai G, Kanaan N, Vu MD, Wu J. Proteasomes modulate balance among proapoptotic and antiapoptotic BCL-2 family members and compromise functioning of the electron transport chain in leukemic cells. *The Journal of Immunology* 2001; 166: 3130-3142.

Luo H, Wu Y, Qi S, Wan X, Chen H, Wu J. A proteasome inhibitor effectively prevents mouse heart allograft rejection. *Transplantation* 2001; 72: 196-202.



Dr. Robert Zhong, Principal Investigator

Dr. Anthony M. Jevnikar, Co-Applicant



University of Western Ontario, London, Canada

Induction of Transplant Tolerance by Antibody against CD45RB

Introduction

We previously demonstrated that antibody targeting of a cell surface molecule, CD45RB, can predictably induce tolerance in animal transplant models and we believe that this approach will have an important role in clinical transplantation. Therefore, the purpose of this project is to characterize the mechanisms of tolerance induced by CD45RB therapy in order to allow its rational use in clinical transplantation.

1. Interaction of mAb CD45RB with cyclosporine (CsA) and rapamycin (Rap)

New immunosuppressive agents are usually assessed in combination with traditional agents during early phase clinical trials. Therefore, it is of great importance to determine whether established immunosuppressive agents can augment or antagonize the effect of anti-CD45RB mAb in preclinical studies. The following data demonstrate the importance of pre-clinical evaluation of drug combinations.

Table 1. Murine cardiac allograft survival using adjunctive immunosuppressive therapy

Group	Treatment	Dose	Survival (days)	Median survival +/- SE (days)
1.	Control	none	8 (3), 9 (6), 11 (3), 14	9.6 +/-0.4
2.	Anti-CD45RB mAb	3 mg/kg iv days -1 to 7	34, 42, 43, 57, 63, 113, 120, 124	74.5 +/-13.4
3.	Anti-CD45RB mAb + Cyclosporine	3 mg/kg iv days -1 to 7 5 mg/kg iv days 0 to 6	19, 20, 28, 29 (2)	25.0 +/-2.3 *
4.	Anti-CD45RB mAb + Rapamycin	3 mg/kg iv days -1 to 7 4 mg/kg iv days 0 to 13	>100 (8)	>100 **

* $p < 0.05$ groups 3 vs. 2

** $p < 0.05$ groups 4 vs. 3

These data indicate that CsA inhibits tolerance induced by CD45RB mAb, while rapamycin has a synergistic effect with CD45RB mAb. The precise mechanism of this phenomenon is currently being investigated.

2. Mechanisms of tolerance induction by CD45RB mAb

Earlier studies have hypothesized that anti-CD45RB mAb inhibited Major Histocompatibility Complex (MHC) – T cell receptor (TcR) interaction through steric hindrance, since CD45RB had been shown to be physically cross-linked to the T cell receptor. However, our recent work has shown that direct MHC class II – TcR interactions are critical for tolerance using anti-CD45RB mAb therapy. Treatment of recipients of renal allografts from MHC class II knock-out mice with anti-CD45RB mAb leads to long term engraftment but is associated with chronic allograft nephropathy in all mice, whereas long-term recipient wild-type control kidneys remain free from injury at 100 days. Furthermore, splenocytes from long term recipients with renal allografts from MHC class II knock-out mice given anti-CD45RB mAb, are unable to transfer tolerance, in contrast to wild-type controls, demonstrating that direct engagement with donor MHC class II is required for tolerance induction.

A potential mechanism involves T cell subset shifts. T cell subsets with differing CD45RB isoform expression have significantly different functional capacities. T cells which heavily express CD45RB are considered CD45RB^{hi} (bright), and T cells with lower CD45RB cell surface expression are considered CD45RB^{lo} (dim). The CD45RB^{hi} T cells produce greater amounts of interleukin-2 (IL-2) and interferon gamma (IFN- γ), while the CD45RB^{lo} cells preferentially make IL-4. Rothstein showed that anti-CD45RB mAb therapy dramatically reduces CD45RB expression in graft infiltrating cells and a CD45RB^{hi} to CD45RB^{lo} isoform shift occurred. Taken together, it was hypothesized that anti-CD45RB mAb therapy induces an isoform shift from CD45RB^{hi} to CD45RB^{lo} cells, and that the presence of the regulatory CD45RB^{lo} graft infiltrating cells may be associated with the Th₁ to Th₂ subset shift. We have recently found that CD45RB isoform shift also occurred in renal allografts from CD45RB mAb-treated tolerant animals.

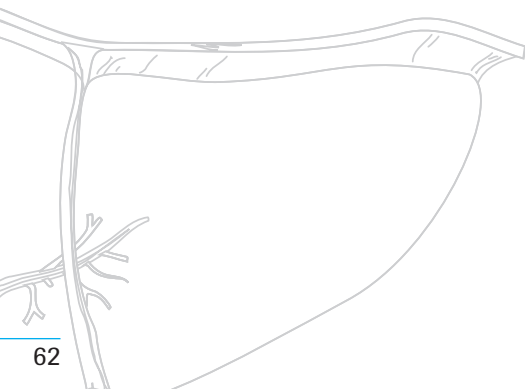
Is there a role for apoptosis in CD45RB induced tolerance? The most stringent model of tolerance is believed to be derived via deletion or apoptosis of allo-reactive T cells. Although a connection between the anti-CD45RB mAb-derived tolerance and apoptosis had never been exhaustively studied, there is evidence to suggest that apoptosis is associated with CD45RB mAb therapy. Klaus *et al.* showed that isoform non-specific CD45 ligation is able to induce programmed cell death in both T and B cells. We have shown that anti-CD45RB mAb markedly reduces leukocyte peripheral blood counts *in vivo*. However, by day 14, the peripheral blood leukocyte counts return to normal levels, despite the absence of graft rejection at this time point. Hence, the immunoregulatory property of anti-CD45RB mAb does not appear to be related to overwhelming depletion of T cells, but may be related to depletion of a specific T cell subset, with subsequent reconstitution of a different, tolerogenic subset. Using annexin V staining, we have shown that anti-CD45RB mAb increases both CD4⁺ and CD8⁺ T cell apoptosis. Additionally, the CD45RB^{hi} cells were found to be more profoundly affected by anti-CD45RB mAb than the CD45RB^{lo} cells, and as a result, there was a shift in the total CD45RB^{lo}:CD45RB^{hi} cell ratio. While it is possible that this selective deletional mechanism contributes to the effector CD45RB^{hi} to regulatory CD45RB^{lo} subset shift and thus

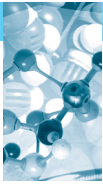
tolerance, the data from our experiments using MHC null donor kidneys strongly suggests that apoptosis is not the sole element responsible for CD45RB mAb-mediated tolerance. However, in the presence of antigen specific activation of T cells, the preferential deletion of CD45RB^{hi} cells might facilitate tolerance induction by a relative overexpression of CD45RB^{lo} regulatory cells, which are antigen specific, and have the capacity to inhibit T cell-mediated rejection. This is currently being studied using *in vivo* models.

The generation of regulatory or suppressor T cells has been implicated as a mechanism for tolerance. However, the surface phenotype of these regulatory cells appears to differ according to the method of tolerance induction. We transplanted C57BL/6 renal allografts into BALB/c mice that were treated with a short course of anti-CD45RB mAb. Splenocytes were harvested from animals surviving more than 100 days, and either bulk splenocytes, CD4⁺ cells, CD8⁺ cells, or B cells were adoptively transferred to naïve BALB/c animals. Indefinite graft survival was achieved in each of three animals (all more than 60 days) transfused with purified CD4⁺ cells, and each of five animals (all more than 60 days) transfused with splenocytes. Adoptive transfer of CD8⁺ cells led to prolonged survival in only one of three animals (56 days), while transfer of B cells was unable to prolong graft survival compared to untreated animals. These data suggest that anti-CD45RB mAb therapy generates immunoregulatory CD4⁺ T cells that have the ability to tolerize naïve T cells. Whether the primary regulatory CD4⁺ T cell has a CD45RB^{lo} phenotype is unknown. We are currently examining the tolerogenic effects of CD45RB^{lo} adoptive transfer from tolerant CD45RB mAb-treated animals into naïve animals.

Publication (waiting for reply regarding ROTRF acknowledgements):

1. Luke P, O'Brien C, Jevnikar A, Zhong R. Anti-CD45 monoclonal antibody-mediated transplantation tolerance. *Current Molecular Medicine* 2001; 1: 521-531.
2. O'Brien C, Luke P, Jiang J, Garcia B, Zhong R, Jevnikar A. Donor MHC class II required for tolerance induction by CD45RB mAb in mouse renal allografts. *American Journal of Transplantation* 2001; 1 (Suppl 1): 224.
3. Luke P, O'Brien C, Zhong R, Jevnikar A. Cell death induced by monoclonal antibody CD45RB (manuscripts submitted)
4. Luke P, Garcia B, Visser AL, Jevnikar A, Zhong R. Monoclonal antibody CD45RB prevents renal allograft rejection and induces tolerance in non-human primates (manuscripts submitted).





5. Final Reports of ROTRF Grantees

Dr. Irma Joosten, Principle Investigator

Dr. Luuk Hilbrands, Co-Applicant

Dr. Elly van de Wiel-van Kemenade, Co-Applicant

University Medical Center St. Radboud,
Nijmegen, The Netherlands



Ex vivo Induction of Regulatory Donor-Specific T cells for Immunosuppression in Transplantation

Background

The *in vivo* generation of immunoregulatory T cells by blocking the B7 (CD86/CD80)-CD28 and/or CD40-CD154 co-stimulatory pathways has great potential for the induction of long-term transplantation tolerance. Already, the direct *in vivo* application of monoclonal anti-CD86 and anti-CD40 antibodies has yielded promising results. However, the *in vivo* use of such agents has a number of potential disadvantages, such as activation of immune components through Fc-receptor interactions, induction of mAb directed antibody formation and a simultaneous blockade of the response against pathogens.

We propose an alternative way of using this co-stimulatory ligand directed mAb approach for the induction of tolerance, namely the *ex vivo* generation of antigen specific immunoregulatory T cells that can be applied as tools in adoptive cell therapy. In view of the time required for modulation of the recipient cells, this approach would be of particular use in transplantations with a graft from a living donor.

Previously, we have shown in a human polyclonal *in vitro* model that combined mAb-blocking of the co-stimulatory ligands CD40 and CD86 leads to allospecific T cell anergy that cannot be reversed by antigenic re-challenge in the presence of IL-2. These anergic T cells suppress the response of naive allospecific T cells in an antigen specific way via linked recognition. Even after five rounds of re-stimulation these cells remain anergic. Using a mouse vascularised cardiac allograft model, we aim to show that the infusion of *ex vivo* anergised T cells can successfully be applied for the prolongation of graft survival.

Results

Our first aim was to demonstrate *ex vivo* induction of T cell anergy by blockade of the B7-CD28 and CD40-CD40L co-stimulatory pathways. Our working hypothesis is that combined blockade of CD86 and CD40, which leaves the negative signalling CD80-CTLA-4 interaction intact, will yield optimum results. In concert with the *in vitro* studies we also set out to show that in our mouse model *in vivo* blockade of CD40L and CD86 leads to prolonged graft survival and possibly even tolerance, whereas this state is abrogated by the simultaneous application of anti-CTLA4 mAb. Finally within the one-year proof-of-concept study we made a start with T cell transfer studies.

To demonstrate energy in the *in vitro* setting, MLC conditions were first optimised with regard to cellular composition, responder-stimulator ratios, and corresponding kinetics so as to ensure consistent and reproducible results.

Primary one-way MLCs were performed by culturing γ -irradiated stimulator splenocytes with T cell enriched responder splenocytes (Figure 1A).

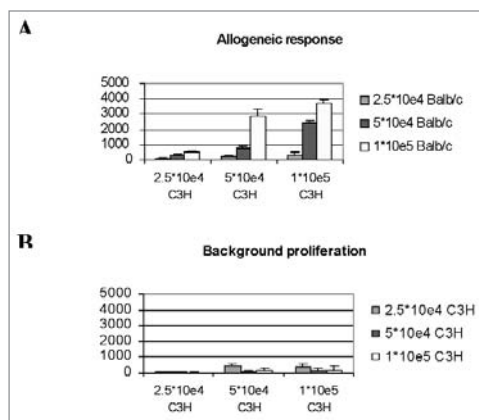


Figure 1A. 2.5×10^4 , 5×10^4 and 1×10^5 C3H responder cells were stimulated with 2.5×10^4 , 5×10^4 and 1×10^5 Balb/c cells (Fig.1 A). The proliferative response of the MLC was examined on day 5 and shows the highest response in a combination of 1×10^5 stimulators and 1×10^5 responders.

B. T cell enrichment of responder cells by depletion of MHC-class II and CD11b positive cells resulted in an abrogation of the background proliferation. Density gradient isolated C3H spleen cells were depleted of MHCII and CD11 positive cells by incubation with magnetic microbeads (MHC Class II (1a) Microbeads and CD11b Microbeads Miltenyi Biotec, Bergish Gladbach, Germany) followed by passage through a LD MACS Cell separation column (Miltenyi Biotec, Bergish Gladbach, Germany).

Responder and stimulator cells were isolated by density gradient centrifugation from spleen cells obtained from Balb/c (H2d) and C3H (H2k) mice. Responder cells (C3H) were T cell-enriched by depletion of MHCII and CD11b positive cells through magnetic cell sorting (Figure 2).

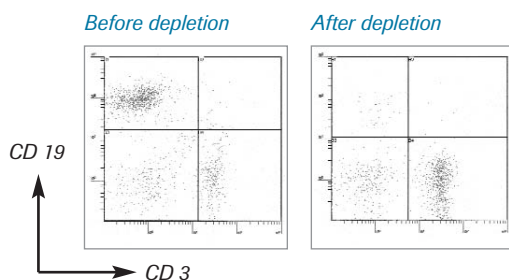


Figure 2. T cell enrichment to reduce background proliferation. FCM analysis before and after T cell enrichment by reduction of the class II and CD11b positive populations in C3H density gradient isolated splenocytes reveals a nearly total depletion of the CD19 positive population. This suggests that B cells are the main source of background proliferation in our system.

T cell enrichment resulted in an abrogation of the background proliferation (Figure 1B). Addition of α CD40L (MR1) and α CD86 (HB253) mAb led to inhibition of the primary proliferative response in a dose-dependent manner. Full inhibition was achieved at doses of around 500 ng/ml (Figure 3).

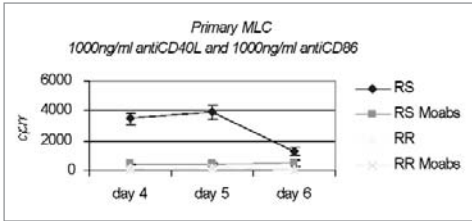


Figure 3. Blocking of primary T cell responses by blocking co-stimulatory pathways. Time-response kinetics of the MLC reveal a maximum response at day 5 (*R*=C3H responder; *S*=Balb/c stimulator). Proliferation was inhibited by blocking co-stimulatory pathways by α CD40L (MR1) and α CD86 (HB253). Concentrations were optimised in the primary MLC by dose-response titrations. Combined mAb blocking of the CD40 and CD86 pathways resulted in successful inhibition of the proliferative response, up to the level of the background proliferation. Here, a representative experiment, using 1000 ng/ml CD40L (MR1) and 1000 ng/ml α CD86 (HB253), is shown.

To generate anergic T cells, MR1 and HB253 were added at the start of a primary bulk MLC. Cells were harvested at day 7 of the primary culture, washed and allowed to recuperate for 2 days. Subsequently, cells were re-stimulated with γ -irradiated stimulator splenocytes. Control T cells responded with secondary proliferative kinetics while T cells from mAb blocked primary cultures responded in a hypo-responsive manner (Figure 4).

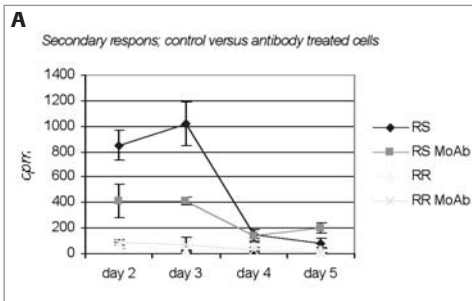
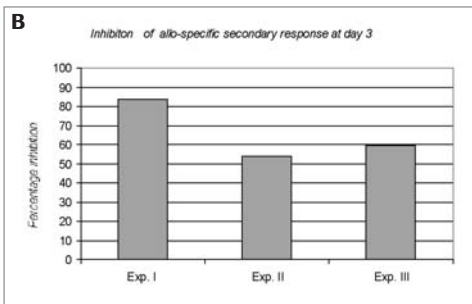


Figure 4. Induction of hypo-responsive T cells in primary MLC by blocking co-stimulatory pathways. Priming in the presence of α CD40L and α CD86 induces hypo-responsiveness.

A. 1000 ng/ml MR1 and 1000 ng/ml HB253 were added at the start of a primary bulk MLC in 96-well culture plates. Cells were harvested at day 7 of the primary culture, washed and allowed to recuperate for 2 days. Subsequently, 2×10^6 cells were re-stimulated with 1×10^6 γ -irradiated (27.5 Gy) stimulator splenocytes. Control T cells responded with secondary proliferative kinetics (max. at D3) while T cells from mAb blocked primary cultures responded in a hypo-responsive manner. The observed hypo-responsiveness was not due to lack of priming, for T cell ignorance during the primary MLC would in result in a response with primary kinetics during a secondary MLC. This was not observed.



B. Hypo-responsiveness was consistently observed in several experiments with inhibition rates of up to 85% as compared to the control cells as analysed on day 3 of culture.

Hypo-responsiveness was consistently observed in several experiments with inhibition rates of up to 85% compared with those of the control cells. The observed hypo-responsiveness was not due to lack of priming, because a neglect of T cells during the primary MLC would result in a response with primary kinetics during a secondary MLC. This was not observed.

The regulatory capacity of the hypo-responsive cells was analysed in an *in vitro* co-culture MLC. From primary MLC, cells were harvested at day seven of culture, washed and allowed to recuperate for two days. Subsequently, γ -irradiated anergic or control cells were added to primary MLC of irradiated stimulator Balb/c cells and C3H responder cells. Up to 74% inhibition of the response was observed compared with an MLC to which were added irradiated T cells derived from an untreated primary MLC (Figure 5).

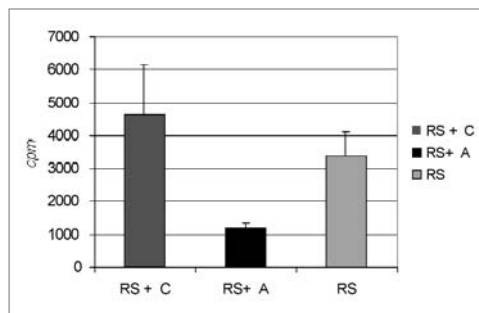


Figure 5. Regulatory capacity of hypo-responsive T cells. The regulatory capacity of the hypo-responsive cells was analysed in an *in vitro* co-culture MLC. From primary MLC, cells were harvested at day 7 of culture, washed and allowed to recuperate for 2 days. Subsequently, 2×10^4 γ -irradiated anergic cells were added to primary MLC of 10^6 irradiated stimulator Balb/c cells and 10^6 C3H responder cells. Inhibition of the response was observed up to 74% as compared to an MLC to which an equal number of irradiated T cells derived from an untreated primary MLC, was added.

Thus, in mice we were able to generate a suppressive T cell population by *ex vivo* blocking of the CD40L and CD86 pathways.

***In vivo* treatment with mAbs in recipients of cardiac allografts**

We have investigated the effect of *in vivo* treatment with mAbs against CD40L and CD86 on allograft survival in the vascularised cardiac allograft model. Eleven recipients have been treated with the combination of anti-CD40L and anti-CD86. Three mice died with a functioning graft at days 16, 49, and 63 after transplantation. In the remaining eight recipients, cardiac graft survival exceeded 90 days. In addition, these long-term acceptors showed donor-specific acceptance of skin grafts. In eight control recipients that did not receive mAbs, the cardiac graft survival varied from 8 to 16 days (MST 11.5 days). To investigate the role of negative CTLA4-signalling in the induction of graft acceptance after treatment with anti-CD40L and anti-CD86, we added the anti-CTLA4 mAb 4F10 to the treatment regimen. In this case, only one out of six recipients accepted the cardiac allograft for more than 90 days (MST 28 days). This suggests that in this model the interaction between CD80 and CTLA4 is indeed required to obtain long-term graft acceptance.

In agreement with previously published findings we observed an interaction between cyclosporine and co-stimulation blocking agents. When cyclosporine was added to the combination of anti-CD40L and anti-CD86, only two out of eight recipients had a cardiac graft survival of more than 90 days (MST 46 days). Tacrolimus did not appear to interfere with the effects of the mAbs. Of the eight recipients that were treated with the combination of tacrolimus, anti-CD40L, and anti-CD86, only one rejected the cardiac allograft within 90 days.

In current *in vitro* studies we are investigating these different effects of cyclosporine and tacrolimus in more detail.

Adoptive cell transfer in recipients of cardiac allografts

The effect of intravenous administration of *ex vivo* tolerised alloreactive cells on cardiac allograft survival time was assessed in a pilot experiment using the Balb/c into C3H model, a transplantation over a full MHC mis-match barrier. Regulatory T cells were generated *in vitro* from spleen cells of C3H mice as described above and viable cells were collected on day six. FCM analyses revealed that transferred cell populations were 95% T cells. In three mice, $2-4 \times 10^6$ cells were transferred intravenously at day 0 of transplantation with Balb/c hearts. No effect on graft survival time was observed. Also, administration of the cells at day one did not have an effect.

Further studies will need to address the number of cells, the time of administration, and the degree of MHC mis-match that can be overcome by this treatment. The effects of simultaneous treatment of the recipient with non-specific immunosuppressive drugs, still essential in clinical transplantation, will be determined, when the effects of regulatory (anergic) cell infusion are known. Finally, we aim to examine the *in vivo* distribution and functional properties of the infused lymphocytes.



Dr. Ezio Laconi, Principle Investigator

Dr. Annibale Donini, Co-Applicant

Prof. Paolo Pani, Research Associate

Dr. Sergio Laconi, Research Associate

Dr. Umberto Baccarini, Research Associate

Dr. Alberto Degrossi, Research Associate



**University of Cagliari & Oncology Hospital “A. Businco”,
Cagliari, Italy**

A Novel Approach for Effective Expansion of Transplanted Isolated Hepatocytes

The present research project relates to the broad field of cell and gene therapy. More specifically, our aim is to explore the potential of isolated hepatocyte transplantation as a means to repopulate a host liver. In fact, many of the disorders treated by liver transplantation are diseases caused by hepatocyte dysfunction, and therefore, it would be unnecessary to replace the entire organ. This is particularly the case for genetic deficiencies of hepatic proteins in which selective replacement of hepatocytes would clearly be therapeutic (1). Within this context, we described a few years ago a new strategy for transplantation of isolated hepatocytes, which uses normal animals and achieves long-term, near-complete liver repopulation (2,3). This model was developed based on two key components: (i) persistent block of the resident hepatocyte cell cycle; (ii) subsequent transplantation of normal hepatocytes, which can selectively expand upon appropriate stimulation, since they are not exposed to the blocking treatment. Retrorsine (RS), a naturally occurring pyrrolizidine alkaloid, was used to inhibit the endogenous hepatocyte cell cycle. Under these conditions we were able to observe greater than 90% repopulation of the resident liver by transplanted hepatocytes within two months post-transplantation (3). In this report, we describe studies aimed at defining the biology and molecular biology of liver repopulation in the RS model.

1. Massive liver repopulation by transplanted hepatocytes in the absence of exogenous growth stimuli (This study was published in *Am J Pathol* 2001; 158: 771-777.)

In one series of experiments we attempted to characterize the best conditions for liver repopulation to occur in this model. Our initial report described massive liver replacement of the RS-treated host liver, when hepatocyte transplantation (Tx) was performed in conjunction with two-thirds partial hepatectomy (PH). However, it was repeatedly observed (3,4) that a low, but significant extent of repopulation was also present in animals not receiving PH (up to about 15% within six weeks, ref. 4). Given the potential relevance of these findings, a long-term study was conducted to follow the fate of transplanted hepatocytes in rats previously given RS and in the absence of any exogenously elicited growth stimulus, such as PH.

Hepatocyte transplantation was performed with modification of our recently published protocol (3). The dipeptidyl-peptidase type IV-deficient (DPPIV⁻) F344 rat model for

hepatocyte transplantation (Tx) was used, as in previous reports (3,4). Briefly, F344 DPPIV⁻ rats, both male and female were given RS followed by Tx (each animal received 2×10^6 freshly isolated hepatocytes from a normal DPPIV⁺ F344 donor rat). Control groups received either RS alone or no treatment. Animals were killed at intervals during the experiment. Results indicated extensive (70–90%) replacement of the recipient liver by donor-derived cells at six to nine months post-Tx, in both male and female rats. Moreover, serum analysis, performed up to one year after hepatocyte infusion, revealed normal values for several parameters related to liver function in the transplanted, repopulated animals (5).

The most relevant conclusion from these data is that massive liver replacement by transplanted normal hepatocytes can take place in rats treated with RS in the absence of any exogenous growth stimulus administered during the process of repopulation. As such, these results represent a significant extension of our previous findings describing total liver replacement in RS-treated rats, when normal hepatocytes were transplanted in conjunction with PH. The kinetics of repopulation in the absence of PH were slower compared to those observed in the original study (3). However, significant liver replacement was already present at two months post-Tx, ($38.2 \pm 6.3\%$), becoming extensive at five months ($65.9 \pm 8.8\%$) and further increasing to greater than 90% between eight months and one year.

We have previously documented that normal hepatocytes transplanted into RS-treated liver express a differentiated phenotype during the process of liver repopulation and perform normal biochemical functions (3). Results presented in this report confirm and extend the above conclusion. In fact, both RS-treated, and RS-treated and repopulated livers, tested normal according to several serum parameters related to hepatocyte function and integrity. It is noteworthy that RS treatment *per se*, when given according to the present protocol for hepatocyte transplantation, does not appear to cause significant impairment of liver function several months after exposure, despite the persistence of extensive areas of megalocytosis (5). Thus, these results provide new insights towards developing strategies for effective liver repopulation by transplanted hepatocytes with reduced toxicity for the host. Extensive liver replacement could in fact be demonstrated in RS-treated animals in the absence any of exogenous growth stimuli, such as PH, thereby avoiding any additional treatment.

2. The repopulated liver: a two-year study (Preliminary findings were published in *Proc Am Assoc Cancer Res* 2001; 42: A3637.)

In this study, the aim was to extend the analysis of the RS model of liver repopulation over a large proportion of the life span of the animals (two years and over). These studies were particularly significant in that, to our knowledge, they were the first of this kind. In fact, in other available models of liver repopulation, animals do not live long enough to address similar issues (6,7). For example, since transplanted hepatocytes undergo several cell cycles in the recipient liver, the possibility for an increased risk of neoplastic transformation in the repopulated liver needed to be examined in detail. Although the available evidence is against such possibility (8), the long-term nature of cancer development was not specifically

addressed in previous reports. Similarly, results from our group and from other laboratories, suggest that liver repopulation is a stable and possibly irreversible phenomenon. However, these studies were designed to extend such information, which is obviously of utmost relevance in a potential clinical setting.

Studies were conducted in both male and female animals. They were treated according to the RS protocol for liver repopulation and killed between 18 and 26 months post-Tx. Massive (85–95%) liver replacement by transplanted hepatocytes was found at 18 months post-Tx, thereby confirming results observed at one year (reported above). Furthermore, the extent of repopulation was similar in groups killed at 24–26 months, with limited (less than 10%) variability among different animals (figure 1). Liver appeared normal both on gross examination and upon histological analysis (figure 2). Dual histochemical-immunohistochemical analysis for DPPIV enzyme activity and PCNA (proliferating cell nuclear antigen) expression, revealed that the transplanted cell population was largely quiescent, with very low (less than 0.1%) proliferative index.

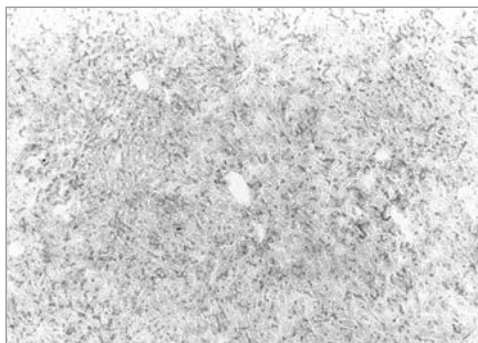


Figure 1. Liver section from a rat treated according to the retrorsine protocol for liver repopulation and killed after two years. Note that almost the entire liver parenchyma expresses the enzyme DPP-IV (orange-rust), a marker of donor-derived hepatocytes. Original magnification: 100 \times .

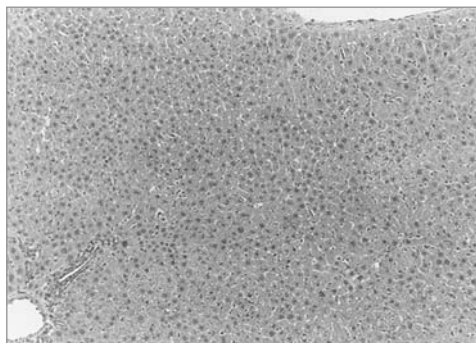


Figure 2. Liver section from a rat treated according to the retrorsine protocol for liver repopulation and killed after two years. Standard H&E staining shows a normal liver histology; greater than 90% repopulation occurred in this animal. Original magnification: 100 \times .

The growth pattern of transplanted cells was also similar to that described in previous reports, i.e. donor-derived hepatocytes integrated into the surrounding parenchyma, forming hybrid bile canaliculi with hepatocytes in the host liver and finally replaced the bulk of the organ. No evidence of a nodular type of growth was seen at any time point considered, including two years and over after Tx. In addition, transplanted cells were never found to over-express the enzyme glutathione-S-transferase 7-7, a marker that is often up-regulated during carcinogenesis in rat liver. Such findings lead to the important conclusion that no increased risk of neoplastic transformation appears to be associated with the process of liver repopulation. While other studies have supported a similar conclusion (8), the present results are of

particular significance in that they were obtained after an extended follow up of the animals, i.e. over two years post-Tx. Such an extended period of follow up was particularly important, given the long-term nature of cancer development.

Appropriate control groups were also considered in our studies. A few (2/10) animals receiving RS only (no Tx) developed hepatocyte nodules at the end of two years (figure 3). The nodules were composed of small hepatocytes, while areas of residual megalocytosis were evident in surrounding liver. No evidence of overt HCC was found on microscopic analysis. Finally, when hepatocytes were transplanted into previously untreated animals, no significant growth of donor-derived cells was seen, in agreement with previous observations from us and from other authors.

Taken together, our data provide the following information on the biology of liver repopulation by transplanted hepatocytes: (i) the process appears to be largely irreversible, in that virtually no variation in the extent of liver replacement was found during more than two years of follow up. This is obviously of utmost significance, in that the irreversible nature of the repopulation process would be a highly desirable prerequisite in a clinical setting. (ii) No increase in the risk of neoplastic transformation appears to be associated with the process of clonal expansion, which is inherent to liver repopulation by transplanted cells. While such observation is intriguing in terms of basic biology, it is also particularly encouraging from a clinical viewpoint and makes one more optimistic of the possible therapeutic impact of normal hepatocyte transplantation.

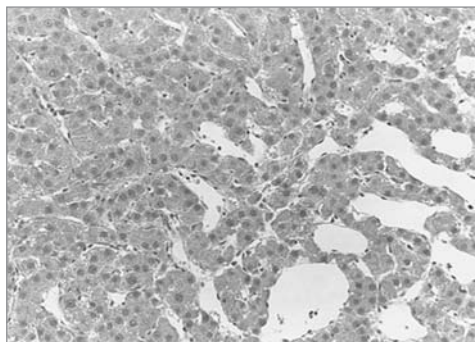


Figure 3. Liver section from a rat treated with retrorsine, without transplantation of normal hepatocytes. A few animals (2/10) in this group developed hepatocyte nodules, as shown in this figure. H&E, 200 \times .

3. Towards the elucidation of molecular mechanism(s) involved in liver repopulation in the RS model.

The experimental evidence obtained so far is consistent with our initial hypothesis (3) that the long lasting block exerted by retrorsine on hepatocyte cell cycle represents a key component for the growth of transplanted hepatocytes in this system. While several agents are known for their ability to transiently inhibit hepatocyte proliferation, the persistent cell cycle block imposed by retrorsine is rather uncommon. Thus, gaining insights into the molecular bases of such a block may help to elucidate the general mechanisms necessary for liver repopulation to occur. Pyrrolizidine alkaloids, including retrorsine, were reported to inhibit G2-M phase of hepatocyte cell cycle. Data from our laboratory have indicated that retrorsine can also affect S phase (9). Based on these considerations, we have analyzed the expression of cell cycle related gene products in resting liver and during liver

regeneration following two-thirds partial hepatectomy (PH) in rats treated with RS. Young adult Fischer 344 rats were given RS according to the protocol for hepatocyte transplantation, while control animals received 0.9% NaCl solution. Four weeks after the second injection, PH was performed and animals from both groups were killed at different time points thereafter. Livers were analyzed for the expression of gene products, which are known to be cell cycle dependent and to play a role in cell cycle regulation. A consistent increase in the mRNA levels of cyclin D1 was found in resting liver exposed to RS compared to untreated controls (figure 4). Furthermore, such levels remained high following PH, with little variation during the first 48 hours. By contrast, expression of cyclin D1 mRNA was low in resting liver of control rats and increased in response to PH, as expected. No major differences between RS-treated and control animals were found in the expression pattern of other cell cycle genes, including HGF, TGF- α , ODC and cyclin E. Additional gene products are currently being examined. Based on these data, it is reasonable to hypothesize that cyclin D1 could play a role in the mechanism

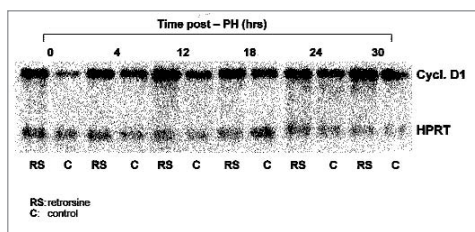


Figure 4. Northern blot of liver mRNA probed for Cyclin D1. Samples were taken from RS-treated and control animals killed at various times following two-thirds partial hepatectomy (PH). Cyclin D1 expression was consistently increased in rats receiving RS.

of liver repopulation by transplanted cells in this model. While endogenous hepatocytes are blocked by RS and are therefore unable to respond to any stimulus, transplanted cells could in fact be driven by the increased cyclin D1 levels to enter the cycle and eventually divide. Interestingly, the levels of cyclin D1 mRNA were already high before PH was performed, suggesting that a growth promoting environment is possibly present in RS-treated liver before PH is performed. Consistent with this finding, normal hepatocytes were able to proliferate upon transplantation into RS-treated rat liver even when no exogenous growth stimuli, such as PH, were applied (see above and ref. 5). Within this framework, it will be important to determine how the expression of cyclin D1 is regulated in animals exposed to RS. While it could serve as a stimulus for the selective expansion of transplanted cells, we have also observed that the latter cells do not grow indefinitely. In fact, repopulated livers were never found to exceed the normal reference mass for that organ, suggesting that the entire process of repopulation is controlled by homeostatic mechanisms which are normally operative in all animals. In addition, the proliferative index of transplanted hepatocytes is very low once massive liver replacement has been attained. This suggests the existence of very fine tuned mechanisms involved in the process of liver repopulation. The level of cyclin D1 could well represent a critical component in this complex network.

In summary, the studies outlined in this report have significantly extended current knowledge of the process of liver repopulation by transplanted cells. We have learned that massive liver replacement can occur in the absence of exogenous growth stimuli, making the procedure

less invasive for the host. We have also observed that liver repopulation is largely (if not entirely) irreversible for at least two-thirds and over of the animal life span. Furthermore, it poses no inherent risk of neoplastic transformation for transplanted cells, as shown after over two years of follow up.

The molecular mechanisms sustaining liver repopulation in this model are beginning to be clarified. A role for cyclin D1 appears to be emerging. Such new information will help in the design of better strategies with potential clinical applicability.

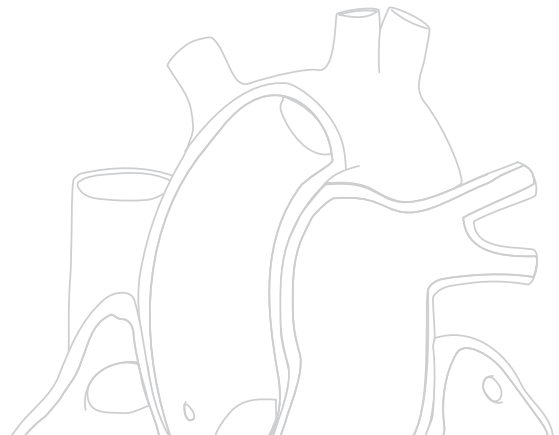
Publications related to this project

Laconi S, Pillai S, Porcu PP, Shafritz DA, Pani P, Laconi E. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. *American Journal of Pathology* 2001; 158: 771-777.

Laconi S, Pani P, Pillai S, Pasciu D, Sarma DSR, Laconi E. A growth constraint environment drives tumor progression *in vivo*. *PNAS* 2001; 98: 7806-7811.

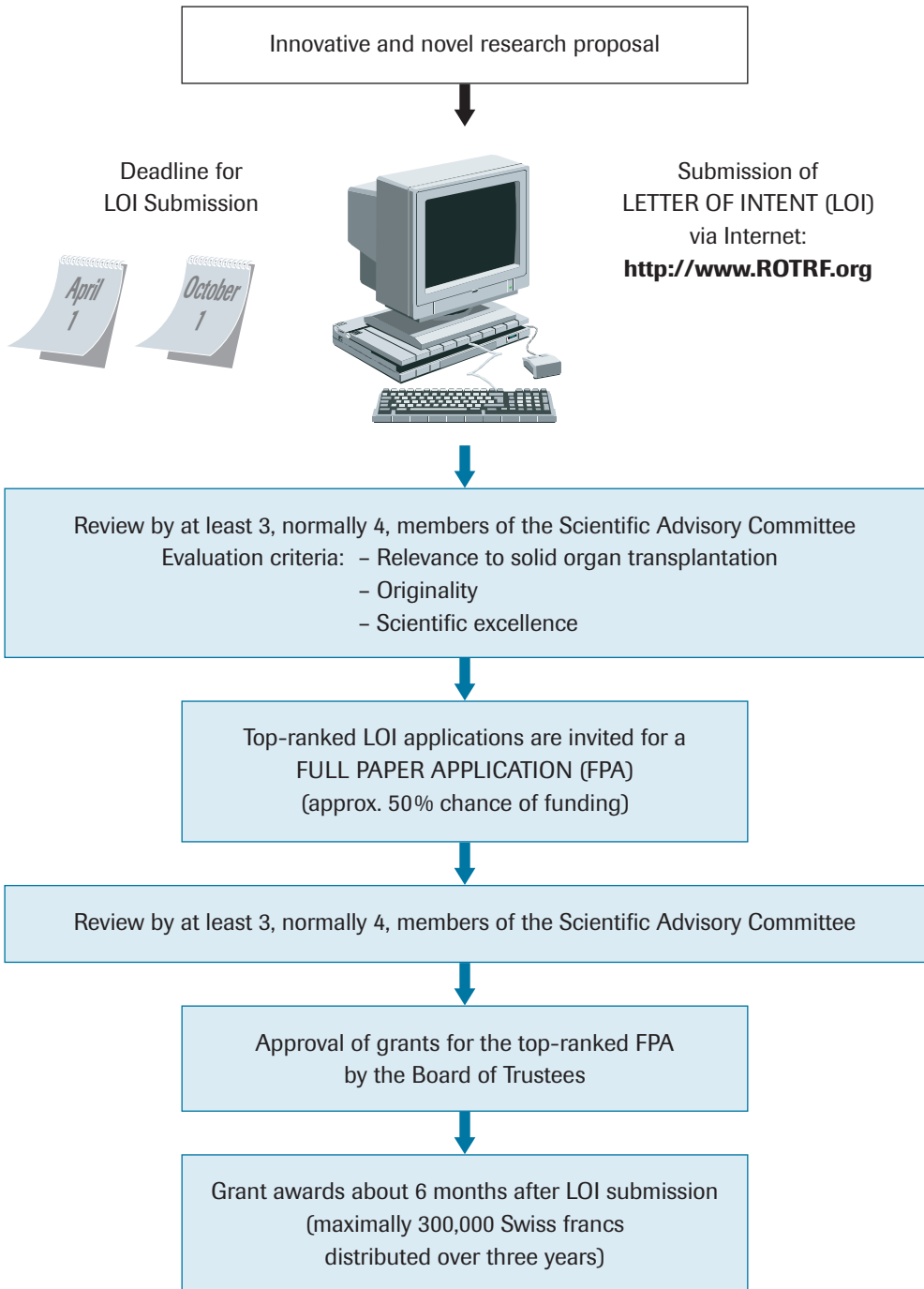
References

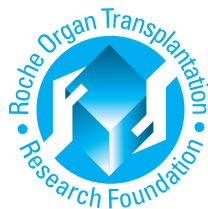
1. Kay MA and Woo SLC. *Trends Genet* 1994; 10: 253-257.
2. Laconi E, Sarma DSR, Pani P. *Carcinogenesis* 1995; 16: 139-142.
3. Laconi E, Oren R, Mukhopadhyay D, Hurston E, Laconi S, Pani P, Dabeva M, Shafritz DA. *Am J Pathol* 1998; 153: 319-329.
4. Pani P, Laconi S, Pillai S, Scintu F, Curreli F, Shafritz DA, Laconi E. *J Hepatol* 1999; 31: 354-359.
5. Laconi S, Pillai S, Porcu PP, Shafritz DA, Pani P, Laconi E. *Am J Pathol* 2001; 158: 771-777.
6. Rhim JA, Sandgren EP, Degen JL, Paimiter RD, Brinster RL. *Science* 1994; 263: 1149-1152.
7. Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou C-N, Finegold M, Grompe M. *Nat Genet* 1996; 12: 266-273.
8. Overturf K, Al-Dhalimy M, Ou C-N, Finegold M, Grompe M. *Am J Pathol* 1997; 151: 1273-1280.
9. Laconi S, Curreli F, Diana S, Pasciu D, De Filippo G, Sarina DSR, Pani P, Laconi E. *J Hepatol* 1999; 31: 1069-1074.





6. How to apply for an ROTRF grant





**To apply, please visit our website,
<http://www.ROTRF.org>**

Questions? Please contact us.

E-mail: admin@ROTRF.org
Tel.: +41 41 377 53 35
Fax: +41 41 377 53 34
Mail: ROTRF, Postfach 222
6045 Meggen, Switzerland