R O T R F

Roche Organ Transplantation Research Foundation

ANNUAL REPORT 2006

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 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 at universities, transplant centres and research institutes. The Foundation supports research in solid organ transplantation, particularly where there is an unmet medical need.

The funding of the foundation consists of donations from F. Hoffmann-La Roche Ltd, with an initial sum of 25 million Swiss Francs over the first five years and renewal donations of 15 and 10 million Swiss Francs for the following five years (a total of 50 million Swiss Francs over 10 years). The funds are distributed as grants of up to 300,000 Swiss Francs distributed over three years. The foundation is legally independent from Roche and is guided solely by the Board of Trustees according to its charter.

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1. Preface

On behalf of the Board of Trustees of the Roche Organ Transplantation Research Foundation (ROTRF), it is my pleasure to announce that in Cycle XV (submission deadline October 2005) and XVI (submission deadline April 2006), ROTRF grants were awarded to 16 conventional and 4 clinical research projects. In total, 2.1 million CHF and 1.5 million CHF were awarded in Cycle XV and Cycle XVI, respectively.

The ROTRF and the Juvenile Diabetes Research Foundation International (JDRF) have joined forces in 2005 and created a new joint grant award. This initiative has attracted the interest of many investigators from around the world. Over 50 Letters of Intent were submitted for this joint initiative during the first submission cycle (deadline 1st April 2006). This grant specifically supports investigators working in the fields of immunology and cell biology, gene therapy, cellular engineering, stem cell research and other approaches that are relevant to both type 1 diabetes and transplantation. In Cycle XVI, three joint ROTRF/JDRF grants, totalling 700'000 CHF, have been awarded to investigators from three continents (USA, Australia and Europe).

The ROTRF continues to support high quality research of investigators working in the area of organ transplantation from around the world. We hope that the interest in these grant awards, ROTRF (conventional and clinical) and ROTRF/JDRF, will continue to attract many high quality and innovative applications.

In 2006 the ROTRF was present at two congresses, the 2006 World Transplant Congress in Boston, USA, and the Annual Congress of the British Transplantation Society in Edinburgh, UK. At these and other national and international congresses, several grantees have presented the results of their research projects highlighting the quality of the work supported by the ROTRF. This is further demonstrated by the quality of the project reports included in the ROTRF Reports, and the papers published in peer-reviewed journals.

Thanks to the generous support from F. Hoffmann-La Roche Ltd since 1998, the ROTRF has funded outstanding research projects which have contributed to the progress of understanding and improving organ transplantation. For this opportunity, the ROTRF is greatly thankful to F. Hoffmann-La Roche Ltd.

The ROTRF Board of Trustees would like to thank the ROTRF Scientific Advisory Committee and the ROTRF grantees for their excellent work and support, which have contributed to the overall success of the Foundation. The ROTRF also wishes to thank the JDRF for their input and enthusiasm in joining with the ROTRF in the new grant award competition.

Finally, ROTRF wishes the newly-granted investigators of cycles XV and XVI success with their research.

On behalf of the Board of Trustees

Philip F. Halloran, MD, PhD, OC

2. Facts and Figures

Funding Cycles XV and XVI – Letters of Intent Submission in October 2005 and April 2006

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that in Cycles XV and XVI, grants have been awarded to *nineteen conventional* and *four clinical research applications,* including three conventional grants in Cycle XVI for the joint ROTRF/JDRF grant award competition. In Cycle XV, 2.1 million Swiss francs (CHF) were allocated to twelve research projects; 2.2 million CHF were distributed to 11 projects in Cycle XVI. The quality of the applications received once again delighted the Board of Trustees and the Scientific Advisory Committee of the ROTRF.

The research funded in both cycles focuses on the understanding of clinical and scientific aspects of transplantation, such as processes involved in the induction of tolerance, sensitisation, graft rejection, development of chronic allograft nephropathy, graft vascular disease, ischaemia, hypoxia, migration of lymphocytes into inflammed allograft tissue, and elucidation of the cellular/molecular pathways of islet allograft destruction in diabetic hosts.

The ROTRF received 83 Letters of Intent in Cycle XV up to the submission deadline (October 2005) from scientists around the world. Of the applications, 45.8% were received from Europe, the major countries being Germany (14.5%), UK (9.6%), and France (4.8%). 48.2% applications were received from North America: United States (43.4%) and Canada (4.8%). Australia/New Zealand (3.6%), South America (1.2%), and Asia (1.2%) accounted for the remaining 6% of the applications. Based on the reviews of the Scientific Advisory Committee (SAC), the Board of Trustees invited 22 applicants to prepare Full Paper Applications. After a thorough review of the Full Paper Applications, grants were awarded to the 12 projects.

In Cycle XVI, 144 applications were received up to the submission deadline (April 2006); 48.6% of the applications were received from Europe and 43.8% from North America. The European submissions were received mainly from the UK (16.7%), Italy (6.9%), Germany (6.3%) and Switzerland (6.3%). 38.9% of the applications came from the USA and 4.8% from Canada. The remaining applications were received from Australia/New Zealand (5.6%) and Asia (2.1%). Following review from the SAC, the Board of Trustees invited 28 applicants to prepare Full Paper Applications and grants were awarded to 11 research projects, including 3 from the ROTRF/JDRF Joint Initiative.

The abstracts of these newly awarded grants (Cycles XV and XVI) are published in the first pages of this Annual Report 2006 and on the ROTRF homepage.

Statistics on Applications to the ROTRF

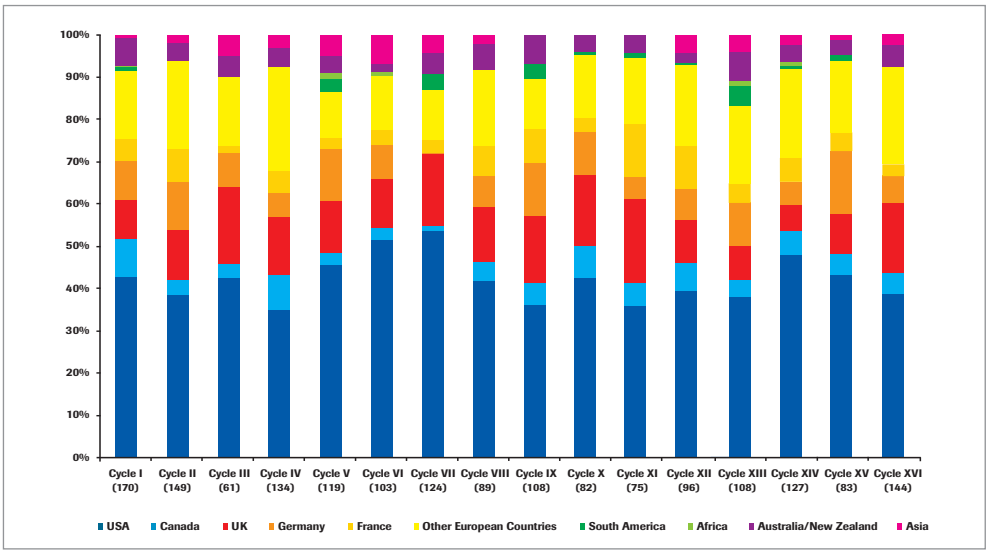


Figure 1. *Geographical distribution of the applicants who submitted Letters of Intent (LOI) during the first sixteen ROTRF funding Cycles. The total number of LOIs submitted per cycle is shown in brackets.*

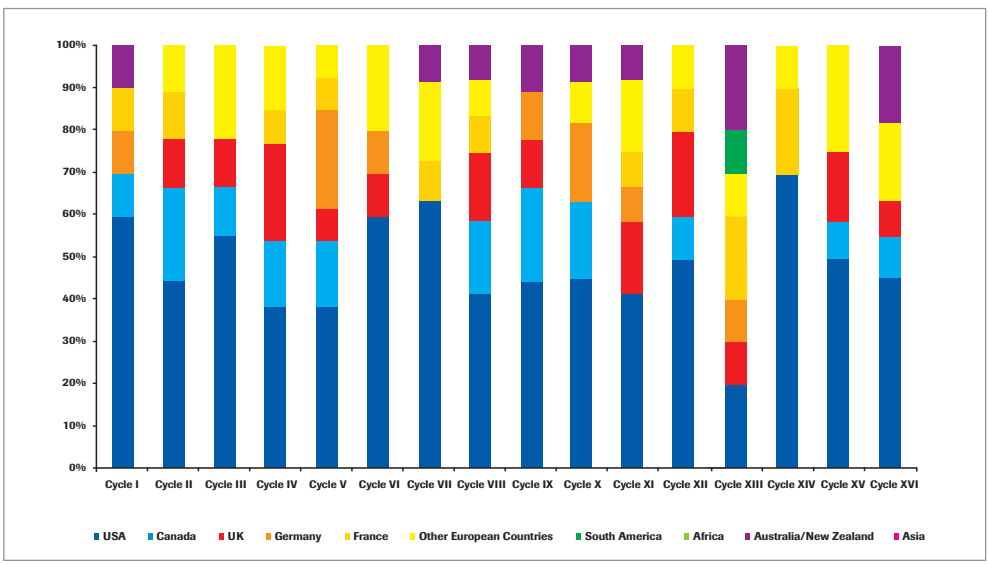
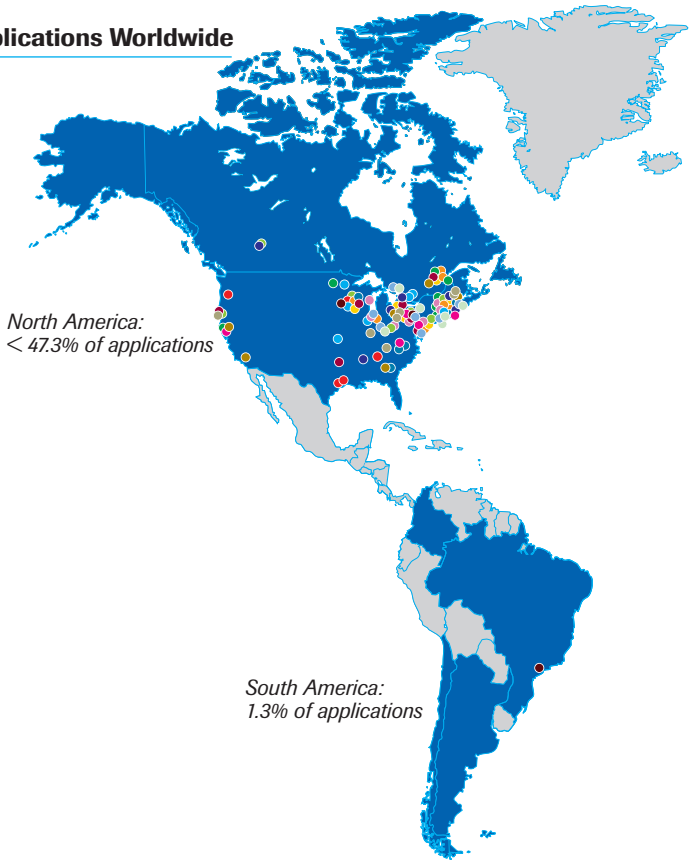


Figure 2. *Geographical distribution of the applicants who were awarded ROTRF grants during the first sixteen ROTRF funding Cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF Applications Worldwide



at least one application ever received

no application received

Grant Awards in:

2006

Cycle XV & XVI

Amsterdam, The Netherlands	*Canberra, Australia*	*London, Canada*	*Parkville, Australia*
Baltimore, USA	*Chicago, USA*	*London, UK*	*Philadelphia, USA*
Bergamo, Italy	*Columbus, USA*	*Newcastle-upon-Tyne, UK*	*Zürich, Switzerland*
Boston, USA	*Helsinki, Finland*	*New Haven, USA*	
	Leeds, UK	*Nijmegen, The Netherlands*	

2005

Cycle XIII & XIV

Atlanta, USA	*Fitzroy, Australia*	*Pittsburgh, USA*	*Toulouse, France*
Boston, USA	*Heidelberg, Germany*	*San Francisco, USA*	*Utrecht, The Netherlands*
Cambridge, UK	*Leiden, The Netherlands*	*São Paulo, Brazil*	
Cleveland, USA	*Madison, USA*	*Sydney, Australia*	
Columbus, USA	*Nantes, France*	*St. Luis, USA*	

2004

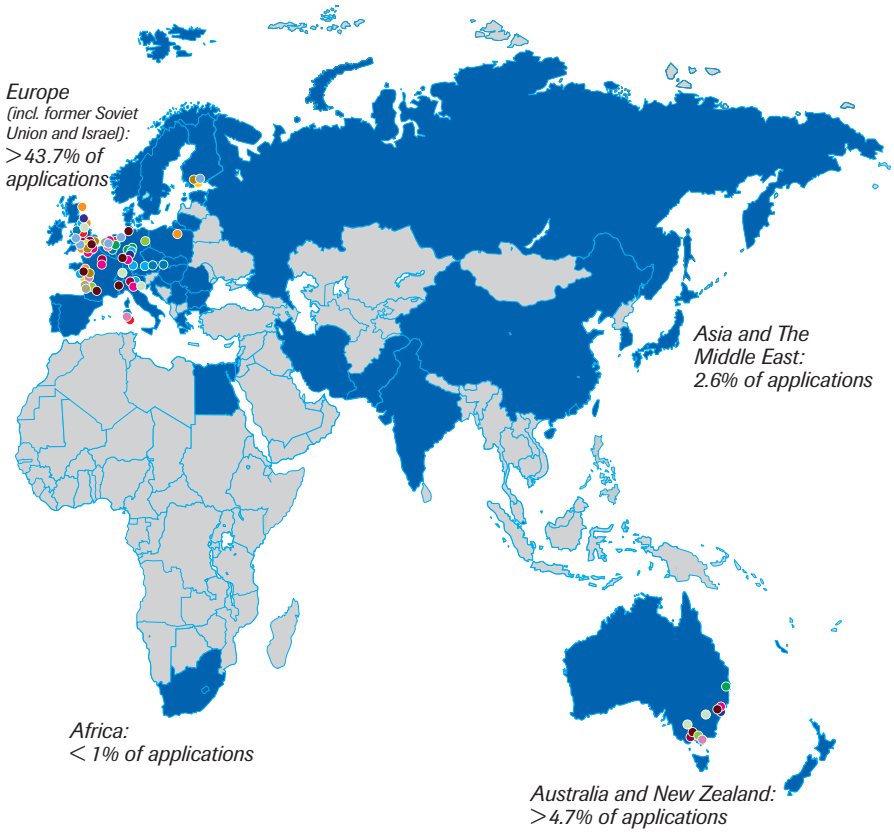
Cycle XI & XII

Augusta, USA	*Chapel Hill, USA*	*Leiden, The Netherlands*	*Paris, France*
Baltimore, USA	*Cleveland, USA*	*London, UK*	*Pittsburgh, USA*
Bergamo, Italy	*Giessen, Germany*	*Los Angeles, USA*	*Stanford, USA*
Boston, USA	*Helsinki, Finland*	*Nantes, France*	*Sydney, Australia*
Cambridge, UK	*Laval, Canada*	*New Haven, USA*	

2003

Cycle IX & X

Birmingham, USA	*Columbus, USA*	*Iowa City, USA*	*New Haven, USA*
Boston, USA	*Edmonton, Canada*	*London, Canada*	*Sainte-Foy, Canada*
Brussels, Belgium	*Heidelberg, Germany*	*Montreal, Canada*	*Stanford, USA*
Charlestown, USA	*Herston, Australia*	*Newcastle-upon-Tyne, UK*	*Sydney, Australia*



2002

Cycle VII & VIII

Baltimore, USA	College Station, USA	Melbourne, Australia	Paris, France
Bergamo, Italy	Columbus, USA	Milwaukee, USA	Pittsburgh, USA
Boston, USA	Heidelberg, Australia	Montreal, Canada	San Francisco, USA
Brussels, Belgium	London, Canada	Nantes, France	
Cagliari, Italy	London, UK	New York, USA	
Chicago, USA	Madison, USA	Oxford, UK	

2001

Cycle V & VI

Augusta, USA	Chicago, USA	Madison, USA	Oklahoma City, USA
Bergamo, Italy	Durham, USA	Manchester, UK	Regensburg, Germany
Boston, USA	Edgbaston, UK	Minneapolis, USA	Toronto, Canada
Brussels, Belgium	Hanover, Germany	Munich, Germany	Vienna, Austria
Chapel Hill, USA	Heidelberg, Germany	Nantes, France	

2000

Cycle III & IV

Atlanta, USA	Dundee, UK	Nantes, France	Portland, USA
Birmingham, UK	Houston, USA	Newcastle-upon-Tyne, UK	Rehovot, Israel
Boston, USA	Laval, Canada	Nijmegen, The Netherlands	Warsaw, Poland
Cagliari, Italy	Madison, USA	Oxford, UK	Winnipeg, Canada
Chicago, USA	Montreal, Canada	Philadelphia, USA	

1999

Cycle I & II

Berlin, Germany	Edmonton, Canada	Melbourne, Australia	New York, USA
Bordeaux, France	Helsinki, Finland	Montreal, Canada	Oxford, UK
Boston, USA	London, Canada	Nantes, France	Pittsburgh, USA
Cincinnati, USA	Madison, USA	New Haven, USA	San Francisco, USA

3. ROTRF Grant Awards in Cycle XV and Cycle XVI

3.1.1 Conventional Research Grant Awards – Cycle XV

Dr Simi Ali, Principal Investigator
Dr Isabel J. Crane, Co-Investigator

University of Newcastle, Newcastle-upon-Tyne, UK

Non-Glycosaminoglycan-Binding Chemokine Receptor Agonists: A Novel Route to Anti-Rejection Therapy?

Solid organ transplantation is often complicated by rejection; this is manifested by vigorous inflammation. The early events in rejection are controlled by small proteins, termed "chemokines", which recruit immune cells from the blood and direct them into the transplanted organ. Recent evidence suggests that chemokines anchor themselves to molecules called proteoglycans, which exist on the surface of cells lining the blood vessels. In addition, they also bind to their specific receptors.

Initial hope that targeting of chemokines may result in the attenuation of inflammation is hampered by the redundancy in chemokine activity. A series of small molecule chemokine receptor antagonists has been created. However, as immune cells can express multiple chemokine receptors, it seems likely that blockade of any single receptor will have an incomplete potential to prevent migration of immune cells promoted by a physiological range of chemokines.

We have recently engineered a chemokine variant which can bind to its receptor but cannot anchor to proteoglycans. Importantly, this molecule can inhibit the recruitment of all white blood cells. In this project we will define the mechanism of action of this new anti-inflammatory molecule and will also determine whether related molecules produce the same effect. In this study we will use a wide range of experiments including *in vivo* experiments and a mouse model of organ transplantation. We anticipate that our work will lead to the development of novel agents for anti-inflammatory therapy.

Significance: These molecules should contribute to the rational design of therapeutic agents to selectively block graft-damaging lymphocytes following transplantation.

Dr Michael Autieri, Principal Investigator
Ms Sheri Kelemen, Technician

Temple University School of Medicine, Philadelphia, USA

The Role of Allograft-Inflammatory Factor-1 in Development of Graft Vascular Disease

Despite advances in organ preservation and immunosuppressive drugs, the vascular narrowing indicative of graft vascular disease (CATV) is the major complication that limits long-term survival of cardiac transplantation. Characteristic cellular changes that typify CATV are activation of vascular smooth muscle cells (VSMC), causing them to migrate from the artery wall to the lumen of the vessel where they proliferate. This is responsible for loss in lumen diameter and organ failure. Recruitment of stem cells to the transplanted organ has also been shown to contribute to this process. The risk of infection and malignancy associated with immunosuppressive therapy support the need to identify a molecular target which directly impacts the VSMC response to injury, rather than suppress the immune response. In the past several years, we have shown that expression of a protein termed allograft inflammatory factor-1 (AIF-1) causes VSMC activation, and is strongly associated with development of clinical CATV. AIF-1 expression also causes granulocyte-colony stimulating factor (G-CSF) expression, which is a migration factor for stem cells and inflammatory cells. VSMC which express AIF-1 are chemotactic for stem cells and monocytes.

We have recently developed a transgenic mouse in which over-expression of AIF-1 is limited to large and medium sized arteries. This mouse represents a novel opportunity to investigate the role of this important protein in development of CATV. We will perform heart transplants into mice, and compare the development of CATV in the AIF-1 transgenic hearts versus the wild-type mouse hearts. We will compare lumen size, and expression of proliferative and inflammatory genes in these hearts. We will also identify and compare the amount of stem cells and inflammatory cells recruited to the arteries in these hearts. Characterization of AIF-1 function will increase our understanding of allograft-induced VSMC activation, of vascular-immune cell cross talk, and stem cell recruitment.

Dr Anita S. Chong
Dr Maria-Luisa Alegre, Collaborator
Dr Chyung-Ru Wang, Collaborator

The University of Chicago, Chicago, USA

Bacterial Infections and Transplantation Tolerance

The ability to successfully alter the immune system to allow specific acceptance of transplanted organs, without the use of continued immunosuppression, is the long-term goal of this research. Mouse models of transplantation tolerance can now be readily achieved for a variety of organs, such as hearts, kidney and islets. However, transplanted organs such as the skin and small bowel are more resistant to tolerance induction. In addition, transplantation tolerance has been difficult to achieve in non-human primates and in the clinic.

Considerable effort in the field is currently focused on understanding how organs resist tolerance induction, and on how tolerance, once achieved, is reversed. We hypothesize that one reason some organs, such as the skin, lung and small bowel, resist tolerance induction is that these organs, upon surgical implantation, are more likely to expose the immune system to bacterial infections. We theorize that the concurrent exposure of the immune system to the allograft and bacterial infection prevents the development of transplantation tolerance. We further hypothesize that systemic bacterial infections is a significant cause for the reversal of long-term transplantation tolerance. The studies we have proposed will test these two hypotheses, define the mechanisms by which bacterial infections alter allograft tolerance, and explore new tolerigenic strategies based on these hypotheses.

Dr Daniel Goldstein, Principal Investigator
Prof. Philip Askenase, Collaborator
Prof. Richard Flavell, Collaborator

Yale University School of Medicine, New Haven, USA

Role of Innate B1 Lymphocytes in Neonatal Transplant Tolerance

Transplantation is a very effective therapy for many end-stage diseases. The success of this therapy has largely been due to medications, which patients must take chronically, that suppress the immune system to avoid rejection of the transplanted organ. However, since these medications also suppress immune responses against infections and malignancy, transplant recipients are at high risk of infection and cancer. Therefore, protocols that can induce immune tolerance (i.e., the ability to manipulate a patient's immune response so that the organ is no longer perceived as foreign and infections and tumors can be fought off without the need of chronic immunosuppressants) would represent a breakthrough in the field.

In this proposal, we will use a neonatal murine skin transplant model to understand the mechanisms of immune tolerance. This model is particularly relevant because immune tolerance is more readily acquired in the neonate as compared to the adult. Although important insights have been made using this experimental system, all of the studies were performed prior to the appreciation of the innate immune system. The innate immune system acts as a rapid, non-specific "first-response" component in the presence of foreign antigens, whereas the adaptive response is slow but specific to the noxious stimulus. Our prior work indicates that the innate system plays an important role in detecting the presence of transplants. However, the impact of innate immunity on transplant tolerance is largely unknown. Given that our preliminary data indicate that innate immune activated neonatal B cells possess unique immunoregulatory properties, this proposal will test the hypothesis that innate immune activated recipient B-1 cells are critical for the induction of neonatal transplant tolerance. We will use both molecular (i.e., antibody inhibition of specific pathways) and genetic (i.e., use of mice with defined genetic deletions) approaches. If our anticipated results indicate that neonatal innate activated B-1 cells are critical for tolerance this may lead to new investigations of tolerance induction in adults that may bring us closer to clinical protocols that induce immune tolerance.

Dr Gregg A. Hadley, Principal Investigator

The Ohio State University, Columbus, USA

Mechanisms Underlying Immune Destruction of Pancreatic Islet Transplants

Despite recent progress in the field of clinical pancreatic islet transplantation, only a small minority of diabetic recipients achieve insulin independence in the long term. While there is compelling evidence that $CD8^+$ T cells contribute to this abysmal success rate, the specificity of such cells, and the efferent pathways by which they compromise long-term graft survival remain obscure. Insight into this important clinical problem is provided by recent studies from the principal investigator's laboratory documenting a pivotal role for the integrin CD103 in promoting destruction of islet allografts by $CD8^+$ T cells. These data suggest that blockade of CD103 as an adjunct to conventional immunosuppressive strategies provides feasible means of preserving long-term function of clinical islet transplants.

The current proposal focuses on four issues critical to advancing this objective. These studies will utilize a clinically relevant mouse model in which islet allografts are transplanted into diabetic NOD mouse recipients. In aim 1, we will determine if $CD103^+CD8^+$ effectors are present within islet allografts at the time of rejection in clinically relevant transplant scenarios. In aim 2, we will determine whether $CD103^+CD8^+$ effectors that infiltrate islet allografts in diabetic hosts recognize foreign or self antigens expressed by the pancreatic islets. In aim 3, we will determine how $CD103^+CD8^+$ effectors compromise the function of islet allografts. In aim 4, we will use mice with targeted disruption of the CD103 gene to provide proof-of-principle that blockade of $CD103^+CD8^+$ effectors as an adjunct to conventional immunosuppressive strategies provides a feasible means of preserving long-term function of islet allografts transplanted into diabetic hosts. The information gained from these studies will elucidate the cellular/molecular pathways of islet allograft destruction in diabetic hosts in clinically relevant transplant scenarios, and reveal novel targets for therapeutic intervention in this important clinical problem.

Dr Irma Joosten, Principal Investigator
Dr Hans Koenen, Co-Investigator
Prof. J. Han van Krieken, Research Associate
Dr Luuk B. Hilbrands, Research Associate
Prof. Peter C. van de Kerkhof, Research Associate

Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Effect of Regulatory T Cell Diversity on the Immune Response

It has recently become clear that a particular population of T cells, the immunosuppressive regulatory T cells (Treg; as opposed to aggressive effector T cells) is crucial in the induction and maintenance of immunological tolerance. In transplantation, the balance between Treg and aggressive effector T cells is important for the eventual outcome; too few Treg numbers will result in transplant rejection. This knowledge can be exploited for the development of new therapeutic designs involving the infusion of substantial numbers of *ex vivo*-expanded autologous Treg. In mouse models, this type of therapy, whereby the cells were re-infused together with the transplanted organ, already successfully prevented graft rejection. Clinical trials with *ex vivo*-expanded cells have been initiated. This treatment can only be successful if sufficient numbers of highly potent suppressors can be obtained. We have now developed an expansion technique that enables us to do this. Quite unexpectedly, we noticed that upon expansion, two different types of Treg subsets emerged. These subsets had distinct phenotypes and suppressive capacity. For successful therapeutic application of Treg we feel that it is imperative to study these subsets in more detail, to understand their behavior in relation to effector cells and notably, to each other. The observed reciprocal expression of a particular receptor – ligand pair (CD27–CD70) indicates that such an interaction might occur. Also we may learn whether the diversity may have any possible clinical implications.

Our objectives are twofold. 1. The two human Treg subsets will be studied under laboratory conditions to analyze if there is a developmental relationship between the subsets, and how they interact. The cells will be characterized in detail, with particular focus on the CD27–CD70 signaling pathway. 2. Since Treg might behave differently under laboratory conditions than in the patient, we need to study how the cells behave *in vivo*. Therefore, we will analyze the actions of Treg subsets in a mouse model. We will again analyze a putative developmental relationship, if they cooperate functionally, where they are localized upon infusion and how long they survive. Together these experiments will teach us more about the biological features of Treg and will enable optimal Treg selection for Treg-based therapy.

Dr Karl Lemström, Principal Investigator
Dr Petri Koskinen, Co-Investigator
Dr Antti Nykänen, Research Associate
Mr Mikko Keränen, Research Associate
Mr Henrik Sandelin, Research Associate
Mr Raimo Tuuminen, Research Associate
Dr Jussi Tikkanen, Research Associate
Mr Rainer Krebs, Research Associate

University of Helsinki, Helsinki, Finland

Role of Hypoxia Inducible Factor-1 in Cardiac Allograft Vasculopathy

Cardiac transplantation is a standard therapy for many end-stage heart diseases and returns patients to normal life. Although the short-term survival of heart transplant recipients has improved, there is a steady decline in graft function over time. Ischemia is an integral part in solid organ transplantation and is associated with impaired long-term graft survival. The recent discovery of transcription factor hypoxia inducible factor-1 (HIF-1) has unfolded a molecular link between hypoxia and regulation of a variety of genes involved in cellular adaptation to hypoxia.

We aim to investigate the role of HIF-1 in cardiac allografts using experimental, pharmacological, gene vector and transgene-based strategies.

Dr Benjamin Medoff, Principal Investigator
Dr Andrew Luster, Research Associate
Dr Umar Mahmood, Research Associate

Massachusetts General Hospital, Boston, USA

The Role of the White Blood Cell Attractant CXCL10 in the Development of Lung Injury Following Lung Transplantation

Lung transplantation is the only effective treatment for patients with severe lung disease, one of the most common causes of death in the world. Unfortunately outcomes after lung transplantation remain relatively poor, compared to other organ transplants. One of the most important causes of death after transplantation is failure of the lung to work after it is initially placed in the recipient. This failure, called primary graft dysfunction or PGD, results from injury to the lung due to the interruption of blood flow during the transplant procedure. Research has suggested that PGD results from the movement of a specific type of white blood cell, called a T lymphocyte, into the transplanted lung after it is reimplanted into the recipient. Chemokines are proteins that control the movement of white blood cells into organs.

We present data showing that a specific chemokine, CXCL10, which is known to control the movement of T lymphocytes, is produced in the lung after transplantation and in the lungs of mice in a model that simulates PGD. We hypothesize that CXCL10 is the primary controller of T lymphocyte movement into the lung immediately after transplantation and thus is a major controller of lung dysfunction after a transplant.

In this proposal we plan to determine the exact role of CXCL10 in the development of PGD. We will do this by altering the expression of CXCL10 in a mouse model of lung injury. We will also confirm our results by studying samples taken from lung transplants in humans. This study has the potential to identify a novel therapeutic target that could prevent an important complication following lung transplantation.

Dr Hao Wang, Principal Investigator
Dr Wei-Ping Min, Collaborator
Dr Bertha Garcia, Collaborator

University of Western Ontario, London, Canada

Induction of Transplant Tolerance in Sensitized Recipients

Although immunosuppressive drugs have increased graft survival and made transplantation a clinical reality, these treatments require life-long administration and are associated with adverse effects, such as cancers and infections. Therefore, induction of immunologic tolerance is an important clinical goal in transplantation. A tolerant patient is someone who accepts the transplanted organ yet is capable of mounting an effective immune response against vaccines and microbial pathogens. Although several immunomodulatory strategies have been successfully used to induce tolerance in rodents, the same strategies have failed in non-human primates and in humans who are constantly exposed to bacteria and viruses or have received a prior stimulus via blood transfusion, previous transplant, or pregnancy. This type of stimulus creates the presence of memory T cells (Tmem). Tmem not only endanger graft survival by causing both acute and chronic rejection but also impede tolerance induction. Using a potent protocol, we have successfully induced stable murine cardiac allograft tolerance through formation of tolerogenic dendritic cells (Tol-DC) and regulatory T cells (Treg), which are responsible for initiating and maintaining immunological tolerance. However, this same therapy failed to induce tolerance in presensitized mice, in a model designed to mimic sensitized patients, owing to significantly increased Tmem.

In this study, we will investigate the interactions among Tol-DC, Treg and Tmem. We will identify an effective strategy to inhibit Tmem-mediated rejection by functionally blocking the activation pathway that is unique for the recall of Tmem. This will restore tolerance, which had been induced by currently available tolerant protocol, through the generation of Tol-DC and Treg. This study will provide insight into understanding the role of Tmem in tolerance induction. Its findings can potentially be translated into improved therapies for preclinical primate studies and eventually sensitized transplant patients, thereby increasing the success of organ transplantation and improving the quality of life for transplant recipients.

Dr Li Zhang, Principal Investigator

University of Maryland, Baltimore, USA

Integrin Mac-1 on Antigen-Presenting Cells Is Key to the Development of Peripheral Immune Tolerance

Organ rejection remains the major risk for patients with organ transplantation. Non-specific suppression of the immune system by drugs like cyclosporine, though very effective, does cause many complications, which adversely affect the quality-of-life of transplant recipients. In this regard, antigen-specific immune tolerance has a great potential in clinical settings to improve the quality-of-life of patients with transplantation and promote the survival of the transplanted organs.

The long-term goal of the proposed study is to understand the mechanism that differentially controls antigen-specific immune activation versus immune suppression. Specific to this application, we propose to study the role of a major surface molecule (termed integrin Mac-1) on antigen-presenting cells in the development of antigen-specific peripheral tolerance. Completion of this project will provide important information regarding the role of this important molecule in the development of antigen-specific immune tolerance. Delineation of the molecular processes required for generation of antigen-specific immune suppression may offer novel therapeutic targets for enhancing immune suppression that will be beneficial to organ transplantation, and prolong the survival of the transplanted organs.

3.1.2 Conventional Research Grant Awards – Cycle XVI

Dr Maria-Luisa Alegre, Principal Investigator
Dr Luciana Molinero, Research Associate
Mr Jason Cosmano, Technician
Mr Ying Wang, Microsurgeon

The University of Chicago, Chicago, USA

Treg Specificity in a Mouse Cardiac Transplantation Tolerance Model

Cardiac transplantation is the only cure for end-stage heart failure but T cell-dependent acute rejection of the transplanted organ invariably occurs in the absence of immunosupression. Short-term treatment with drugs that prevent T cell activation during the peri-operative period can successfully lead to permanent acceptance of the graft in mice, and to a state of transplantation tolerance. We and others have shown that administration of anti-CD154 mAb in combination with donor-specific transfusion (DST) results in long-term acceptance of cardiac transplants and development of donor-specific tolerance. This tolerance correlates with the induction of donor-specific regulation, a phenomenon in which tolerant mice cannot reject a second transplant from a donor genetically identical to the original donor. This inhibition is mediated by regulatory T cells (Tregs), which suppress the function of graft-reactive T cells. In contrast, tolerant mice retain the ability to reject a second heart transplant from a mouse genetically distinct to the original-donor, suggesting that the immune system of the tolerant mouse is not globally immunosuppressed.

However, the mechanism by which tolerance becomes donor-specific is not clear and attempts at identifying the donor-reactive regulatory cells have been largely unsuccessful. Our preliminary results indicate that cardiac transplants in tolerant but not rejecting mice, attract Tregs that may be responsible for maintenance of tolerance. The surface molecules expressed on intragraft Tregs are different from those on Tregs from the spleen or lymph nodes, suggesting that a specific subset of Tregs is attracted to the tolerated graft. We hypothesize that intragraft Tregs are enriched in cells capable of suppressing the immune system in a donor-specific manner. In this project, we propose to test the specificity of the Tregs from the graft and sequence their antigen-recognition receptors.

Dr Masato Imai, Principal Investigator
Dr Simon C. Robson, Co-Investigator
Dr Beat Kunzli, Research Assistant
Dr Csizmadia Vilmosne, Technician

Beth Israel Deaconess Medical Center, Boston, USA

NTPDase-1/CD39 Ameliorates Transplant Arteriosclerosis

Advances in immunosuppression have largely controlled acute allograft rejection and organ failure after clinical transplantation. However, delayed graft loss still poses a barrier to prolonged organ survival. Indeed, graft survival rates after one year have not improved over the past three decades. Graft loss is due to chronic rejection and the development of transplant arteriosclerosis (TA). TA is characterized by formation of diffuse thickening of the inside of blood vessels. Donor: recipient mismatch and history of acute rejection are risk factors for chronic rejection. However, reinforcement of conventional immunosuppression does not prevent TA. Certain white blood cells, called natural regulatory T cells, appear to inhibit inflammation and vascular injury in atherosclerosis models in mice.

There are chemicals called nucleotides that are important mediators within the vasculature and immune system. NTPDase-1/CD39 is the dominant vascular and immune ecto-enzyme that removes these nucleotides, e.g., ATP. However, this biochemical activity is lost from the vasculature during injury and in graft rejection. CD39 up-regulation is seen in working grafts. We have previously shown that viral-mediated overexpression of CD39 in cardiac grafts limits vascular injury and suppresses thrombosis. Transgenic mouse cardiac allografts overexpressing CD39 are also resistant to antibody rejection responses and platelet sequestration.

Our preliminary data indicate that CD39 overexpression in allografts, soluble NTPDase protein treatment and recombinant CD39 adenovirus vectors significantly suppress intimal hyperplasia. Certain white blood cells, called natural regulatory T cells, appear to inhibit inflammation and vascular injury in conventional atherosclerosis models in mice. CD39 is a selective surface marker of such regulatory T cells and CD39 null cells are defective in that they cannot block allograft rejection.

This proposal will elucidate whether NTPDase-1/CD39 has beneficial effects on the development of TA. We propose that NTPDase-1/CD39 supplementation will suppress TA and control elements of chronic rejection.

Prof. James McCluskey, Principal Investigator
Prof. Jamie Rossjohn, Research Associate
Dr Whitney Macdonald, Research Associate
Dr Anthony Purcell, Research Associate
Dr Scott Burrows, Research Associate

The University of Melbourne, Parkville, Australia

Understanding Why Organ Transplants Are Rejected by the Host Immune System

Long-term transplant rejection in recipients of kidney and other solid organ transplants is due to an attack on the grafted organ by the T cells of the immune system. This attack results from mistaken identity of the transplanted tissue that resembles an infection to the unsuspecting immune system. The molecular recognition events that occur in transplant rejection are very poorly understood. This research aims to determine exactly how and why this case of mistaken identity occurs at a molecular level.

The project will first determine the three-dimensional structure of several protein receptors that T cells normally use to detect common viral infections but which are also known to attack tissue grafts from unrelated individuals. We will also identify the exact molecules that are recognised on transplanted organs during immune rejection and compare these to the molecules that are normally recognised during viral infections. Once these molecules are identified we plan to determine their shape at the atomic level by X-ray crystallography. By comparing exactly how the same receptors that recognise viruses also recognise foreign tissues we hope to learn more about the basic mechanisms by which T cell receptors interact with host tissues and organ grafts.

The molecular details by which host T cells recognise transplanted tissues may allow us to design ways of monitoring immunological rejection events in transplanted patients and perhaps permit us to design decoy molecules that could subvert the rejection process.

Dr Wei-Ping Min, Principal Investigator
Dr Hao Wang, Collaborator
Prof. Garcia Bertha, Collaborator
Prof. David White, Collaborator

University of Western Ontario, London, Canada

Protection of Donor Organs in Transplantation

Currently, people who receive organ transplants must take drugs for the rest of their lives to prevent their own immune system from rejecting the transplanted organ. Despite these potent anti-rejection drugs, almost all transplanted organs ultimately fail due to slow progressive rejection. This 'chronic rejection' largely stems from the transplanted organs becoming partially damaged during the preservation and transportation stage or during the transplant surgery itself. To date, we have no way to prevent chronic rejection in patients.

We know that certain molecules are associated with organ damage. In this project, we will use a newly developed gene-blocking method to reduce the genes that precipitate organ damage. We will then look at how blocking these genes can increase kidney storage time prior to transplantation as well as decrease injury to the organ, and how this, in turn, prevents chronic rejection.

This project aims to develop an organ storage solution that contains new gene inhibition components, called small interfering RNA (siRNA). We believe that siRNA can suppress those genes responsible for organ damage. Kidneys will be stored in this new solution before transplantation in order to prolong organ preservation time, to reduce organ injury during storage and transplantation, and to prevent immune rejection after transplantation.

If successful, this new organ storage solution will offer better protection for transplant organs both before and during transplant surgery. By reducing organ injury, this storage solution will prevent chronic rejection and ultimately improve long-term kidney transplant survival.

Prof. Yorgo Modis, Principal Investigator
Dr Ryuta Kanai, Postdoctoral Fellow
Ms Christina Newman, Technician
Ms Kaury Eisenman, Graduate Student
Prof. Ruslan Medzhitov, Consultant

Yale University, New Haven, USA

The Structural Basis of Innate Immune Sensing and Signaling by Toll-Like Receptors

We rely on our innate immune system as the first line of defense against invading pathogens. The most important molecular sentinels of the innate immune system are the toll-like receptors (TLRs). Upon exposure to a microbe, TLRs recognize pathogen-associated molecular structures. This recognition event triggers an inflammatory response, which normally serves to fight infection by recruiting and activating various components of the adaptive immune system. However, TLR signaling causes inflammation following exposure to microbes during or after organ transplantation, especially in transplanted lungs, which are continually exposed to the environment. There is also mounting evidence that TLRs can cause inflammation even in the absence of microbes, in response to endogenous ligands released upon tissue injury, including injuries associated with solid organ transplantation. The combination of microbe-driven and endogenous TLR signaling in organ transplant recipients results in local inflammation in damaged or exposed regions of the grafted organ. Inflammatory compounds at these sites ultimately lead to the recruitment of armed T cells to the graft. It is these T cells that are principally responsible for graft destruction and acute graft rejection.

We propose to determine a three-dimensional structure of a TLR bound to the ligand that it recognizes. Knowing the three-dimensional structure of a molecule provides a deep understanding of how the molecule functions, and structures often suggest methods to control molecular function. Observing the structural changes that TLR undergoes as it recognizes microbial components will help us understand how this recognition event is translated into an inflammatory signal. We propose a rational, structure-based strategy to guide the design of novel TLR inhibitors. By reducing pathogen-induced inflammation, these inhibitors will provide new means to prevent inflammation and induce graft tolerance in solid organ transplant recipients.

Dr Julian Pratt, Principal Investigator
Dr Marie Parker, Research Associate
Prof. John Kirby, Collaborator
Dr Ryzard Smolenski, Collaborator

Leeds University, Leeds, UK

Ischaemic Epigenetics in the Transplanted Kidney

Organ transplantation is a severe insult to the donor organ, exacerbated by the duration and temperature of the storage conditions. We hypothesise that upon transplantation, extensive damage to DNA occurs caused by ischaemia/reperfusion injury, with significant effects on genes expressed by the donor tissue.

One form of such damage is potentially the demethylation of specific cytosines in the DNA sequence which normally perform regulatory functions to limit gene expression. Where so-called 'aberrant demethylation' occurs it is already known to have profound effects in cancerous tumours. Since potential exists for similar changes to occur in transplanted kidneys, we wish to investigate the extent to which the donor genome is modified and to assess the consequences of such modifications.

3.2.1 Clinical Research Grant Awards – Cycle XV

Dr Anthony Dorling, Principal Investigator
Prof. Terence Cook, Co-Investigator

Imperial College London, London, UK

Understanding How Antibodies and B Cells Contribute to the 'Chronic' Rejection of Transplanted Kidneys

Chronic allograft nephropathy (CAN) resulting in loss of the transplant kidney is an important problem; – by 10–12 years after transplantation, approximately 50% of patients have returned to dialysis. A significant proportion of cases are due to ongoing immunologically mediated damage, or 'chronic rejection' (CR) and many of these have evidence of antibody-mediated pathology as evidenced by the deposition of complement component C4d in the peritubular capillaries or glomeruli of transplant biopsy specimens alongside the features of CAN. There is no established treatment for CR, although optimisation of immunosuppression to include tacrolimus and mycophenolate mofetil (MMF) achieves short-term stabilisation of function in approximately 4 out of 5 patients.

In late 2006, we are starting a randomised trial of anti-CD20 therapy in patients who fail to stabilise after initiation of an MMF/tacrolimus-based regime, with the goal of establishing the efficacy of anti-CD20 in this group. This application will fund scientific analyses of the patients entered into this trial, with the objective of analysing the contribution on non-HLA-specific antibodies and to determine whether B lymphocytes can be shown to be acting as antigen presenting cells (APC) for the allogeneic anti-graft T cell response. We hope to determine laboratory and biopsy criteria for predicting responsiveness to standard therapy or anti-CD20.

Additionally, we hypothesise that there are a subgroup of patients with CR without evidence of C4d deposition, in whom B cells may be playing an important role in pathology. We hypothesise that this subgroup develops circulating anti-HLA antibodies as a marker of this process. This application will fund laboratory studies in this group to define biopsy criteria for diagnosis, antibody specificities and to determine whether B lymphocytes are acting as APC for the allogeneic anti-graft T cell response, with the ultimate aim of establishing a rational basis for targeting therapy towards B cells in this subgroup.

Finally, it is now well established that anti-graft antibodies are not always associated with progressive graft deterioration, but can also be associated with 'accommodation', in which the transplanted kidney becomes resistant to the pathological effects of antibody. This application will fund a detailed study of our transplant recipients with stable function, to determine the nature and specificity of the antibodies in patients with accommodation and to examine the hypothesis that B cells in these patients also act as APC for allogeneic T cells, but in contrast to those with CR, these interactions take place in the context of effective peripheral immunoregulatory mechanisms, whereas in CR we hypothesise we will be able to demonstrate an absence of peripheral regulation.

Prof. Rudolphina J.M. ten Berge, Principal Investigator
Dr Ajda T. Rowshani, Co-Investigator
Prof. René A.W. van Lier, Research Associate
Dr E. Eldering, Research Associate
Prof. J.J. Weening, Research Associate
Dr S. Florquin, Research Associate
Prof. J.P. Medema, Research Associate
Prof. F. Baas, Research Associate
Dr A. van Kampen, Research Associate
Prof. A.H. Zwinderman, Research Associate
Prof. E.C. Hack, Research Associate

Academical Medical Center, Amsterdam, The Netherlands

Immunological Injury and Mechanisms of Self Defense in Subclinical Renal Allograft Rejection

Subclinical renal allograft rejection is defined as the presence of granzyme (Gr) positive T cell infiltrate in the graft causing tubulitis which, in contrast to acute rejection, does not lead to acute dysfunction. Subclinical rejection causes chronic allograft nephropathy and long-term graft failure. Two converging theories may explain this clinically silent state of cytotoxicity: one is that the offending T cells don't execute their cytotoxic potential.

The other theory, which is the main focus of the present proposal, refers to the resistance of kidney graft tubular epithelial cell (TEC) as a key factor.

Recently, we have shown that SERPINB9 expression by TECs is significantly higher in subclinical rejection than in acute rejection. High SERPINB9 expression correlates with the presence of GrB^+ T cells. These data point to a protective role of SERPINB9 conferring TECs resistance to injury. SERPINB9 is the only known human serpin specifically inhibiting GrB, the main effector molecule during rejection.

Hypothesis: In subclinical rejection, strong expression of granzyme inhibitors by TECs protects them against T cell-mediated cytotoxicity. Genetic polymorphism may underlie the interindividual differences in expression of protective proteins like SERPINB9 by donor-derived TECs.

Overall aim: to elucidate the role of granzyme inhibitors in subclinical rejection.

Key objectives: to study 1) regulation and cytoprotective function of SERPINB9 in TECs *in vitro;* 2) expression and regulation of protease inhibitors of the SERPIN family, potentially involved in inhibition of other cytotoxic molecules in TECs *in vitro* and in allograft biopsies with subclinical rejection, acute rejection or non-rejection; 3) design of a non-invasive diagnostic tool to distinguish subclinical from acute rejection by analysis of mRNA for SERPINB9 and other candidate protease inhibitors in urinary cells; 4) genetic polymorphisms for SERPINB9 by single nucleotide polymorphism analysis of genomic DNA derived from spleen cells of donors belonging to recipients with subclinical, acute and no rejection.

Significance: This proposal will provide new insights into the pathogenesis of subclinical rejection. Identification of new molecular therapeutic targets may lead to new therapies by amplification of naturally occurring mechanisms of defence. Invention of a non-invasive diagnostic tool for acute and subclinical rejection would mean a great improvement in patient care.

3.2.2 Clinical Research Grant Awards – Cycle XVI

Dr Henrik Petrowsky, Principal Investigator
Prof. Pierre-Alain Clavien, Co-Investigator
Dr Wolfram Jochum, Research Associate
Dr Rolf Graf, Research Associate
Mr Udo Ungetuem, Technician

University Hospital Zurich, Zürich, Switzerland

Protective Mechanisms of Pentoxyfilline for Liver Surgery and Liver Transplantation

The present project investigates the potential protective effects of pentoxyfilline for the remnant liver after major liver resection under inflow occlusion (=ischemia). The study analyses human serum and liver tissue samples in terms of TNF signalling. The biochemical profiling will be correlated with the postoperative liver function and regeneration. This study simulates to a certain extent the situation of living-related or split-liver transplantation where a partial graft is transplanted. Often, these grafts are too small, causing failure of regeneration and graft failure (small-for-size syndrome). If the experimental effects of pentoxyfilline are also observed in the setting of major liver resection, this finding might open the doors for a new pharmacological protective strategy to improve the outcome of living-related or split-liver transplantation and lower the risk for living donors.

Dr Andrea Zachary, Principal Investigator
Dr Keith Melancon, Co-Investigator
Dr Mary Leffell, Co-Investigator
Dr Dessislava Kopchaliiska, Research Associate

Johns Hopkins University, Baltimore, USA

Detecting Humoral Sensitization

Patients may become sensitized to the transplantation antigen, called HLA antigens, through pregnancy, transfusion and transplantation. Once sensitized, these patients have greater difficulty finding a compatible donor and generally wait longer for a transplant. The frequencies of sensitized patients are higher in women and African-Americans. However, the evidence of sensitization, antibody against HLA, may diminish over time making it difficult to recognize that a patient is sensitized. When this happens, a patient may have an unexpected rejection episode that can damage the transplanted organ.

We hope to address the problems of unrecognized sensitization as well as investigate why African-Americans have higher rates of sensitization, using a technique developed in the Immunogenetics Laboratory at the Johns Hopkins University. This technique places a label on the cells responsible for the antibody to HLA and provides an opportunity to count them and characterize them. Thus, we may be able to determine if there is a certain number of such cells that predicts that a patient will have a rejection episode after transplantation. It may also allow us to identify differences between African-Americans and Caucasians that account for the higher rates of sensitization among African-Americans. In turn, this information may provide the opportunity for early intervention that could extend the life of a transplanted organ and improve the chances for a sensitized patient to be transplanted successfully.

4. ROTRF/JDRF Joint Initiative Grant Awards

4.1 ROTRF/JDRF Joint Grant Awards – Cycle XVI

Dr Ariela Benigni, Principal Investigator
Dr Marina Morigi, Co-Investigator
Dr Andrea Remuzzi, Co-Investigator

Mario Negri Institute for Pharmacological Research, Bergamo, Italy

Generation of New Blood Vessels and Immunomodulation without Drugs to Improve Survival of Islet Grafts

Diabetes is a major health problem, affecting millions of people worldwide. It is caused by the failure of the body's insulin-producing cells, the pancreatic islets. Although diabetes can be treated by regular insulin injection, long-term treatment can lead to many debilitating side effects including blindness, kidney failure, cardiovascular disease and loss of limbs. A much better treatment option is to cure diabetes by transplanting islets from a human donor. One problem is that islets need to "grow" a new blood supply after they are transplanted into a new host. This is a slow process, and many of the transplanted islets die in the meantime because they are starved of oxygen and nutrients. Moreover, ideally, the objective of clinical islet transplantation is to cure diabetes mellitus without the need of chronic immunosuppressive drug therapy. Indeed, although immunosuppressive medications have made allograft possible, there are many potential side effects associated with their use, including risks of cancer and infections. In addition, many of the immunosuppressive drugs needed to prevent graft rejection turn out to be diabetogenic. Therefore, promotion of islet revascularization through locally increased expression of growth factors, such as vascular endothelial growth factor (VEGF), and induction of donor-specific tolerance (immunosuppression without the need of antirejection drugs) to islet grafts could facilitate the applicability of islet transplantation as a viable clinical therapy for diabetes. Bone marrow-derived mesenchymal stem cells (MSCs) are capable of releasing VEGF. These cells have also profound immunomodulatory function.

Thus, the aim of this project is to test the hypothesis that when MSCs are co-transplanted with pancreatic islets they form a biological barrier which supports islet engraftment by secreting growth factors and prevents graft rejection by eliminating or regulating the host immune response in diabetic rats. If successful, this strategy would allow implementation of the islet transplant program in humans opening new perspectives for this effective therapy to cure millions of people with diabetes.

Dr David M. Rothstein, Principal Investigator

Yale University, New Haven, USA

How Experimental Agents Prevent Islet Transplant Rejection by Inducing Different Types of Regulatory T Cells

Transplantation is the treatment of choice for end-stage disease of organs including heart, kidney, and pancreas. Despite great progress, life-long therapy with immunosuppressive medications is required to prevent rejection. However, these medications increase the risks of infection and malignancy, in addition to risks of kidney damage, high blood pressure, and cardiovascular disease. As such, pancreas or islet transplantation is frequently reserved for diabetic patients who already display significant damage to their kidneys or other organs. Therefore, approaches that can induce immune tolerance (i.e. limited therapy designed to "trick" the immune system into accepting the transplant as self rather than foreign tissue) would not only promise to improve outcomes, but would lower risks of infection, cancer and kidney damage, currently associated with chronic immunosuppression. For example, this would make islet transplantation accessible to younger diabetic patients, before the onset of damage to other organs, such as the kidney.

Various approaches can induce transplant tolerance in experimental animals. Many such approaches appear to augment regulatory T cells (Treg) in the immune system. These cells inhibit immune responses, and might dampen autoimmunity or prevent transplant rejection. Thus, there has been great interest in understanding how these cells can best be manipulated for therapeutic gain. However, understanding has been limited because there are different types of Treg that are difficult to identify and appear to act in distinct manners. In this proposal, we will use novel genetically engineered mice whose regulatory cells fluoresce red. This will allow us to confirm whether individual tolerance-inducing strategies actually induce Treg and if so, how this occurs. This will provide critical insights into the generation and function of Treg and ultimately allow us to understand how they can best be manipulated for the induction of immunological tolerance in islet transplantation.

Dr Charmaine Simeonovic, Principal Investigator
Prof. Christopher Parish, Co-Investigator
Dr Craig Freeman, Collaborator
Ms Sarah Popp, Research Assistant
Mrs Debra Brown, Technician

The Australian National University, Canberra, Australia

Immune Destruction and Protection of Pancreatic Islet Transplants

Type 1 diabetes (T1D) is an autoimmune disease in which the insulin-producing β cells of pancreatic islets are destroyed. The imperfect control of hyperglycemia by exogenous insulin therapy inevitably leads to microvascular disease, e.g. kidney disease. The clinical transplantation of pancreatic islets potentially offers an improved treatment for T1D because insulin can be delivered physiologically, as required. Clinical islet transplantation has progressed considerably in recent years with implementation of the Edmonton protocol for preventing immune destruction of the transplants. Despite this progress, the heavy use of immunosuppressive drugs has severely limited its application to only adult patients whose diabetes has been difficult to control. In the long term, however, islet function is eventually lost and insulin therapy is again required. This graft failure is due to toxicity of the immunosuppressive drugs used to prevent immunological rejection of the transplant and recurrence of autoimmune disease. It is therefore essential that better anti-graft rejection/destruction strategies are developed to eliminate the need for toxic drugs and thus preserve the health status of the patients and the integrity of the transplant.

Our study will ascertain whether (i) the enzyme heparanase contributes to the rejection and/or autoimmune destruction of islet transplants by arming activated leukocytes with a degradative mechanism for facilitating their invasion into transplanted islets and whether (ii) inhibition of heparanase with PI-88 can protect islet grafts from these destructive immunological processes. PI-88 has now entered Phase III clinical trials as an anti-cancer drug; it is safe for patients, well-tolerated and has minimal side-effects. Our objectives are therefore to identify a non-toxic therapy (PI-88) for protecting islet transplants from rejection and/or autoimmune damage. Ultimately our goal is to optimize islet transplantation as a safe treatment for T1D in patients of all ages and to re-establish a normal lifestyle in those transplant recipients.

5. Progress Reports of ROTRF Grantees

5.1 Conventional Research Grants

Dr Ginny L. Bumgardner, Principal Investigator
Ms Anna Marie Hummel, Research Associate
Mr Thomas Pham, Research Associate

The Ohio State University, Columbus, USA

Analysis of a Novel Strategy which Suppresses Aggressive (CD4-Independent) CD8$^+$ T Cell-Initiated Hepatocyte Rejection

The overall purpose of this project is to determine the mechanisms by which the immunotherapeutic strategy targeting both LFA-I and CD40/CD40L costimulation induces long-term suppression of (CD4-independent) CD8$^+$ T cells. In recently published work we demonstrated that the efficacy of this immunotherapeutic strategy for suppression of (CD4-independent) CD8-dependent alloimmune responses is not due to persistence of treatment antibody, inhibition of lymphocyte recruitment to the graft site, induced apoptosis of CD8$^+$ GICs or CD8$^+$ T cell ignorance or deletion. Instead, this dual immunotherapy suppresses CD8$^+$ T cell activation and maturation into cytolytic effectors. The latter was demonstrated by suppression of expression of T cell activation markers (CD69, CD25) and T cell integrin CD103 and the absence of *in vivo* allospecific cytolytic function and expression of CD4$^+$ by CD8$^+$ GICs in treated animals compared to untreated rejector mice.

Long-term allograft survival by this strategy does appear to be associated with some evidence of regulation *in vivo* since these hepatocellular allograft recipients with long-term allograft survival are resistant to rejection by adoptively transferred naïve CD8$^+$ T cells (sufficient to initiate hepatocyte rejection in control mice). The failure to precipitate rejection following naïve CD8$^+$ T cell transfer into long-term hepatocyte allograft acceptor mice and the presence of a subset of CD8$^+$ GICs in these mice with a regulatory phenotype (CD8$^+$CD25^{hi}) suggest that that this immunotherapeutic strategy may induce the development of a regulatory CD8$^+$ T cell population. This mechanism is further supported by the observation that persistence of graft survival in these treated mice with long-term allograft acceptance is dependent on recipient CD8$^+$ T cells. The latter was demonstrated in studies showing that recipient CD8$^+$ T cell depletion prior to adoptive transfer of naïve CD8$^+$ T cells results in hepatocyte rejection (Fig. 1) and the development of *in vivo* allospecific cytotoxicity. In future studies we also plan to deplete CD25$^+$ cells from hepatocellular allograft recipients with induced long-term survival in order to determine whether or not CD8$^+$CD25$^+$ T cells are critical for the maintenance of hepatocellular allograft survival induced by this therapeutic strategy.

In ongoing studies we are examining the expression of FoxP3 by $CD8^+CD25^+$ T cells in the spleen and graft-infiltrating cells in recipients with long-term hepatocellular allograft acceptance induced by this strategy. Thus far the results suggest that this strategy does not induce the expression of FoxP3 on recipient $CD8^+$ T cells. We are also pursuing studies to evaluate whether or not this strategy induces the development of linked antigen recognition. In order to do this we will transplant hepatocytes by kidney subcapsular injection and treated with anti-CD154 and anti-LFA-1 mAbs. Recipients with long-term hepatocellular survival at 60 days will undergo nephrectomy of the kidney bearing hepatocellular allografts. Following confirmation of hepatocellular graft loss these recipients will be rechallenged with donor, third party or F1 (donor x "third party") type hepatocytes. Acceptance of donor and F1 type hepatocellular transplants (but rejection of "third party" hepatocyte transplants) in the absence of additional immunosuppression will suggest the development of linked antigen recognition.

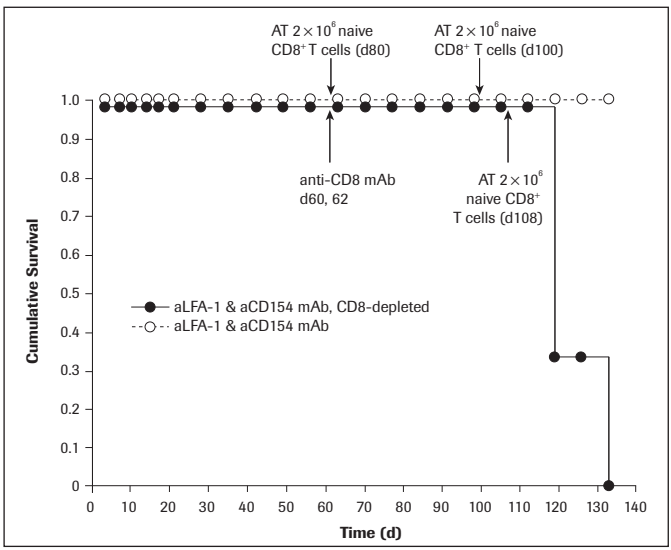


Figure 1. Adoptive transfer of naïve $CD8^+$ T cells into CD4 KO recipients with long-term hepatocellular allograft survival induced by short-term treatment with anti-LFA-1 and anti-CD154 mAbs. *CD4 KO ($H\text{-}2^b$) hosts were transplanted with allogenic hepatocytes (hAlAT-FVB/N, $H\text{-}2^a$) and were treated with anti-LFA1 mAb (300 µg, d0–6) and anti-CD154 mAb (1 mg. d0, 2, 4, 7). After 60 days ($n = 5$) or 100 days ($n = 3$) of stable hepatocyte function, five recipients with long-term function received adoptive transfer of $2x10^6$ naïve $CD8^+$ T cells. Two mice died of causes unrelated to transplantation 30 days following $CD8^+$ T cell adoptive transfer with functioning hepatocellular allografts. Five of the remaining six recipients had ongoing hepatocellular allograft function > 60 days despite adoptive transfer of naïve $CD8^+$ T cells; (unfilled circles; one recipient lost graft function 56 days following adoptive transfer). Another group of anti-LFA-1 and anti-CD154 mAb-treated recipients with stable hepatocyte allograft function were depleted of $CD8^+$ T cells 60 days following transplantation (anti-CD8 mAb i.p., day 60 and day 62, $n = 3$). Hepatocytes continued to survive for an additional 48 days following CD8-depletion. At 108 days following transplantation, these recipients received adoptive transfer of 2×10^6 naïve $CD8^+$ T cells. Hepatocytes were rejected in all recipients with MST of 11 days ($n = 3$); (filled circles).*

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Mechanisms of Antibody-Mediated Chronic Rejection in Mouse Cardiac and Kidney Allografts

The goal of this project is to define the pathogenesis of antibody-mediated chronic rejection, using murine heart and kidney allografts. The first specific aim was to develop the passive transfer model using complement-fixing IgG2a anti-H-2K^{k} Mab in B6 RAG1$^{-/-}$ mice with B10.BR heart grafts. We have accomplished this aim and published the results of our pathological analysis[1]. RAG1$^{-/-}$ recipients received twice-weekly injections of complement-fixing IgG2a anti-H-2K^{k} MAb over 14–28 days. Graft samples were scored histologically and histochemically at intervals up to 56 days for arterial stenosis, complement deposition (C4d), and the phenotype of the infiltrate. Chronic allograft arteriopathy was scored by morphometric analysis using Weigert's elastin-stained sections. Serum levels of the MAb were measured at corresponding intervals. Isotype-matched non-reactive MAb was used as a control. These experimental groups were also compared to groups with male-to-female heart transplants, B10.BR hearts to wild-type B10.A recipients and heart isografts.

We found that passive transfer of complement fixing antibody to MHC class I expressed by the allograft was able to initiate chronic arteriopathy lesions and C4d deposition in the microvasculature in the absence of T cells. Isotype controls were negative. A minimum threshold of antibody exposure was needed to cause arteriopathy, but this dose did not cause acute antibody-mediated rejection. While the development of arteriopathy initiated by antibody was associated with C4d deposition as long as the antibody remained in the circulation, arteriopathy lesions progressed and persisted in the absence of continued antibody or C4d deposition. A similar sequence of transient C4d deposition and antibody production occurred in wild-type B10.BR recipients of B10.A hearts. We also documented a T cell-mediated form of chronic allograft arteriopathy in male cardiac grafts to female recipients (C4d was always negative). These observations may explain the imperfect correlations between arteriopathy, C4d and donor-reactive antibody in clinical studies.

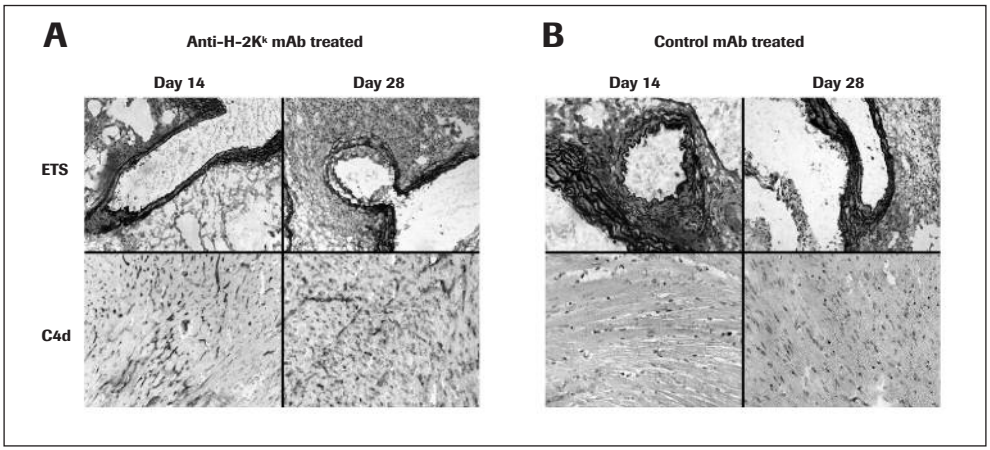

Figure 1. Representative histology of chronic allograft arteriopathy and C4d deposition in B6.RAG1 KO recipients of B10.BR cardiac allografts given donor-specific monoclonal antibody. *The recipients were given 30 μg of donor-specific mAb (anti-H-2Kᵏ, IgG2a) or control mAb (HOPC-1), beginning on day +1 after transplantation and continuing twice a week thereafter until the animals were sacrificed at 14 days or 28 days after transplantation.* ***(A)*** *anti-H-2Kᵏ mAb,* ***(B)*** *control IgG2a mAb. Sections were stained with Weigert's elastin stain (ETS) for the presence of arteriopathy, and anti-C4 staining for C4d deposition (C4d), respectively. These sections are representative for at least 5 independent cardiac grafts[1].*

The next specific aim will seek to determine the dependence of antibody-mediated arteriopathy on the complement system and Fc receptors. For this purpose we have started experiments using passive transfer of non-complement fixing IgG1 Mab anti-H-2Kᵏ into B6RAG1$^{-/-}$ recipients and are in the process of breeding B6 C3$^{-/-}$RAG1$^{-/-}$ recipients. Other planned experiments will include FcγRIIb/III-blocking Mab in the passive transfer model and anti-NK1.1, and examination of endothelial expression of molecules potentially involved in accommodation. Further delineation of Fc receptors can be pursued using FcγRI-deficient recipients into a B6RAG$^{-/-}$ background. The other major goal of the coming year is to establish conditions by which passive transfer of antibody is able to promote chronic arteriopathy and glomerulopathy in life-sustaining B10.BR kidneys into B6RAG$^{-/-}$ recipients.

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Increase of Islet Engraftment by Mobilizing Bone Marrow-Derived Endothelial Progenitor Cells

Studies during this period were conducted to evaluate the contribution of bone marrow-derived endothelial progenitor cells (angioblasts) to islet allograft revascularization and survival. To this end, a steroid-free immunosuppressive protocol adapted from the "Edmonton Protocol" for clinical transplantation (Daclizumab, rapamycin and tacrolimus) has been implemented in allogeneic models of rodent islet transplantation. However, the anti-proliferative effects of rapamycin have been clearly established, which potentially may affect islet revascularization.

Studies were conducted initially in a syngeneic model of islet revascularization after transplantation. Briefly, 450 islet equivalents recovered from FEV/N mice were infused intraportally into STZ-induced syngeneic recipient.

To mobilize angioblast into the peripheral circulation and therefore promote islet revascularization[1], recipients received either vehicle or GM-CSF (0.5 μg/day/7 days starting 4 days before the transplant). Animals received rapamycin diluted in peanut oil, 1 mg/kg/day by gavages for 30 days. Although all animals achieved normoglycemia after the transplant ($n = 6$ per experimental group) a trend to higher non-fasting glucose levels that did not reach statistical significance was demonstrated in all animals given rapamycin. In this regard, rapamycin-treated recipients presented a lower glucose disposal rate assessed 30 days after the transplant (no immunosuppression + vehicle-treated recipients = 2.21 ± 0.44; no immunosuppression + GM-CSF = 3.22 ± 0.61; rapamycin + vehicle = 1.44 ± 0.32; rapamycin + GM-CSF = 1.85 ± 0.28, $p < 0.05$). Next, animals were euthanized and the islet graft vascular density was evaluated as described[1]. The islet vascular density of rapamycin-treated recipients was significantly lower compared with vehicle-treated controls (vascular density [number/mm^2], no immunosuppression + vehicle-treated recipients = 226 ± 79; no immunosuppression + GM-CSF = 654 ± 116; rapamycin + vehicle = 134 ± 56; rapamycin + GM-CSF = 215 ± 24). Overall, these results suggest that rapamycin is detrimental to the intrahepatic islet revascularization process.

Next, we evaluated whether rapamycin affects localization of angioblasts into the islet revascularization site. To this end, we used our model of angioblast localization into angiogenic sites in the context of islet transplantation[1]. Briefly, FEV/N mice underwent bone marrow (BM) transplant from transgenic mice constitutively expressing β-galactosidase (β-gal) encoded by LacZ under the transcriptional regulation of an endothelial cell-specific promoter, TIE-2. Reconstitution of the transplanted BM yielded FEV/N-TIE-2-LacZ mice in which expression of LacZ is restricted to BM-derived cells expressing TIE-2-LacZ and is not observed in other somatic cells, as we and others previously described[1]. Reconstituted STZ-diabetic animals received 450 syngeneic (FEV/N) islets into the protal vein. β-galactosidase activity was used to assess localization of angioblasts in the whole liver and was performed 4 weeks after the transplant. Rapamycin did not significantly reduce β-galactosidase activity in vehicle-treated animals (2496 ± 334 RLU/Sec/mg total protein) and GM-CSF-treated recipients (3501 ± 421 RLU/Sec/mg total protein) compared with non-immunosuppressor controls (vehicle = 2876 ± 661, GM-CSF = 3767 ± 561 RLU/Sec/mg total protein).

These results suggest that rapamycin does not seem to interfere with localization of angioblast into sites of islet revascularization but prevents formation of new blood vessels, a critical step for adequate islet engraftment and function. Studies are underway in our laboratory to evaluate the effect of angioblast mobilization despite rapamycin-based immunosuppressive therapy in allogenic islet transplantation and the effect of angioblast infusion in the outcome of islet grafts.

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A Genetic Strategy to Accelerate Revascularization of Islet Xenografts

The objective of this project is to develop a genetic strategy to accelerate revascularization of pancreatic islet xenografts by inducible, graft-specific expression of the pro-angiogenic factors VEGF-A ± angiopoietin-1. If successful, this will reduce the xenogeneic islet mass required to reverse diabetes, and consequently reduce the immunological and 'mechanical' burden on the recipient. VEGF expression at the appropriate time may have the additional benefit of increasing the yield of islets per donor.

The specific aims are as follows:

1. To generate transgenic mice with inducible islet-specific expression of human VEGF-A and/or Ang-1.
2. To use the transgenic mice as donors to determine whether transient local expression of these factors improves the revascularization and engraftment of islets transplanted under the kidney capsule or into the portal vein.
3. To show that islet- or pancreas-specific expression of these factors during development increases the number of islets (and thus the potential yield of islets per donor) in adult mice, and to determine whether islets thus produced function normally following transplantation.

Significant progress has been made in aim 1. Full-length cDNAs for human VEGF-A_{165} (576 bp) and Ang-1 (1497 bp) were generated by RT-PCR and cloned into the pCI-Neo expression vector. COS-1 cells were transfected with the pCI-Neo constructs and expression was confirmed by ELISA (Fig. 1A) and Western blot analysis (not shown) of 24 hour culture supernatants. The cDNAs were then cloned separately into the pTRE-Tight (Tet-Responsive Element) vector. Transactivator plasmids were prepared by cloning the rtTA2^{S}-M2 (Tet-ON) cDNA under the control of either the 9.5-kb β cell-specific RIP7 promoter or the 4.3-kb pancreas-specific *Pdx1* promoter. The insulinoma cell line βTC3 was co-transfected with various combinations of transactivator and response plasmids and cultured for 24 hours in the presence or absence of 25 ng/ml doxycycline (DOX). Using either the RIP7 or *Pdx1* promoter to drive Tet-ON, strong DOX-inducible expression of both VEGF-A_{165} (Fig. 1B) and Ang-1 (Fig. 1C) was observed. Importantly, no expression was detected in the absence of DOX. The induced level of both transgenes was approximately threefold higher when the RIP7 promoter was used to drive expression of the Tet-ON transactivator (Fig. 1B, C).

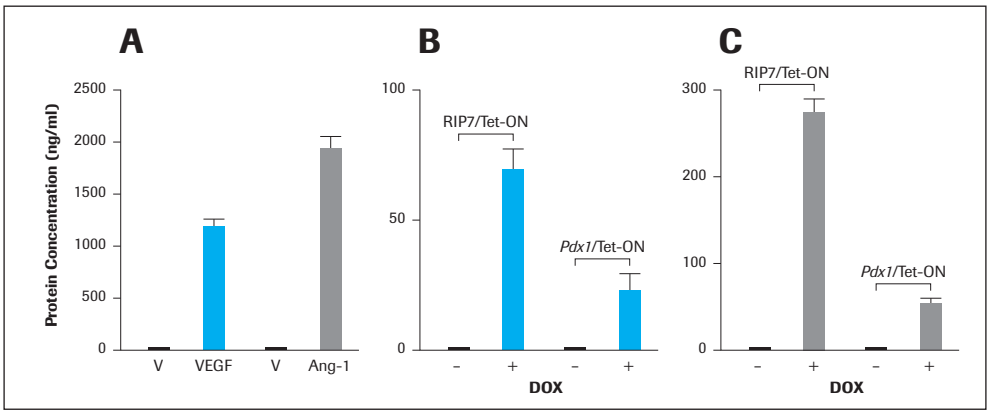

Figure 1. Expression of human VEGF-A$_{165}$ (blue bars) and Ang-1 (grey bars) in culture supernatants 24 hours after transfection, detected by ELISA. (A) *COS-1 cells transfected with pCI-Neo/VEGF-A (VEGF), pCI-Neo/Ang-1 (Ang-1), or pCI-Neo vector control (V).* ***(B)*** *βTC3 cells transfected with TRE/VEGF-A plus either RIP7/Tet-ON or Pdx1/Tet-ON, cultured with (+) or without (–) doxycycline.* ***(C)*** *βTC3 cells transfected with TRE/Ang-1 plus either RIP7/Tet-ON or Pdx1/Tet-ON, cultured with (+) or without (–) doxycycline.*

Having demonstrated the functionality of the transgenic constructs, we have commenced microinjection to generate transgenic mice. Initial rounds of microinjection with RIP7/Tet-ON plus TRE/VEGF-A or TRE/Ang-1 have generated 16 and 26 mice, respectively. These mice are currently being screened and microinjection is continuing.

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^{1}H Magic-Angle-Spinning NMR Spectroscopic Assessment of Human Donor Livers Pre- and Post-Transplantation

NMR-based metabonomic studies of bile and liver tissue biopsies from liver transplant donor-recipient pairs (DRPs) have been conducted. Extensive spectroscopy has been applied to samples to characterise metabolite profiles, involving standard 1-D, CPMG, diffusion-edited, J-resolved, ^{1}H-^{1}H and ^{1}H-^{13}C experiments.

Comparisons of biopsies from six donor livers (A–F) confirmed the high lipid content of E and F confirmed by histology, and it was observed that these two livers possessed higher levels of total and unsaturated triglyceride and lower levels of phospholipid and low MW compounds.

Lipid mobility studies revealed a higher proportion of mobile triglyceride in biopsies E and F, typical of cytosolic droplets[1]. Similarly, livers A–D showed evidence of both mobile triglyceride and less mobile, fatty acyl phospholipid groups. After transplantation, tissue glycerophosphocholine levels decreased (suggesting reduced cell turnover) in all livers except E, which later developed post-graft dysfunction (PGD). Elevated tissue glycerophosphocholine levels may represent an early indication of PGD.

Sequential bile samples from DRPs were analysed. Recipient bile contained higher levels of amino acids, lactate, citrate and succinate than donor bile. Pre-transplantation levels of phospholipids increased over time in donor E. The analysis of post-transplantation bile showed increasing levels of phospholipid and bile acids, with decreasing levels of UW solution (preservative), alanine, lactate, creatine, citrate and succinate.

UPLC-MS studies of bile composition were also performed. A UPLC-MS assay was developed to separate and confirm the mass of 18 bile acids in concentrations ranging from 0.1 μM to 10 mM. Subsequently the method was used to analyse gallbladder bile excreted by a fatty liver, the composition of the gallbladder bile and the liver biles at different time-points from thirteen donors, and the corresponding bile compositional changes post-transplant, (total of 76 samples). This method was repeated from sample preparation to data treatment and found to be reproducible[9, 11].

Pattern recognition analysis of the bile signatures showed differentiation of gallbladder, pre- and post-transplant bile. PLS-DA models showed >95% confidence in grouping samples of either donors or recipients of unsuccessful versus successful transplant. Samples from fatty livers were readily observed as outliers.

Chemometric models constructed from bile acids and related molecules achieved characterisation of the three classes of bile samples and time trajectories for each patient were explained mainly by glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurodeoxycholic acid (TDCA) and taurocholic acid (TCA) bile acids. Post-transplant the clearance of the immunosuppressant methylprednisolone from bile was identified as an indicator of liver function, the highest amount found in the recipient who developed PGD.

The bile acid composition has been established for 13 DRPs of liver transplant and a biomarker of post-transplant liver function identified. LC-NMR-MS identification of further candidate biomarkers of the donor liver quality is currently being carried out.

HR-MAS spectroscopy has been completed for seven further sets of biopsy samples from DRPs. NMR analysis of the corresponding bile samples is underway. Future analyses will expand the dataset for the graft function assessment work outlined above and link bile and biopsy datasets for NMR and UPLC-MS in combined analysis.

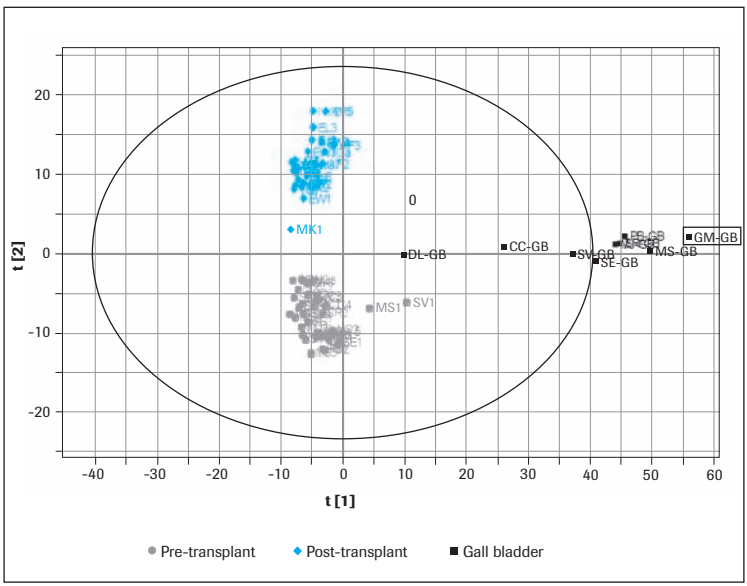

Figure 1. PLS-DA plot of the 76 bile samples. *We can observe from this plot several outliers, the GM-GB sample from gallbladder belongs to a liver that was rejected; biles MS1 and SV1 were extracted from the liver immediately after the gallbladder was removed and were contaminated with gallbladder bile. More importantly AR5 post-transplant (50 minutes after transplant was performed) showed the highest amount of methylprednisolone, – this liver transplant was unsuccessful.*

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

In vivo models of immune modulation are the most useful models for studies in transplantation because of their relevance to clinical transplantation. HLA-G is a human MHC molecule that is thought to regulate immune responses during pregnancy. We have generated HLA-G transgenic mice in which allogeneic skin graft rejection is significantly delayed. In these mice, dendritic cells were deficient and induced T cell hyporesponsiveness. 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.

For the past year, our major goal has been to determine if dendritic cells from HLA-G transgenic mice, and HLA-G tetrameric complexes are able to induce hyporeactivity to allogeneic skin grafts. We compared the effect of wild-type dendritic cells, and dendritic cells from HLA-G transgenic mice on graft rejection and cytotoxic T cell development. We used an *in vivo* model to deliver tetramers into recipient mice followed by allogeneic skin grafts. We designed special microspheres, coated with HLA-G tetramer or with anti-PIR-B mAb (to cross-link the PIR-B receptors) and injected them into CBA mice prior to their receiving allogeneic skin grafts from C57BL/6 mice. Injection of microspheres alone did not affect the time of rejection of allogeneic skin (Table 1). In contrast, microspheres coated with either HLA-G tetrameric complexes or anti-PIR-B mAb significantly prolonged skin allograft survival. In addition, considerably lower cytotoxic T cell activities directed against donor C57BL/6 target cells were obtained in mice treated with miscrospheres coated with HLA-G tetramer or with anti-PIR-B mAb that in control groups of mice treated with microspheres coated with isotype match IgG1 or microspheres only.

Treatment of graft recipient[a]	No. rejected/ no. grafted[b]	Graft survival time (days) Range	Mean (MST)[c]	*P*
without microspheres	8/8	10–13	12.00 ± 1.07	
HLA-G microspheres	6/6	19–23	21.17 ± 1.47	0.0019[d]
anti-PIR microspheres	6/6	20–24	22.50 ± 1.64	0.0027[e]
IgG1 microspheres	7/7	11–14	12.29 ± 1.11	
microspheres only	9/9	10–15	12.67 ± 1.58	

Table 1. Allogeneic skin survival is prolonged on HLA-G-tetramer and anti-PIR-B-treated recipient mice.[a]*CBA/Ca mice were recipients of allogeneic skin transplant.* [b]*Donor allogeneic skin from C57BL/6 mice.* [c]*The results shown are the mean of survival time (MST) ± SD of values obtained in each group.* [d]*Compared to group of mice with microspheres only (Mann-Whitney test).* [e]*Compared to group of mice with IgG1 microspheres.*

We demonstrate that HLA-G tetrameric complexes inhibit function of murine dendritic cells, similar to what occurs in HLA-G transgenic mice. The proposed mechanism of this inhibition is based on the interaction between HLA-G and PIR-B. To facilitate *in vivo* studies of interaction between HLA-G and PIR-B we generate transgenic mice expressing PIR-B receptor on dendritic cells. Transgene-positive founders were identified by PCR using oligonucleotides specific for the PIR-B construct. The mice are fertile and breeding well. The FACS analysis using a specific anti-PIR-B mAbs revealed no transgene expression on CD11c populations of cells. Now we are in progress of establishing a different line of CD11c-PIR-B transgenic mice using a modify cassette for the expression of inhibitory receptor PIR-B on murine dendritic cells.

Significance

Our findings provide support for the fact that HLA-G is an important tolerogenic molecule for the prolongation of allograft survival. In addition, they support the important inhibitory function of PIR-B in dendritic cells *in vivo* and open strategies for targeting dendritic cells in the prevention of graft rejection.

Publications

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2. Wei JI, Zhang W, Liang S, et al. Expression of human inhibitory receptor Ig-like transcript 2 (ILT2/LILRB1/LIR1/CD85j) *in vivo* facilitates the regulatory activity of $CD4^{+}CD25^{-}$ programmed death-1+ T cells. *J Immunol* 2006; 176:S167.
3. Horuzsko A. Murine models of HLA-G-based tolerance to allografts. *4th International Conference on HLA-G* 2006; *(Abstract)*.

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Visualizing the Role of Individual CD4$^+$ T Cell Clones in Response to Allograft

We have produced unique mice that express a miniature TCR repertoire (TCR^{mini} mice). In this *in vivo* model a whole repertoire of TCRs can be reliably examined at the level of T cell clones, because the diversity of this repertoire depends on natural rearrangements of only one Vα2 segment to two Jα26/Jα2 segments (TCRα mini-locus) and one transgenic TCRβ chain. Subsequently, our ROTRF application proposed to use this model to investigate how TCR affinity to specific alloantigen (the A^b bound with covalent peptide) and T cell lineage commitment, would affect the fate of CD4$^+$ T cells during response to grafted organ. To determine the role of individual CD4$^+$ T cells in this process, we used the sequence of the CDR3α region as a marker of T cell clones. During the first year of research, we discovered that transplanted hearts expressing class II MHC bound with single allogenic peptide are not acutely rejected by TCR^{mini} mice regardless of increased frequency of CD4$^+$ precursors specific for this peptide. In addition, a number of TCRs expressed on CD4$^+$CD25$^+$Foxp3$^+$ (T_R) cells found in animals with transplanted allogenic hearts were also found on CD4$^+$CD25$^+$Foxp3$^+$ thymocytes.

This last result indicates the role of thymus-derived T_R cells in tolerance induction to a transplanted organ. We also gathered experimental evidence indicating that the thymus-derived T_R cells express a broad TCR repertoire that is unlikely to be limited to self-derived antigens. This last conclusion is supported by single-cell RT-PCR analysis of over 1600 individual TCRs expressed on naïve Foxp3- (T_N) and Foxp3$^+$ T_R cells in TCR^{mini} mice (Fig.1). Importantly, this high diversity of TCRs on Foxp3$^+$ T_R cells was established in the thymus and was not significantly enriched in the periphery. In fact, we have found that T_R and T_N cells express largely non-overlapping repertoires. Our results imply that thymus-derived T_R cell clones are significantly involved in tolerance induction to endogenous and exogenous antigens.

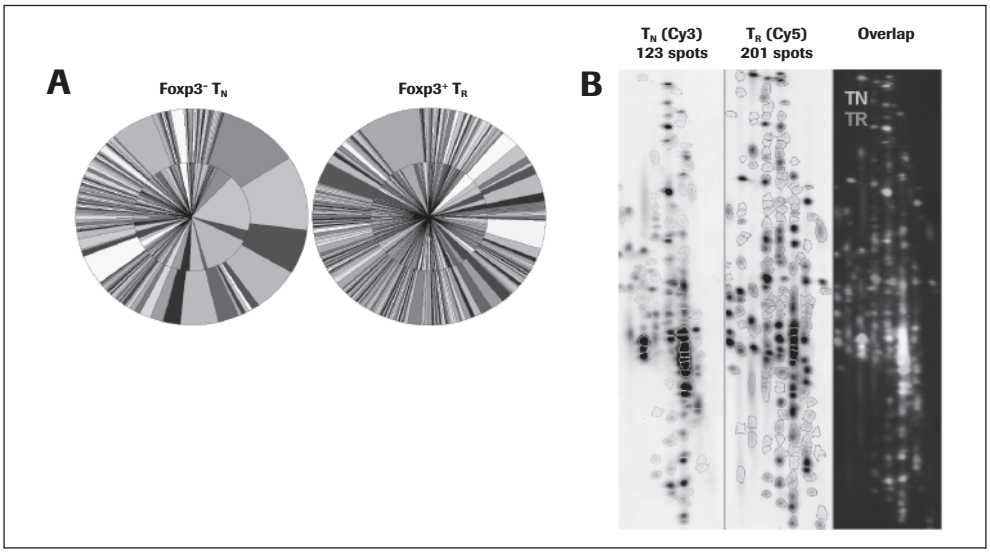

Figure 1. Foxp3⁺ T_R cells express a more heterogeneous and non-overlapping repertoire of TCRs than Foxp3⁻ T_N cells. (A) *Pie-chart representation of TCRα sequences obtained from single-cell RT-PCR. Each CDR3 sequence is represented by a different color and the size of a pie slice represents the frequency of a TCRα sequence. The outer and inner pie circles represent sequences from lymph nodes and thymii, respectively.* ***(B)*** *Diversity of TCRα CDR3 from T_R and T_N cells analyzed by 2D-F-SSCP. Spots migrating with different speed in SSCP gel represent different VαJα rearrangements. Overlapping spots between two populations are shown (right panel).*

Publication

1. Pacholczyk R, Ignatowicz H, Kraj P, et al. Origin and TCR diversity of CD4⁺CD25⁺Foxp3⁺ T cells; *(Submitted)*.

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The Role of Plasmacytoid Dendritic Cells in the Expansion and Function of $CD4^+CD25^+$ Regulatory T Cells

Naturally occurring $CD4^+CD25^+$ regulatory T cells (Treg) play a major role in peripheral tolerance of self-reactive T cells as well as in allograft tolerance. Therefore, using *ex vivo*-expanded antigen-specific Treg holds great promise in the field of transplantation and autoimmune diseases. However, the mechanisms of activation and homeostasis of Tregs and their antigen-presenting cell (APC) requirement are poorly understood and need to be dissected. Our preliminary results obtained in the rat indicate that, unlike conventional dendritic cell (cDC) subsets, mature plasmacytoid dendritic cells (pDC) induced strong proliferation of allogeneic $CD4^+CD25^+$ T, in the absence of exogenous IL-2. Adoptive transfer on the day of transplantation of low numbers of *ex vivo*-expanded $CD4^+CD25^+$ T cells to syngenic hosts induced significant prolongation of heart allograft survival.

Specific aims of this study are:

1. To determine the optimal conditions for using donor pDC-expanded Treg *in vivo* to control allograft rejection;
2. To determine the molecular mechanism by which pDC induce expansion of Treg;
3. To reproduce our *in vitro* data in human and primates with the aim to develop a pre-clinical model of adoptive transfer of *ex vivo*-expanded Tregs.

During the first year of ROTRF funding, we have focused on aims 2 and 3. Indeed, the identification of the molecules involved in the stimulatory activity of pDC on Treg (aim 2) might provide new valuable tools to expand Treg *in vivo* and *ex vivo*. PDC-induced Treg expansion is IL-2-independent and partially dependent on CD86 expression by pDC, yet conventional mature DC (cDC) expressed similar levels of CD86 as pDC. Importantly, while proliferating vigorously when stimulated by allogeneic pDC, Treg strongly suppressed the production of cytokine, including IL-2, by $CD4^+CD25^-$ T cells but not efficiently their proliferation. To address the molecular basis of functional specialization of DC subsets with regard to their Treg stimulatory capacity, we performed global gene expression analyses on immature versus mature DC. We have identified a number of genes specifically overexpressed in mature pDC

as compared to cDC, and confirmed their expression in pDC. The function of the related molecules is now being assessed *in vitro* (year 2). We will also generate new mAb against rat pDC as the pDC-specific 85C7 clone we generated previously is not depleting (year 2). Meanwhile we are setting up mouse models to reproduce our data in this species *in vitro* and *in vivo* using DO11.10 transgenic T cell adoptive transfer experiments (year 2). As part of aim 3, we sought to reproduce our data using human blood DC and T cells. Human $CD4^{+}CD25^{high}$ T cells are strongly hypoproliferative, and neither LPS-stimulated $CD11c^{+}$ DC or CpG or virus-stimulated pDC induce proliferation of allogenic Treg *in vitro* in the absence of exogenous IL-2. However, in the presence of IL-2, pDC appeared to be slightly better APC than myeloid DC for Treg.

During the second year of ROTRF funding we will concentrate on aim 1 and on the *in vivo* mechanisms of allograft prolongation induced by donor pDC-expanded Treg.

Prof. Robert I. Lechler, Principal Investigator
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Promoting Transplantation Tolerance: Adoptive T Cell Therapy Using Customised $CD4^+$ $CD25^+$ Regulatory Cells

Immunosuppressive drugs have been successfully used to prevent acute allograft rejection after organ transplantation. The long-term administration of non-specific immunosuppressive agents has significantly prolonged allograft survival, and this is inevitably accompanied by increased mortality and morbidity. Thus, strategies to achieve donor-specific transplantation tolerance are needed[1]. Thymus-derived $CD4^+CD25^+$ Tregs have been established to play a key role in mediating dominant transplantation tolerance in experimental models, and these cells can have indirect anti-donor allospecificity[2–6]. Furthermore, many studies have provided compelling evidence that donor-specific transplantation tolerance can be achieved in naïve animals by adoptive transfer of $CD4^+CD25^+$ Tregs from tolerised animals[2–6]. This has led us to address whether $CD4^+CD25^+$ Tregs with indirect allospecificity could be used as potential reagents for adoptive cell therapy to promote donor-specific transplantation tolerance.

In this progress report we first describe the generation and expansion of murine C56BL/6 $CD4^+CD25^+$ Tregs with antigen specificity for K^d peptide. Using bone marrow-derived autologous dendritic cells pulsed with the K^d peptide, we generated T cell lines from purified $CD4^+CD25^+$ Tregs from C56BL/6 mice. The T cell lines expressed a high level of CD25 and low levels of CD45RB and CD69. They maintained the expression of CD62L, GITR, CTLA-4 and more importantly FoxP3. The $CD4^+CD25^+$ T cell lines were anergic after TCR stimulation and produced little cytokine such as IL-2 and IFN-γ. Importantly, they were more potent than freshly isolated $CD4^+CD25^+$ T cells in suppressing proliferation and cytokine secretion by effector $CD4^+$ T cells. Furthermore, the $CD4^+CD25^+$ T cell lines could be expanded to large cell numbers and maintained in culture for up to 1 year. Currently, we are investigating the *in vivo* efficacy of the K^d-specific $CD4^+CD25^+$ T cell lines in combination with short-term rapamycin treatment for the induction of cardiac transplantation tolerance in wild-type C57BL/6 mice carrying a fully mismatched BALB/c heart.

Finally, we have extended this approach into a human system. Similar $CD4^+CD25^+$ Treg lines, with indirect allospecificity for HLA-A2 have been generated from peripheral blood $CD4^+CD25^{high}$ and $CD4^+CD25^{dim}$ cell populations against autologous HLA-DRB1*0101 DCs pulsed with an HLA-A2 (103–120) peptide. Antigen-specific cells for the A2 peptide have

been successfully isolated using a DR1:A2 (103–120) tetramer by flow cytometric cell sorting. Using our established *in vitro* expansion protocol for human $CD4^+CD25^+$ Tregs, we are exploiting the conditions to expand these A2-specific $CD4^+CD25^{high}$ and $CD4^+CD25^{dim}$ Tregs to large numbers, and to compare their efficacy in suppressing the proliferation of A2-specific effector $CD4^+CD25^-$ T cells.

These data will provide an invaluable platform for pilot clinical studies in transplant patients.

Publications

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2. Sakaguchi S. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; 6:345.
3. Jiang S, Herrera O, Lechler RI. New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Curr Opin Immunol* 2004; 16;550.
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5. Waldmann H, Cobbold S. Exploiting tolerance processes in transplantation. *Science* 2004; 305:209.
6. Jiang S, Lechler RI. Regulatory T cells in the control of transplantation tolerance and autoimmunity. *Am J Transplant* 2003; 3:516.

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Modulation of TRAF-Dependent Signaling in EBV$^+$ B Cell Lymphomas

Epstein Barr virus (EBV) is a B lymphotrophic herpes virus associated with multiple human malignancies including B cell lymphomas in immunocompromised solid organ and bone marrow recipients. We propose that EBV itself contributes to immune modulation of recipient B and T cell responses, thereby promoting the outgrowth of malignant B cell lymphomas in transplant recipients. The objective of this research is to understand how EBV modulates the response of B cells to signaling through cell surface molecules that control B cell activation, growth, and apoptosis.

Specific Aim 1: Determine the role of EBV in modulating the signaling pathways used by TNFR family members to activate responses in B cell lymphomas

We first analyzed the expression of TNFR proteins and their cellular signaling adaptor proteins, TRAF, in paired B lymphoma cell lines that were either uninfected or infected *in vitro* with EBV. We determined that latent EBV infection does not significantly alter the expression of CD27, CD40, TNFR1, Fas nor of TRAF2, 5, or 6. In contrast, whereas CD40 signaling lead to up-regulation of ICAM-1 expression and IL-10 production in uninfected cells, CD40 activation in EBV-infected cells resulted in reduced ICAM-1 expression and IL-10 production. Moreover, recruitment of TRAF2 and TRAF3 proteins to CD40, upon signaling with agonist anti-CD40 antibodies was diminished in EBV-infected B cells compared to uninfected cells. CD40 signaling inhibited proliferation of uninfected B cell lymphomas but did not alter proliferation in infected cell lines. Finally, induction of apoptosis through the Fas receptor was blocked in B lymphoma cells harboring a latent EBV infection. This resistance to Fas-mediated apoptosis was associated with a defect in formation of the death-inducing signaling complex. Together, these data indicate that EBV modulates signaling through B cell membrane proteins to promote growth and survival.

Specific Aim 2: Determine the role of latent membrane protein 1 (LMP1) and TRAF signaling pathways in modulation of B cell activation

LMP1 is a constitutively active viral protein expressed on the surface of latently infected cells and acts as a functional mimic of CD40. We hypothesized that LMP1 signaling would usurp cellular signaling pathways utilized by TNFR family members and thereby modulate their function. To establish an inducible LMP1 signaling model, we generated stable transfectants of uninfected B lymphoma cells expressing nerve growth factor receptor (NGFR)/LMP1 chimeric molecules. Crosslinking of NGFR led to activation of LMP1, TRAF recruitment, NFκB and p38 MAPK activation, and IL-10 production. As with latent EBV infection, we found that LMP1 activation during CD40 signaling inhibited recruitment of TRAF2 and TRAF3 proteins to CD40. However, LMP1 signaling did not alter IL-10 production or ICAM up-regulation in response to CD40 signaling. In contrast, LMP1 signaling was able to inhibit Fas-induced apoptosis by 45–60% in uninfected B cell lymphomas. Together, these data indicate that LMP1 can modulate the distribution of cellular signaling intermediates utilized by TNFR in B cell lines. However, the functional outcome of concomitant LMP1 signaling and TNFR signaling depends upon the specific cellular receptor that is activated. Moreover, LMP1 alone does not fully recapitulate the effect on TNFR function observed in latently infected B cells suggesting that other viral proteins also play a role.

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2. Lambert SL, Martinez OM. Latent membrane protein 1 of EBV signals for IL-10 production in B cell lymphomas. *American Association of Inmunologists* 2006; *(Abstract)*.
3. Snow AL, Lambert SL, Natkunam Y, et al. Epstein Barr virus can protect latently infected B cell lymphomas from death receptor-induced apoptosis; *(Submitted)*.

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Prevention of Allograft Rejection by Specific Tolerance Induction Using $CD4^+CD25^+$ Regulatory T Cells

Background and significance

Although very effective, immunosuppressive drugs given to prevent graft rejection are toxic and only partially block chronic rejection. Alternative treatments are thus explored, such as the use of the $CD4^+CD25^+$ regulatory T cells (Treg). Indirect evidence suggest that Treg specific for donor allo-antigens (allo-Ag) would be very efficient in preventing graft rejection. The goal of our project is to explore the possibility of preventing rejection of allogeneic skin or pancreatic islets by injecting Treg, previously selected *in vitro*, to recognize specifically donor-type allo-Ag through the direct and/or indirect pathways.

Progress made on the specific aims and achieved results

1. Generation of Treg specific for allo-Ag presented by the direct and indirect pathways

In vitro generation of BALB/c Treg specific for C3H allo-Ag presented by the direct pathway (dir-Treg) was performed by stimulating purified Treg by allogeneic antigen presenting cells (APC). Selecting the rare Treg specific for allo-Ag presented by the indirect pathway appeared a much more difficult task. We have recently described a protocol allowing the selection, among the diverse repertoire of polyclonal Treg, of rare Treg specific for a nominal Ag. This method required specific culture conditions using the splenic $CD8^+$ dendritic cells as APC[1]. Using a similar culture protocol, we were able to generate BALB/c Treg specific for C3H allo-Ag presented by the indirect pathway (indir-Treg). After an initial phase of selection, both dir-Treg and indir-Treg exhibited high expansion in culture (Fig. 1A). *In vitro* experiments showed that dir-Treg and indir-Treg have suppressive functions and were specific for allo-Ag presented by the direct and indirect pathways, respectively (Fig. 1B, C).

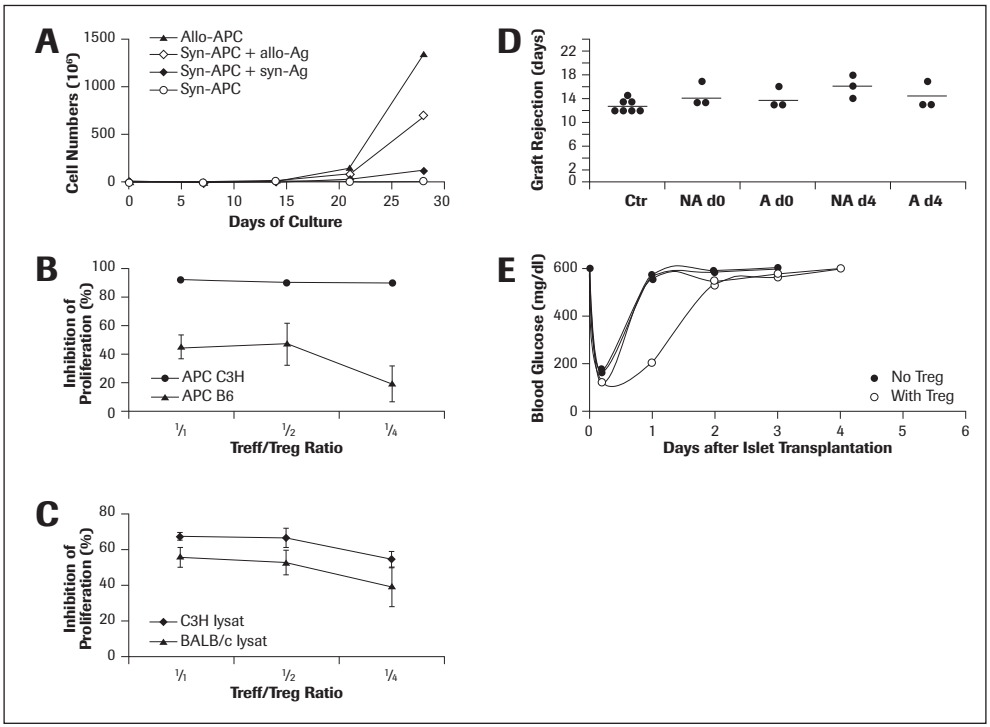

Figure 1. Generation and functional activity of Treg specific for allo-Ag presented by the dir-Treg and indir-Treg pathways. (A) *BALB/c Treg were stimulated by irradiated C3H splenocytes (Allo-APC) or syngeneic CD8⁺ dendritic cells pulsed with C3H lysat (Syn-APC+allo-Ag) in order to generate dir-Treg and indir-Treg, respectively. In control cultures, BALB/c Treg were stimulated by syngeneic CD8⁺ dendritic cells pulsed with a BALB/c lysat (Syn-APC+syn-Ag) or without lysat (Syn-APC). Living cells excluding trypan blue were counted every week. After 4 weeks of culture, expanded Treg were harvested 7 days after the last re-stimulation for in vitro and in vivo analyses (B–E).* ***(B)*** *Suppressive activity of dir-Treg. Expanded dir-Treg, co-cultured at the indicated ratio with BALB/c effector T cells (Teff), were stimulated by irradiated C3H (APC C3H) or third party C57BL/6 (APC B6) splenocytes. Since Treg suppression requires activation via their T cell receptor, the fact that significant inhibition of T cell proliferation was obtained in the presence of C3H APC, but not B6 APC, indicated that expanded dir-Treg were indeed specific for C3H allo-Ag presented by the direct pathway.* ***(C)*** *Suppressive activity of indir-Treg. Expanded indir-Treg, co-cultured at the indicated ratio with effector T cells specific for an islet Ag (Teff), were stimulated by syngenic dendritic cells pulsed with the cognat islet Ag and C3H lysat or BALB/c lysat. Data showed that indir-Treg have suppressive activity and are specific for C3H allo-Ag presented by the indirect pathway. Some reduced Treg suppression was also observed in the culture with BALB/c lysat probably because during the phase of Treg selection, cells specific for Ag released by stressed cells had been selected.* ***(D)*** *Expanded dir-Treg were analyzed for their capacity to prevent allogeneic skin rejection. C3H tail skins were grafted on the back of anesthetized non-irradiated BALB/c mice. Then, mice were not injected (ctr) or injected intravenously with 10 million non-activated (NA) or pre-activated (A) dir-Treg the same day (d0) or 4 days later (d4). Whatever the conditions, the administration of dir-Treg specific for C3H allo-Ag presented by the direct pathway did not delay rejection of C3H skin grafted in BALB/c mice.* ***(E)*** *Auto-immune diabetes was obtained in BALB/c mice as previously described[7]. Then, mice were grafted with C3H islet Ag. Graft rejection was revealed by checking blood glucose level. Normalization of glucose level 5 hours after transplantation showed that grafted islets were functional. In control mice, islet transplants were rejected within 24 hours. Injection of 10 million dir-Treg + 10 million indir-Treg + 10 million islet-specific Treg had no effect in this model with extremely rapid auto-immune and/or allogeneic islet rejection.*

2. Capacity of Treg specific for allo-Ag to prevent graft rejection

We tested the capacity of these allo-specific Treg to prevent rejection of allogeneic skin. BALB/c control mice rejected allogeneic C3H skins between 12 to 14 days after transplantation (Fig. 1D) whereas syngeneic skins were not rejected (not shown). Unfortunately, the intravenous administration of dir-Treg had no effect since allogeneic skin was rejected as in controls. Even when dir-Treg were pre-activated, which would increase their suppressive function, their injection had no effect (Fig. 1D). The absence of effect of dir-Treg could be due to their rapid death after injection. However, injection of dir-Treg 4 days after transplantation did not delay skin rejection (Fig. 1D). We are currently analyzing the effect of indir-Treg.

After transplantation of pancreatic islets, the graft is attacked not only by alloreactive T cells but also by auto-reactive T cells that have already rejected patient's own islets. We thus performed transplantation of allogeneic islet in mice that have an auto-immune diabetes. In our model, very aggressive autoreactive T cells rejected islet grafts extremely rapidly, probably within 24 to 48 hours. Not surprisingly, injection of allo- and islet-Ag-specific Treg (mix of dir-Treg, indir-Treg and islet-specific Treg, 4 million each) had no effect (Fig. 1E). In further experiments, short-term administration of immunosuppressive drugs will be performed to delay islet rejection, combined with Treg administration.

Publications

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Interactions between Porcine Ligands and the Human Lymphocyte-Activating Receptor NKG2D

Refinements to our method of measuring cell-cell adhesion have allowed us to confirm and extend our preliminary data such that it now forms the basis for a manuscript[1].

Target cells HeLa (known to express the human NKG2D ligand MICA), PK15 (pig renal epithelial) or PIEC (pig endothelial) were seeded into 96-well plates and cultured to confluence. CHOP cells transfected with either NKG2D alone, DAP-10 alone, both constructs, or an empty-vector control, were labeled with CFSE, and added to the target cells. To determine the specificity of binding, transfected CHOP cells were pre-incubated with a blocking anti-NKG2D mAb (1D11) or IgG1 isotype control Ab (MOPC21). Plates were analyzed using a fluorescence plate reader, and all experiments were performed in triplicate. Results are shown in figure 1. In summary, binding was greatest to the double-transfectants. There was some binding to NKG2D alone, but DAP-10 alone did not increase binding above background. Binding of transfectants to all cell lines was blocked by the anti-NKG2D antibody 1D11, but not by the isotype-control antibody.

Ligand expression in porcine cells and tissues was also examined by binding of the human NKG2D-Ig fusion protein (NKG2D-Fc). NKG2D-Fc was used to stain HeLa cells, PK15, PIEC, and CHOP cells. As a negative staining control, soluble mouse ThB receptor with a human IgG portion was used (ThB-Fc). Flow cytometric examination of the stained cells showed that NKG2D-Fc is able to bind to HeLa, PK15 and PIEC, and not the CHOP cells. There was no staining of any cell line with ThB-Fc. Similarly, NKG2D-Fc but not ThB-Fc bound to renal tubules of a rejecting pig-baboon xenograft, explanted on day 5. NKG2D-Fc did not bind to a normal pig kidney, suggesting that expression of these ligands is induced under conditions of cellular stress.

Since the commencement of the project, we have been collecting tissues from a porcine preclinical model of ischaemia-reperfusion injury and transplantation, incorporating brain-death, organ procurement, storage and re-implantation. The first stage of these experiments,

examining events during the 6 hours following brain-death, is now complete. RT-PCR analysis shows that expression of mRNA for NKG2D ligands is up-regulated by 6 hours after brain-death. The pattern of ligand up-regulation varies between tissues, with MIC2 being expressed by livers, and ULBP1 by kidney and pancreas. The extent of ligand up-regulation is influenced by the management of the brain-dead donor, and in particular is increased by heavy use of noradrenaline. These results were the subject of an oral presentation at the Annual Scientific Meeting of the Transplantation Society of Australia and New Zealand in April 2006, and have also been accepted for presentation as a poster at the 2006 World Transplant Congress[2, 3].

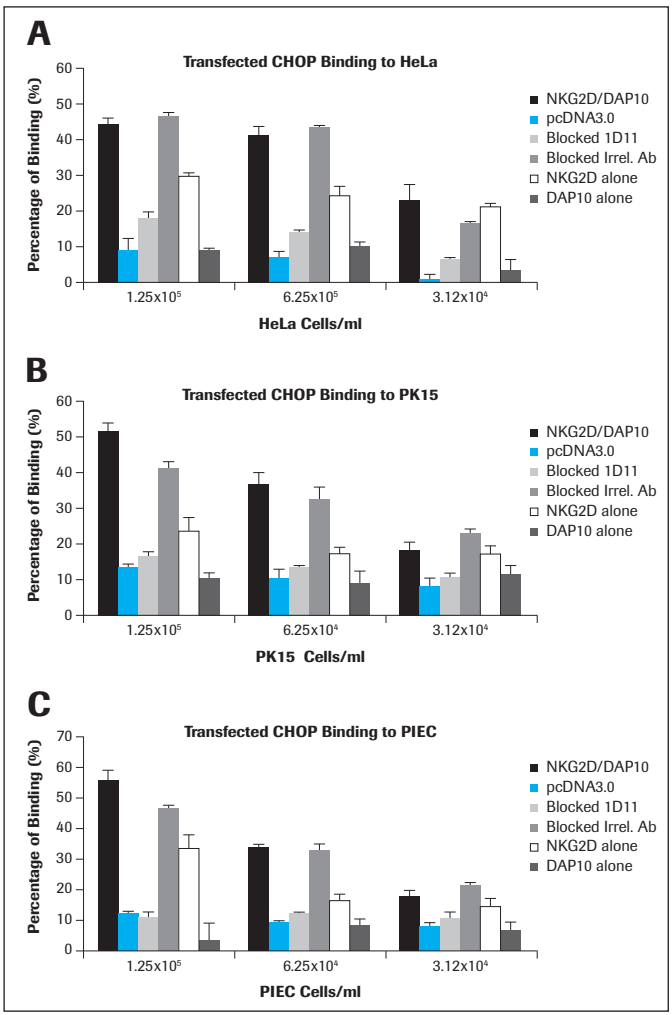

Figure 1: Specificity of binding of CHOP cells.

All the necessary constructs have been prepared for the soluble recombinant forms of the porcine ligands, and the first of these proteins (ULBP1) is now being produced from insect cells.

Publications

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2. Tran P, Hing A, Hicks M, et al. Expression of NKG2D ligands following brain-death. Oral presentation, *Annual Scientific Meeting of TSANZ* 2006; *(Abstract)*.
3. Tran P, Hing A, Hicks M, et al. Expression of NKG2D ligands following brain-death. Poster presentation, *Am J Transplant* 2006; 6:753; *(Abstract)*.

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Correction of Diabetes Using Primary Liver Cells

Aims

1. To determine if the expression of an insulin cassette alone and together with a β-cell transcription factor, induces storage of insulin and permanent reversal of diabetes in non-obese diabetic (NOD) mice (0–36 months).
2. To determine if we can achieve better physiological control of insulin production from livers of NOD mice, if we transfer insulin under the control of a glucose- and insulin-sensitive promoter (13–36 months).
3. To determine if the insulin-secreting cells in NOD mice are subject to autoimmune attack (10–36 months).

Research Outcomes

Aims 1 & 3

As mentioned in our report last year, early studies indicated that, we were successful in reversing diabetes in NOD mice for a period of 2 months with the expression of the insulin cassette alone in animals in which the onset of diabetes was marked by glucosuria and non-fasting blood glucose levels exceeding 11 mM on two occasions. However, subsequent studies indicated that in more overtly diabetic animals, with blood glucose levels exceeding 14 mM, we experienced less success and significant animal attrition. It was determined that this was related to the transfer of some contaminants from the vector production process that detrimentally affected these less robust animals. We spent some months developing a method of vector isolation that involves tangential flow filtration, which concentrates the vector removing contaminants.

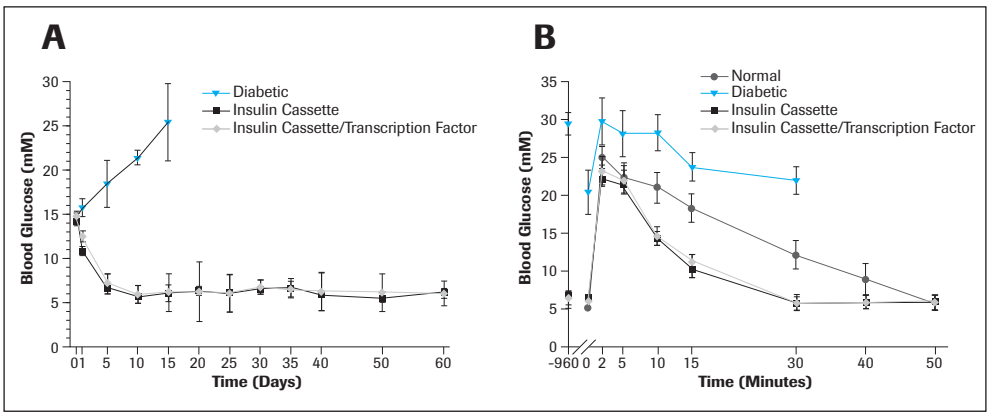

Figure 1. (A) *Blood glucose levels of diabetic mice, diabetic mice treated with the insulin cassette or the insulin/transcription factor cassette, 2 months previously.* ***(B)*** *Plasma glucose levels following intravenous glucose tolerance tests (IVGTT's) on the same animals, plus a normal control (n = 6, ± SEM).*

Figure 1A indicates that reversal of diabetes is seen almost immediately in the animals transduced with the insulin cassette and the insulin/transcription factor cassette. Normal blood glucose was maintained for 2 months, when the animals were sacrificed. By comparison, the blood glucose levels of the untransduced diabetic control animals continued to increase, as did the blood glucose of animals transduced with the empty vector (results not shown). Figure 1B shows that blood glucose levels of transduced animals during an (IVGTT) fell to normal levels more rapidly than non-diabetic animals. This is most likely because the diabetic process had already commenced to a certain extent in the non-diabetic controls, affecting the IVGTT response but not daily blood glucose levels. Immunohistochemical staining of the liver was positive for insulin and to a lesser extent glucagon and somatostatin indicating a level of pancreatic transdifferentiation. PCR analysis indicates insulin expression was restricted to the liver and we are currently examining expression of β-cell transcription factors in the liver. No evidence of autoimmune attack has been observed by microscopy. We are currently pursuing animals over longer time points, however, indicators are that we have accomplished the stable reversal of autoimmune diabetes with the insulin cassette alone.

Aim 2

In vitro studies with the glucose- and insulin-sensitive promoter indicate that on transduction of primary hepatocytes with 1–3 glucose-responsive elements (GRE) in the presence of 10^{-7} M insulin and increasing glucose concentrations from 0–25 mM, one GRE produced a significant increase (1.2-fold), this was further increased for two GRE (5.1-fold), but further increases were not seen for three GRE. We will shortly commence animal experiments with the GRE2 promoter in our system.

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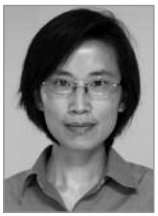

Visualizing Regulatory T Cell Control of Islet Transplant Rejection

In the grant proposal, we hypothesize that regulatory T cells (Tregs) with precise antigen specificities can prevent both allo- and autoimmune rejections of islet graft in diabetic individuals through bystander suppression.

We proposed the following specific aims to test the hypothesis:

Aim 1. Analyze LN and intragraft activation of allo-reactive T cells during alloimmune rejection of islet and skin allografts.

Aim 2. Determine the functions of allo-specific Tregs in controlling alloimmune rejection of islet and skin grafts in conventional and autoimmune mice.

Since grant funding, we have begun analyzing the anatomy of the allo-immune responses as outlined in specific aim 1 and started to characterize various Tregs from transgenic mice bearing TCR specific for allo-antigens.

Specific descriptions of the progress are as follows:

1. LN priming of allo-reactive T cells

We have assessed proliferation of direct and indirect allo-reactive T cells in islet and skin allograft recipients. Cells from TCR75 TCR transgenic mice recognize allogeneic peptide from BALB/c MHC class II I-E^d presented by C57BL/6 MHC class II I-A^b, whereas T cells from 4C TCR transgenic mice recognize BALB/c MHC class II I-A^d directly on BALB/c antigen-presenting cells (APC). In both skin and islet transplant settings, the TCR75 cells proliferated extensively whereas the proliferation of 4C cells was limited (islet data in Fig. 1A). We hypothesized that this is due to the limitation of donor-derived APC in the draining LN. To determine the number of resident dendritic cells (DC) in islets, we imaged islets purified from B6.CD11c.YFP transgenic mice. In steady state, each islet contained 5-15 DC (Fig. 1B), and after transplantation, these DC can be observed through the kidney capsule (Fig. 1C), however very few (less than 5) can be detected in the draining LN. Consistent with this finding, we found that the 4C T cells did not swarm and arrest; whereas in the same LN, majority of the TCR75 cells arrested (Fig. 1D,E). We are now determining the proportion and kinetics of donor-derived DC in the graft draining LN. This is crucial to the understanding of the activation of direct allo-reactive effector T cells.

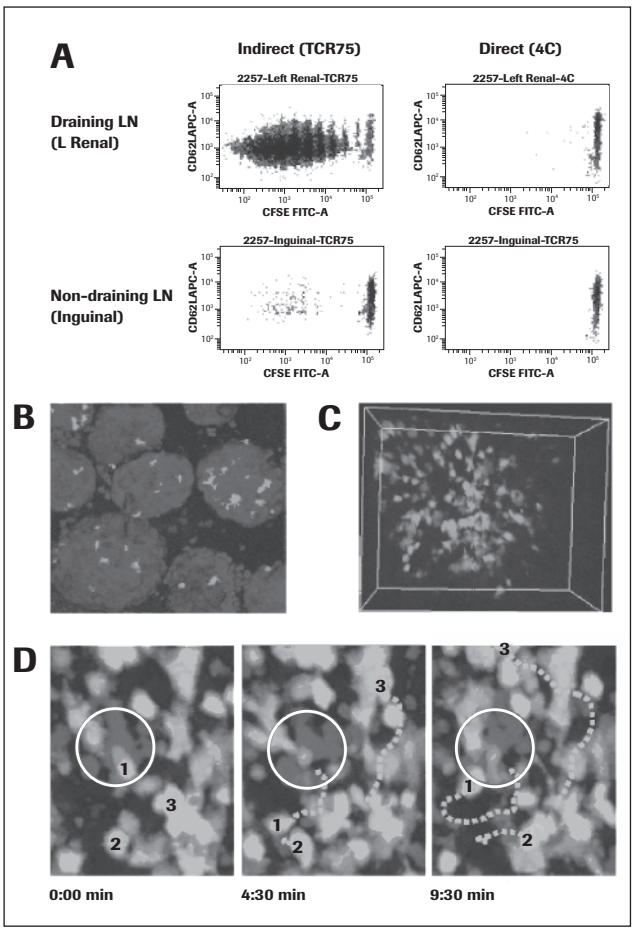

Figure 1. (A) *Proliferation of indirect (TCR75) and direct (4C) allo-reactive T cells. CFSE-labeled TCR75 and 4C CD4⁺ T cells were injected into C57BL/6 mice recipients of BALB/c allografts under the kidney capsule. Draining and non-draining LN's were harvested on day 4. Proliferation as assessed by CFSE dilution was determined by flow cytometry.* ***(B)*** *Islets from CD11c-YFP transgenic mice were purified and stained with vital DNA dye Hoechst 33342 and imaged on a confocal microscope.* ***(C)*** *Islets from CD11c-YFP transgenic mice were transplanted under the kidney capsule and graft-associated DCs were visualized by in vivo two-photon microscopy.* ***(D)*** *Early activation of indirect and direct alloreactive T cells in draining LN of islets allograft. BALB/c islets were transplanted under the kidney capsule of C57BL/6-CD11c-YFP recipients. Direct alloreactive T cells (4C yellow and numbered) and indirect T cells (TCR75, red and circled) were administered on day 3 and the draining LN explant was imaged on day 4. Three time-lapse images taken at 0, 4.5, and 9.5 minutes are shown, track for three 4C cells are indicated by dotted white lines and arrested TCR75 cells are highlighted in the circles.*

2. Efficacy of allo-reactive Tregs in preventing graft rejection

Previously, we have found that 4C Tregs could extend cardiac allograft survival for a few weeks. To determine if the limit in protection is due to insufficient numbers of Tregs transferred or intrinsic deficiency of direct allo-reactive Tregs, we increased the cell number from 5 million to 30 million to each recipient, and the rejection kinetics remained unchanged (data not shown). This result together with the very low number of donor DC present in graft-draining LN suggest that the limitation in protection by direct allo-reactive Tregs is likely due to insufficient activation of these cells *in vivo*. To assess the efficacy of indirect allo-reactive Tregs, we are now expanding Tregs from TEa TCR transgenic mice, as to our surprise the TCR75 mice have very few Tregs.

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Induction of Allograft Tolerance with Regulatory T Cells in Mice Harboring a Humanized Immune System

$CD4^+CD25^+$ regulatory T lymphocytes play a central role in maintenance of peripheral immunological tolerance. Given their crucial physiological function, we anticipated that these cells could be used to induce tolerance to allografts. In our protocol, we cultured host-type $CD4^+CD25^+$ cells freshly isolated from unmanipulated mice in the presence of donor-type antigen-presenting cells (APC). *Ex vivo* stimulated regulatory T cells are then injected into sublethally irradiated mice that receive simultaneously a fully allogeneic bone-marrow graft. Grafted cells survive for more than 100 days. When the mice are subsequently grafted with fully allogeneic hearts, the allografts are protected from rejection.

The research project financed by the Roche Organ Transplantation Research Foundation concerns the identification of effector mechanisms as well as cellular aspects of the induced tolerance to alloantigens. We have evaluated the role of the immunosuppressive cytokine IL-10 in regulatory T cell-mediated tolerance to bone-marrow allografts. Regulatory T cells from IL-10-deficient (or control wild-type) mice were *ex vivo* cultured with donor-type APC and injected into sublethally irradiated hosts that simultaneously received donor-bone marrow. Thus treated mice readily accepted the bone-marrow allografts. We also used IL-10-deficient mice as hosts for allogeneic bone-marrow. These mice received IL-10-deficient regulatory T cells. Five out of eleven thus treated mice lost their allogeneic bone-marrow grafts, indicating that IL-10 participates in the regulatory T cell-mediated immunological tolerance to allografts. Similar results have since been obtained for cardiac allografts. We also investigated the role of TGF-β in the protection of bone-marrow allografts by regulatory T cells. For this purpose we made use of T cells expressing a dominant negative mutant of the TGF-β receptor II ("dnTbRII"). While wild-type alloreactive T cells could be controlled by regulatory T cells, T cells insensitive to TGF-β could not. We conclude therefore that TGF-β plays a crucial and non-redundant role in the protection of allografts by regulatory T cells in our model.

In terms of cellular aspects, we studied the specificity-requirement of the regulatory T cells. In mice treated with regulatory T cells *ex vivo* cultured with donor-type APC, cardiac allografts survived for the 100-day observation period. However, histological analysis of the grafts after 100 days revealed substantial fibrosis, atherosclerosis, and mononuclear cell infiltrates. When regulatory T cells were used that had been cultured with (donor x host) F1 APC, no such chronic rejection was observed (Fig. 1).

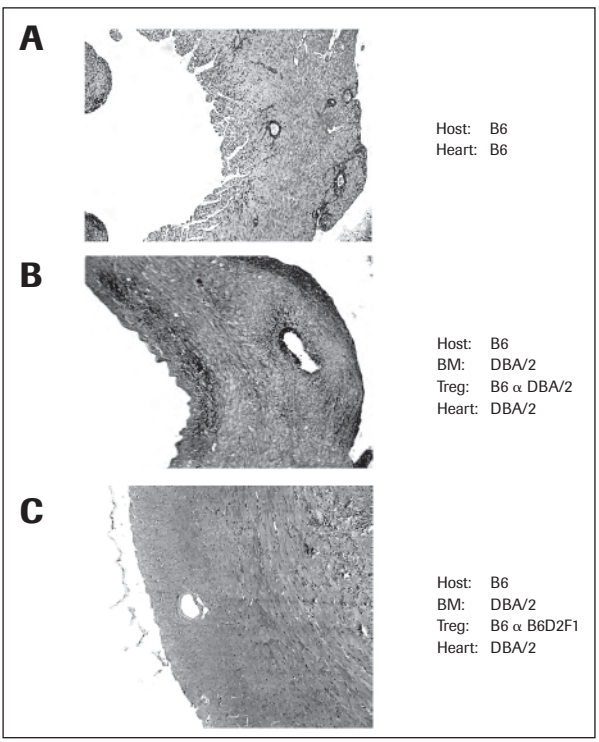

Figure 1. Prevention of chronic rejection requires regulatory T cells specific for indirectly presented alloantigens. *B6 hosts were* ***(A)*** *sublethally irradiated and transplanted 3 weeks later with a B6 heart,* ***(B)*** *sublethally irradiated and grafted with DBA/2 bone marrow plus Treg prestimulated with donor-type APC, and 3 weeks later transplanted with a donor-type heart,* ***(C)*** *sublethally irradiated and grafted with DBA/2 bone marrow plus Treg prestimulated with (donor x host)F1 APC, and 3 weeks later transplanted with a donor-type heart. In all cases hearts survived for the 100-day observation period. At 100 days hearts were analyzed by histology (Masson staining).*

These data therefore show that the specificity of the regulatory T cells plays a crucial role in the protection of allografts: cells specific for directly presented alloantigens will protect cardiac allografts from acute rejection, but prevention of chronic rejection requires regulatory T cells specific for indirectly presented alloantigens.

We are presently evaluating other molecular aspects of regulatory T cell-mediated tolerance to alloantigens and have more recently also used allogeneic skin grafts. Preliminarily these very encouraging results indicate that highly immunogenic grafts can be protected from acute and chronic rejection by a combination of transplantation of donor bone-marrow and donor-specific regulatory T cells.

Publications

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4. Santolaria T, Joffre O, Hudrisier D, et al. Induction of transplantation tolerance with $CD4^+CD25^+$ regulatory T lymphocytes. Poster presentation, *Nantes Actualités Transplantation* 2006; *(Abstract)*.
5. Santolaria T, Joffre O, Esquerré M, et al. Induction of transplantation tolerance with $CD4^+CD25^+$ regulatory T lymphocytes. Poster presentation, *Société Française d'Immunologie* 2005; *(Abstract)*.

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Immunosuppression and Tolerance Induction by Selective Inhibition of CD28

Aim 1: Characterization of regulatory cells in tolerance to rat kidney allograft

1a, b. Defining the phenotype of our suppressive non-T B7$^+$ regulatory cells[1]

The proliferation of alloreactive T cells in the blood of tolerant recipients could be restored by depletion of CD6$^-$ (non-T) CD80$^+$, CD86$^+$ or CD80$^+$CD86$^+$ cells. Therefore, blood non-T regulatory cells co-express CD80 and CD86 in tolerant recipients. Since removal of NKRP-1$^+$ cells also restored the proliferation of alloreactive T cells in the blood of tolerant recipients, we further tested the abundance, phenotype and function of non-T NKRP-1$^+$ CD80/86$^+$ cells in the blood. Myeloid cells with this phenotype were found accumulated in the blood of tolerant recipients, as compared with controls (61% increase; $p < 0.05$). Their phenotyping (5-color LSR FACs analysis) revealed the expression of CD161 (NKRP-1), CD80/86 (B7), CD172a (SIRP α), and CD11a, CD11b with a fraction of them expressing CD4. These cells are probably different from those initially identified *(also non-T B7$^+$, but not necessarily expressing NKRP-1)* since their suppressive activity is not donor-specific and is NO- and not IDO-dependent. Therefore, our results so far suggest the co-existence of two types of non-T regulatory cells, working through the action of IDO (CD6$^-$B7$^+$) and NO (CD6$^-$B7$^+$NKRP1$^+$), in a specific and non-specific manner, respectively.

1c. Western blot for the expression of IDO

CD6$^-$B7$^+$ cells from the blood of tolerant and control recipients were sorted and expression of IDO analysed by Western blotting (Fig. 1). The overexpression of IDO, together with our previous observations that 1-MT inhibited the suppression and induced rejection of otherwise tolerated kidneys *in vivo* (together with the blockade of NOS) reinforce the assumption that IDO participated in the tolerance observed in these animals.

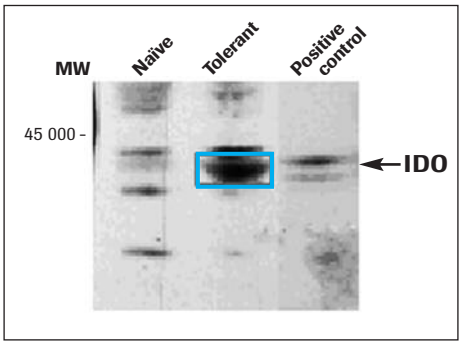

Figure 1. Western blot analysis of IDO expression by blood CD6$^-$B7$^+$ cells from naive and tolerant recipients. *Revelation was performed with a rabbit antibody against a rat IDO peptide.*

1d. Adoptive transfers

$1.5x10^8$ spleen cells from tolerant recipients were transferred to four freshly grafted recipients. The deaths of the recipients occurred at days 12, 12, 12, and $>$ 100. Since our model suffers from minor technical failures (breakdown of urethral anastomoses) resulting in the death of the recipient on day 12, we cannot yet come to any conclusion. Additional experiments have been planned. Nevertheless, the one animal experiencing prolonged survival suggests that transferable regulatory cells do exist in this model.

1e. Analysis of mRNA transcripts

We have first analyzed the transcriptome of tolerated whole rat kidneys versus syngeneic grafted controls using Applied Biosystems pan-genomic rat-DNA chips ($n = 3$ in each group). A similar analysis on sorted CD6$^-$CD80/86$^+$ blood cells, which is actually part of aim 1, will be the next step. The ongoing analysis revealed 333 differentially expressed genes, 82 being overexpressed more than 2 times (genes related to B and T cells, macrophages, TGFβ, TLRs, IL-13, FgL-2, TRIM/cyclophilin and Forkhead) and 49 being down-regulated more than 2 times (transcription factors, genes of the WNT pathway, G proteins).

Aim 2: Evaluation of sc28AT in baboon kidney allograft

For organisational reasons, we have planned to initiate these experiments in September 2006.

Publications

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2. Haspot F, Séveno C, Coulon F, et al. Anti-CD28 antibodies-induced transplant tolerance involving TCR$^-$ Class II$^-$ CD80/86$^+$ regulatory cells and tryptophan degradation. Oral presentation, *5th Annual Meeting of the Federation of Clinical Immunology Societies* (FOCIS) 2005; *(Abstract)*.
3. Haspot F, Séveno C, Coulon F, et al. Anti-CD28 induced transplant tolerance involves TCR$^-$ Class II$^-$ CD80/86$^+$ cells and is in part related to tryptophan degradation. Oral presentation, *Am J Transplant* 2005; 5:384; *(Abstract)*.
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Activation of Macrophages and Endothelium by Non-Complement Fixing Antibodies in Allograft Rejection

Alloantibodies are a clinically significant component of the immune response to organ transplants. In our previously reported model, passive transfer of a subthreshold dose of 25 μg of IgG2b Allo-mAbs or a single 100–200 μg dose of non-complement (C) activating IgG1 did not restore acute rejection of B10.A (H-2a) hearts to C57BL/6 (H-2b) Ig KO recipients. However, a combination of these Allo-mAbs caused acute graft rejection. Histologically, rejection was accompanied by augmented release of von Willebrand factor from endothelial cells and deposition of C4d in arteries and capillaries. Real-time PCR revealed higher expression of intragraft MCP-1 RNA transcripts in rejecting recipients treated with a combination of IgG2b+IgG1 compared to non-rejecting groups. To investigate a range of mediators stimulated by the interaction of IgG1 and IgG2b Allo-mAbs with endothelial cells, supernatants of mouse endothelial cells cultured in the presence of Allo-mAbs were screened in a cytokine microarray assay and then in ELISA. In these assays we identified high expression of MCP-1 and KC. Rantes and TIMP-1 were also detected. Co-cultures of endothelial cells sensitized with IgG1+IgG2b with macrophages stimulated high levels IL-6 in addition to MCP-1, KC, Rantes and TIMP-1.

To test our hypothesis that macrophage activation results from engagement of FcRs on macrophages by antibodies, endothelial cells were stimulated with IgG1 Allo-mAbs in the presence of lymph node/spleen cells from C57BL/6 WT and FcγRIII KO mice exposed for 10 days to B10.A skin transplants. The levels of both MCP-1 and IL-6 were significantly lower in co-cultures of endothelial cells with spleen or lymph node cells from FcγRIII KO skin recipients compared to co-cultures with WT cells. Blocking of FcγRIII with 2.4G2 mAb significantly inhibited IL-6 production by macrophages that were subsequently cultured with IgG1 immobilized on tissue culture plates.

To evaluate the effects of complement-mediated activation of macrophages, we used CR3-deficient C57BL/6 mice. CR3 is the primary receptor for C3 split products on mouse macrophages. CR3 binds multiple ligands including C3b/iC3b and acts as a signaling partner for FcRs. CR3 also augments FcR-mediated cytotoxicity toward tumor cells. In our *in vitro* model, mouse endothelial cells sensitized with IgG1+IgG2b were co-cultured with peritoneal

macrophages or alloantigen primed lymph node cells from CR3 KO or WT skin graft recipients in the presence of normal mouse sera (NMS). The levels of MCP-1 and IL-6 were significantly lower in co-cultures with cells from CR3 KO than with cells from WT (Fig. 1). We conclude that CR3 participates effectively in reciprocal activation of endothelial cells and macrophages by interaction with C3b/iC3b generated in the presence of IgG2b alloantibodies and NMS. Since CR3 can bind to integrins (ICAM, extracellular matrix proteins) as well as C3b/iC3b we will test CR3-mediated activation of macrophages in C3 deficient serum.

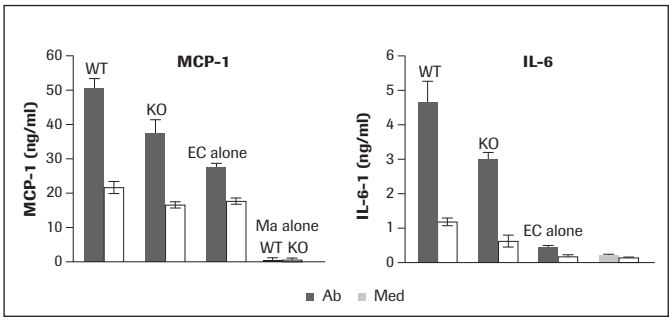

Figure 1. *The effect of IgG1+IgG2b mAbs on MCP-1 and IL-6 production in 48 hour co-cultures of SVEC4-10 endothelial cells with CR3 KO C57BL/6 peritoneal macrophages in the presence of 10% normal mouse sera. Solid columns (Ab), opened columns (Medium, isotype controls).*

Our findings indicate that non-C-activating Allo-mAbs can augment injury to allografts by C-activating Allo-mAbs. Both, C-activating and non-C-activating Allo-mAbs stimulate endothelial cells to produce chemokines which in turn activate macrophages. They are also activated via engagement of Fc domain of antibodies and C3b/iC3b by macrophage FcR and CR3, respectively.

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Potency of Embryonic Stem Cells to Induce Graft Tolerance

In Specific Aim 1 of our proposal we proposed to determine the immunogenicity and susceptibility of embryonic stem cells (ESC) to NK and CTL cytotoxicity. Further, we proposed to study the role of FasL on ESC engraftment. We have now shown that ESC are not susceptible to NK killing or lysis by alloreactive T cells. The results for NK cells are rather surprising as we have also now shown for the first time that NK cells express RAE-1, retinoic acid expression antigen 1, which is a ligand for NK2GD (Fig. 1).

We further proposed to examine the role of FasL that is constitutively expressed by ESC. Our data on Fas-deficient mice, the lpr/lpr, show that lack of Fas-FasL interaction results in poor ESC engraftment[1].

In Specific Aim 2, we set out to establish stable mixed chimerism with ESC. Non-manipulated ESC have proven to be less suitable for the induction of mixed chimerism. So far, the level of established mixed chimerism has been in the order of 5–10 %, but has been lost by 28 days, suggesting rejection. Skin grafting in these animals has showed prolonged graft survival, but subsequent rejection too. Thus, our strategy has been to use BMP4-treated ESC that pre-direct ESC differentiation into the hematopoietic lineage. Further, the role of a number of transcription factors is being studied, in particular that of HoxB4. ESC have also been transduced with cre-recombinase to allow the study of cell fusion in mice transgenic for the floxed loxP sites with a lacZ reporter gene. At the conclusion of these experiments, we are confident that we will be able to establish stable mixed chimerism that will allow successful transplantation of cardiac and skin allografts without the need for immunosuppression.

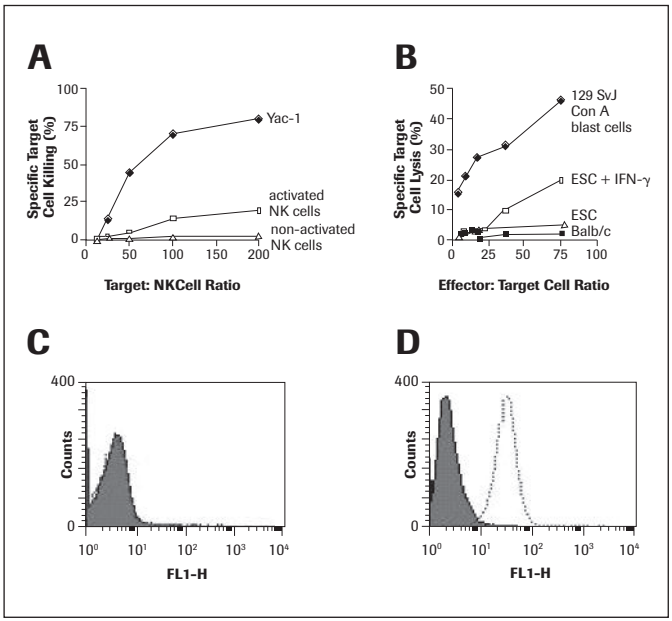

Figure 1. ESC express RAE-1 and can be susceptible to NK cells and CTLs under IFN-γ stimulation. *To determine ESC susceptibility to NK cells, ESC were used as target cells in a cytotoxicity assay. The Yac-1 cells were positive controls. NK cells were not lysed by NK cells, however, modest killing was observed when NK cells had been treated with IFN-γ* ***(A)****. Anti-129SvJ CTL were raised and tested for their cytotoxicity against ESC. Con A blast cells were lysed, but not ESC nor the Balb/c third-party target cells. After IFN-γ treatment, ESC were more susceptible to CTL killing* ***(B)****. To determine whether ESC express RAE-1, splenocytes* ***(C)*** *and ESC* ***(D)*** *were tested by flow cytometry for RAE-1 expression. ESC, but not splenocytes express high levels of RAE-1, a ligand for NK cell receptors[2].*

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8. Mueller T, Scoville HAC, Bonde S, Zavazava N. $CD117^+$ cells derived from embryonic stem cells induce stable mixed chimerism in allogeneic mice. Oral presentation, *American Transplant Congress* 2005; *(Abstract)*.
9. Zavazava N. Immunobiology and homing pattern of murine embryonic stem cells. Oral presentation, *American Transplant Congress* 2005; *(Abstract)*.

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The Toll-like Receptor 4 and Liver Ischemia Reperfusion Injury

The question we have focused on this year is to determine the roles of different TLR4 downstream signaling and effector pathways in liver ischemia-reperfusion, in particular, the type-I interferon pathway. As one of the major pathways activated by TLR4 engagement, it is MyD88 independent but interferon regulatory factor 3 (IRF3) dependent, as shown in our previous publication. Additionally, chemokine CXCL10 (IP-10) was induced significantly as the result of this particular pathway activation, which was correlated closely with the development of liver inflammation and hepatocellular injury. Further characterizations of CXCL10 induction during liver IR showed that it was a rapid (within 1 hour of reperfusion and peaked at 4 hours) and local (no induction detected in spleens of liver IR mice) response, restricted within ischemic lobes (much reduced induction in adjacent lobes). As CXCL10 belongs to the CXCR3 ligand family which includes two other members, their induction in liver IR injury (IRI) was examined. Neither CXCL9 (Mig) nor CXCL11 (I-TAC) was induced during the first 6 hours of liver reperfusion. The functional significance of CXCL10 in liver IRI was tested in either WT mice with neutralization antibodies (rabbit polyclonal from Cell Sciences, or rat monoclonal from Dr. A. Luster) administered post-ischemia but prior to the onset of reperfusion (0.2 mg/mouse), or using CXCL10 KO mice. In both cases, liver IRI was reduced as shown by both histology and serum ALT levels (Fig.1). Interestingly, liver inflammation (measured by quantitative RT-PCR of intrahepatic TNF-α, IL-1β, IL-6, MCP-1 gene induction) was diminished only in CXCL10 KO mice, but remained the same in antibody-treated as in untreated mice (Fig.1). Thus, there are at least two potential mechanisms responsible for CXCL10 functions in liver IRI: promoting liver inflammation, and/or executing hepatocellular damages.

To explore the first mechanism, liver IR-induced gene expression (including the above plus IL-10, IL-12) was further characterized kinetically, and compared between WT and CXCL10 KO mice. While the induction of the proinflammatory set of genes was mostly reduced, the induction of immune regulatory IL-10 was intact in CXCL10 KO mice. The functional significance of IL-10 induction in KO mice was tested by its neutralizing antibodies administered at the onset of reperfusion. Interestingly, blocking IL-10 function fully restored liver inflammation and recreated liver IRI in CXCL10 KO mice (Fig.1). This indicates that

CXCL10 KO mice are capable of mounting inflammatory responses. In the absence of CXCL10, the liver organ is less sensitive to IR insult, of which IL-10 plays a key regulatory role. The role of CXCL10 in promoting liver inflammation is specific to IRI, as livers respond to LPS stimulation the same in KO and WT mice in producing proinflammatory cytokines/ chemokines.

Thus, we have demonstrated a critical chemokine downstream of liver TLR4 activation during IR: CXCL10, whose constitutive expression promotes the induction of liver inflammation by IR. Targeting this particular chemokine may provide a novel strategy for clinics to ameliorate liver IRI.

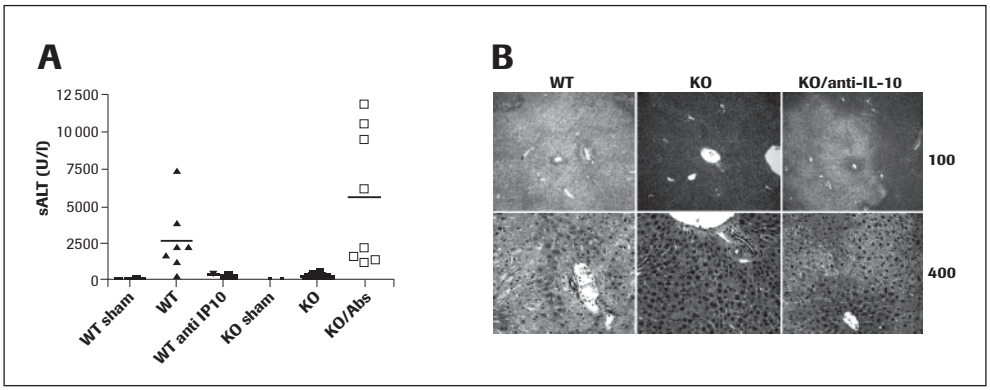

Figure 1. (A) Serum ALT levels: *Groups of WT, WT treated with anti-CXCL10 antibodies, CXCL10 KO, or CXCL10 treated with anti-IL-10 antibodies mice were subjected to either sham-operation or 90 min liver partial warm ischemia. Serum ALT levels were measured 6 hours post-reperfusion.* ***(B) Liver histology (H/E) under low or high magnifications:*** *Livers were harvested at 6 hours post-reperfusion.*

5.2 Clinical Research Grants

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Identification of Prognostic Markers and Targetable Genes in Protocol Renal Transplant Biopsies

Introduction

Chronic allograft nephropathy (CAN) is the major cause of renal graft loss. Interstitial fibrosis is prominent in renal biopsies of patients with CAN. Myofibroblasts, the main effector cells responsible for fibrosis, can originate from tubular epithelial cells by epithelial-to-mesenchymal transition (EMT). In keeping with the original working plan, we investigated whether expression levels of EMT-related and fibrosis-related molecules are of prognostic relevance in protocol renal allograft biopsies.

Aims of the project

1. To determine the predictive value of mRNA expression levels for graft outcome in comparison to that of histological and clinical variables.
2. To test predictive value of the corresponding protein using prognostic markers at the mRNA level.
3. To determine the site of expression of the mRNA transcript in the tissue.

Progress to date

Of the patients, 130 participated in a prospective study. Sufficient protocol renal biopsy material at 6 and 12 months after transplantation was available from 80 patients.

Aim 1. As described in the original working plan, intrarenal mRNA expression levels of transforming growth factor-β, (TGF-β), S100A4, Smad interacting protein-1, Smad 1, Smad 2, Smad 5, bone morphogenic protein-7, hepatocyte growth factor, S100A9, prolactin receptor, and interleukin-10 were quantified with QPCR. The biopsies were scored according to Banff criteria and the extent of interstitial fibrosis was assessed by quantitative Sirius red-staining. Data on donor age, delayed graft function, acute rejection episodes and renal function

(glomerular filtration rate; GFR) at 6 and 24 months were collected. Patients showing an increase of GFR between 6 and 24 months had significantly ($p < 0.05$) higher mRNA expression levels of TGF-β and S100A4 in their 6-month biopsies compared to patients with a decrease of GFR over time (Fig. 1A, B). None of the clinical and morphologic data within the first 6 months post-transplantation discriminated the two patient groups.

Aim 2. Immunohistochemistry was performed for S100A4 using peroxidase staining. The extent of area stained positive by the antibody in the biopsies was not associated with outcome. Following the proposed research plan, EMT was further studied at the protein level using double-labeling immunofluorescence for S100A4 and the tubulus marker cytokeratin-8/18 (Fig. 1C).

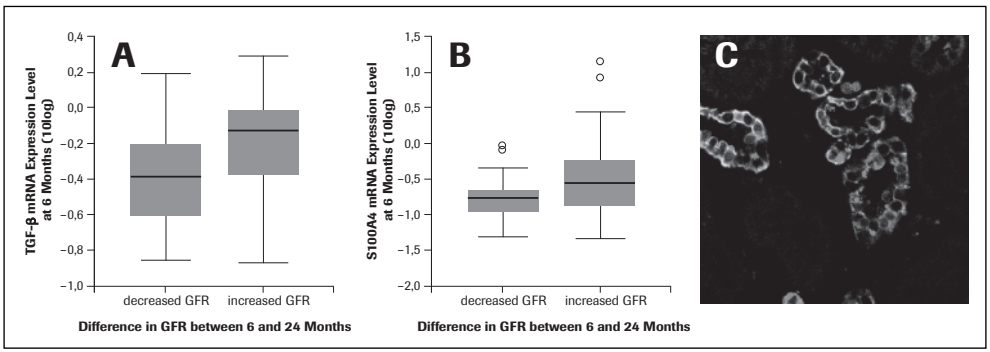

Figure 1. *Box and whisker plots of log transformed (10log) mRNA levels for* ***(A)*** *TGF-β, and* ***(B)*** *S100A4 at 6 months, respectively. mRNA expression levels were significantly increased for both molecular markers in those patients showing an increase in GFR between 6 and 24 months, $p < 0.05$.* ***(C)*** *Merged image depicting colocalization of S100A4 and cytokeratin 8/18 in the renal tubules. GFR: calculated by the Cockcroft formula; (o) indicates outlier.*

Significance

Strikingly, increased mRNA expression of EMT-related markers in protocol renal transplant biopsies taken at 6 months predicted improvement of graft function over time. The current results show that the prognostic value of mRNA levels exceeds that of clinical and morphologic variables. Furthermore, the results may suggest that EMT does not necessarily contribute to fibrosis but could rather represent an increased tubular repair and connective tissue remodeling for maintaining stable graft function.

Objectives for the next year

Aim 1 of the project, as proposed in the original research plan, has been largely addressed. To complete the work indicated in the project plan, in the current patient cohort additional genes that are related to EMT and to fibrogenesis will be studied by QPCR. Follow-up time will be extended to four years post-transplantation. Prognostic markers at the mRNA level will be studied by immunohistochemistry and RNA *in situ* hybridization.

Publications

1. Roos-van Groningen MC, Scholten EM, Baelde JJ, et al. Prognostic relevance of the expression of epithelial to mesenchymal transition-related molecules in protocol renal allograft biopsies; *(Submitted)*.
2. Roos-van Groningen MC, Scholten EM, Baelde JJ, et al. Protein assessment of epithelial to mesenchymal transition-related molecules in protocol renal allograft biopsies; *(In preparation)*.
3. Roos-van Groningen MC, Scholten EM, Baelde JJ, et al. The prognostic relevance of epithelial to mesenchymal transition related markers in protocol renal allograft biopsies. *Transplantation* 2006; 82:462.
4. Roos-van Groningen MC, Scholten EM, Baelde JJ, et al. The prognostic relevance of epithelial to mesenchymal transition related markers in protocol renal allograft biopsies. *J Am Soc Nephrol* 2006; *(Abstract)*.
5. Roos-van Groningen MC, Scholten EM, Baelde JJ, et al. The prognostic relevance of epithelial to mesenchymal transition related markers in protocol renal allograft biopsies. Oral presentation, *Am J Transplant* 2006; 6:463; *(Abstract)*.

Dr Luis A. Fernandez, Principal Investigator
Dr Jon S. Odorico, Co-Investigator
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Characterization of Human Islet from Donation after Cardiac Death: Assessment of the Liver's Microenvironment after Islet Transplantation

Isolation of functionally potent human islets is technically difficult and is compounded by donor-specific variables, cold and warm ischemia, and isolation-induced stresses that lead to intracellular redox state imbalance, excess reactive oxygen species production, mitochondria dysfunction and apoptosis. We have developed a comprehensive quality control assessment protocol to augment the standard FDA-approved assays, providing a more complete and clinically relevant diagnosis of overall islet graft quality.

Methods

Human islet quality was assessed after 24-hour *in vitro* culture by:

1. flow cytometry analysis of single cells stained with fluorescent probes for membrane integrity (ToPro3), apoptosis (Annexin V and VADFMK), mitochondrial membrane potential (JC1) and reactive oxygen species (ROS) (DHE);
2. luminol-based chemiluminescence assay of ROS levels in the basal state and after stimulation by 16.7 mM glucose or the mitochondrial complex I inhibitor rotenone;
3. fluorescent probes and glucose-induced reduction of pyridine and flavin nucleotides and FuraRed fluorescence measured and quantified in a 15-minute kinetic assay on a FACSVantage™ flow cytometer and compared to INS-1 cells and rat islets;
4. HPLC quantification of adenine and pyridine nucleotides to determine basal energy and redox state;
5. glucose responsiveness as measured by static insulin secretion assays;
6. long-term function with reversal of diabetes by transplant under the kidney capsule of streptozotocin-induced diabetic immunodeficient mice.

Results

Our preliminary results demonstrate that a cut-off value for each assay could be established which clearly distinguishes high- from low-quality islet preparations. High-quality islets were characterized by cells with viability (80%), apoptosis (12%), necrosis (10%), and DHE+ (flow cytometry; 10%); low basal ROS levels in parallel with high levels induced with glucose (2x) and rotenone (3x; luminol-based chemiluminescence); ATP/ADP 3 and NADH/NAD 0.25 (HPLC); glucose-induced insulin secretion stimulation index 3 (static incubation): reversal of diabetes in the NOD-SCID mice. Comparative analysis of INS-1, rat and human islet cells using this method revealed that the majority of human islet preparations showed functional impairments and redox state imbalance that were not predicted simply by the level of apoptosis or insulin secretion. The predictability of these tools correlates with clinical observations. Those patients with a score above the thresholds stated above have an average reduction in daily insulin requirements of 25 ± 13 units per day after a single islet preparation with normalization in HbA1c and stable glucose control after a mean follow-up of 6 months post-transplant.

Quality Control Assessments	**Transplanted Preps (*n* = 4)**	**Rejected Preps (*n* = 1)**
Yield (IEQ)	984,839 ± 236,063	390,000
Purity	87.5 ± 5.0	90.0
Packed Cell Volume (ml)	4.1 ± 1.0	ND
Dose (IEQ/ kg patient body weight)	12,678 ± 2563	ND
Cellular Integrity		
Viability (fluorescent microscopy)	91.5 ± 4.7	ND
Viability (flow cytometry)	84.6 ± 9.9	59.7
Apoptosis (flow cytometry)	11.9 ± 9.7	17.8
Necrosis (flow cytometry)	3.5 ± 0.9	22.5
Rotenone Index [100 nM] (luminal-based ROS)	6.0 ± 3.1	2.3
Glucose Index [16.7 mM] (luminal-based ROS)	3.0 ± 1.4	1.1
ATP/ADP (HPLC)	4.1 ± 1.1	3.3
Functional Potency		
GSIS (static incubation S.I.)	5.6 ± 2.1	1.6
Patient Outcomes		
Average reduction in daily insulin requirements (units/day)	25.3 ± 13.0	

Table 1. Clinical outcomes of islet isolations evaluated using the UW-Madison Expanded Release Criteria Assessment Protocol (preliminary results).

In summary, we have developed a novel set of diagnostic tests of islet viability and functional potency that significantly exceed the sensitivity, accuracy, and precision of the methods currently approved by the FDA as release criteria assays. These new methods are critical components of both our quality control program and improvement plan and will assist in our understanding of islet biology and improvement of higher quality of islets for transplantation. Further correlation with clinical outcome is needed.

Conclusions

A comprehensive assessment of islets prior to transplantation constitutes a powerful tool to minimize primary non-function and predict clinical outcome. More importantly, application of these tools could guarantee the transplantation of islets of the highest quality which will impact the need for repeat infusions to achieve insulin independence.

Publications

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Detection and Prevention of Patients with High Risk of Chronic Allograft Nephropathy Development

Background

Tubulointerstitia are affected in chronic allograft nephropathy (CAN). However, there is little information on proximal tubular dysfunction (PTD) in this situation. In this project we exploit the morphologic pattern and molecular fingerprints of PTD, which we have shown to be an early marker of CAN, and correlate it with graft prognosis.

Specific aims and progression report

1. Is PTD present in stable renal transplant patients before tubulointerstitial fibrosis development?
To answer this question 67 renal transplant patients were included in this phase of the project. Urinary retinol binding protein (uRBP) was collected serially during 3-consecutive outpatient visits, and at the last consultation a protocol biopsy was performed. Preliminary results demonstrated that 33 of 67 patients presented higher uRBP (> 0.400 mg/l; 1.40 ± 0.83 vs. 0.12 ± 0.09 mg/l, $p < 0.001$), where the serum creatinine levels were more similar than normal uRBP levels (1.47 ± 0.34 vs. 1.42 ± 0.34, $p = 0.628$). We intend to enrol 100 patients and to follow them for 3 years, and perform graft biopsies at time zero. Any clinical suspicion of CAN will be investigated by graft biopsy.

2. Can we observe any morphological alteration in proximal tubular cells in patients with PTD?
Immunohistochemistry for epithelial-mesenchymal-transdifferentiation (EMT) markers associated with morphometric analyses for tubulointerstitial fibrosis was performed (50 biopsies at time zero in patients with higher and normal levels of uRBP). Preliminary results demonstrated that 42% patients have PTD. After taking into consideration the histological findings, 23/50 were considered normal according to Banff criteria. Of the patients, 60% had no tubule injuries and 34% had no interstitial abnormalities. Cytokeratin positive staining was

present in 70.7% of the biopsies from patients with normal uRBP and in 70.3% of those with high uRBP ($p = 0.970$). Additionally, we did not see any statistical differences in α-smooth muscle actin and vimentin staining in both groups (23% vs. 27% and 1.14% vs. 1.31%). We aim to increase the enrolled population and investigate other markers of EMT.

3. Which genes are activated in the graft during this early point of CAN development?

Tissue fragment collected from protocol biopsies from patients with higher ($>$ 0.400 mg/l) or normal levels of uRBP was kept in RNA later and snap frozen for further mRNA extraction and hybridization in GE microarray membranes.

Perspectives

Besides these aims, 166 patients were also enrolled for a prospective study where we addressed the question whether PTD could be reversed by individualization of immunosuppression, namely removal of calcineurin inhibitor and introduction of mycophenolate mofetil. Of 166 patients, 31 presented sustained higher levels of uRBP, and after a protocol biopsy, they were randomized into two groups according to previously described individualization of immunosuppression.

6. Final Reports of ROTRF Grantees

Dr David Briscoe, Principal Investigator
Dr Stuart Robertson, Co-Investigator

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Vascular Endothelial Growth Factor in Acute and Chronic Rejection

Vascular endothelial growth factor (VEGF), a potent angiogenesis factor, has recently been identified to have proinflammatory properties. It has direct effects on the recruitment of T cells and monocytes, and it can facilitate leukocyte recruitment via its ability to induce the expression of endothelial cell adhesion molecules and chemokines. Hypoxia, which is a characteristic component of transplantation is well established to be a potent physiological stimulus for the expression of VEGF. We, and others, have reported that the immune response (via cytokine-mediated responses and cell surface interactions) also facilitates the expression of VEGF.

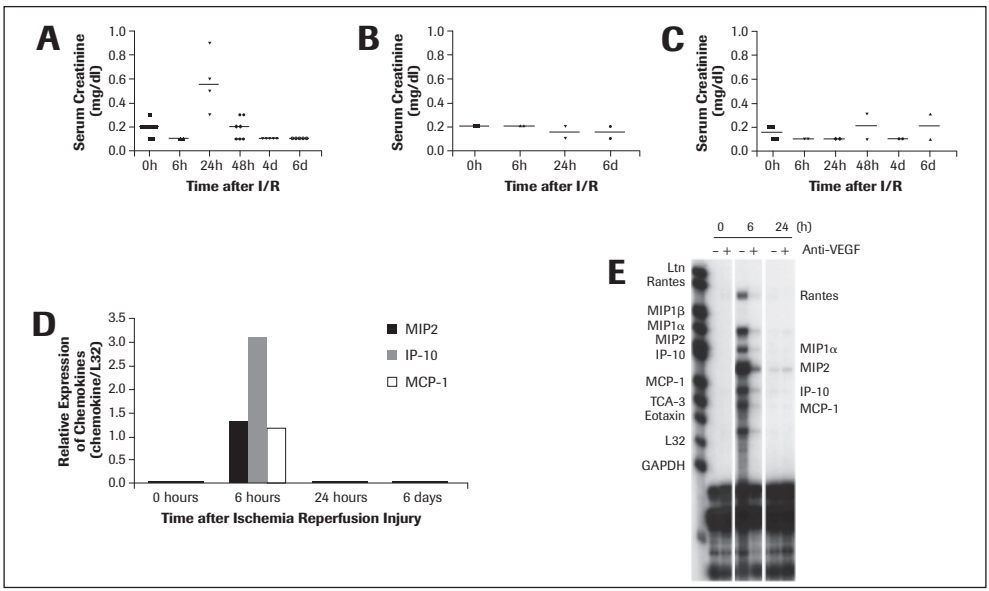

Figure 1. *Renal ischemia was induced by clamping of the left renal pedicle for 45 minutes; and serum creatinine was monitored at 2, 6, 12, 24, 48 hours and daily following release of the clamp. Animals were untreated* ***(A)****, were treated with a blocking anti-VEGF anti-serum on day –1, and days 0, 1 and every other day* ***(B)*** *or with PTK 787 (a known VEGF post-receptor kinase inhibitor 100mg/kg/d,* ***(C)****. mRNA was generated from kidneys harvested at 2, 6, 12, 24 and 48 hours following ischemia and RNase protection was performed to assess the time-dependent patterns of chemokine expression in association with reperfusion injury* ***(D, E)****. Chemokine expression was evaluated in untreated animals* ***(D)*** *and animals treated with anti-VEGF* ***(E)****. The expression of chemokines was quantified by densitometry relative to GAPDH* ***(D)****. Representative of 3–5 animals/group.*

With the support of the ROTRF, we first evaluated the expression and function of VEGF in association with ischemia-reperfusion (I/R) injury. We found that VEGF is expressed at early times in association with I/R; and that blockade of VEGF or VEGF receptor inhibits leukocyte recruitment, proinflammatory chemokine expression and acute renal failure associated with I/R injury. In addition, in models of rejection, we found that VEGF is delivered into allografts by leukocytes (especially monocytes) and it mediates chemokine expression to further augment the inflammatory process. Blockade of VEGF inhibited chemokine expression as well as leukocyte recruitment, but this function of VEGF required the ability of the graft to express the chemokine MCP-1.

Collectively, our studies supported by this grant have enabled us to develop models and studies that will further characterize the proinflammatory function for VEGF in the early post-transplant period
a) in association with I/R injury, and
b) in association with immune-mediated injury to allografts.

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Molecular Virology of Post-Transplant Malignancy: A Novel Large Animal Model

Post-transplant lymphoproliferative disorder (PTLD) is a common complication following solid organ transplantation. PTLD is often associated with Epstein-Barr virus (EBV) infection, generally with marked B cell proliferation and expression of viral proteins. In the absence of protective cellular immune responses, B cell proliferation progresses to malignant, non-Hodgkin's lymphoma. The pathogenesis of this disorder remains uncertain. In our novel model, PTLD develops in MHC-inbred miniature swine undergoing splenic transplantation or allogeneic HSCT, preceded by 10-100-fold increase in porcine lymphotropic herpesvirus-1 (PLHV-1) viral load. PLHV-1 is a γ-herpesvirus with homology with EBV and human herpesvirus-8 (HHV-8).

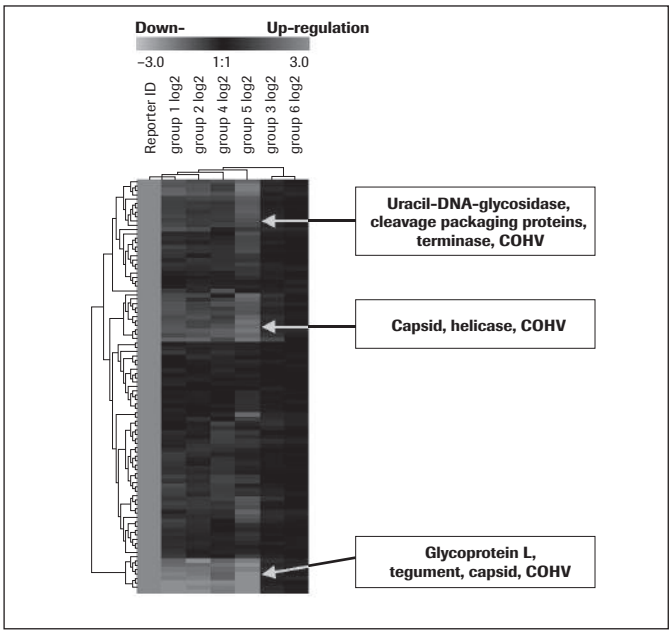

Figure 1. Microarray visual image of combined 5'- and 3'- reporters of open reading frames from PLHV-1. *Image compares mRNA expression from six PLHV-infected miniature swine with post-transplantation lymphoma to six without PTLD. Black indicates no significant change.*

Oligonucleotide microarrays were designed based on the open reading frames (ORFs) of PLHV-1. After allogeneic spleen transplantation, PLHV-1 ORF expression was compared by co-hybridization of cDNA from lymph nodes from PLHV-1+ swine either with or without PTLD. In PLHV+ animals, consistent up-regulation (9 ORFs) or down-regulation (4 ORFs) of PLHV-1 mRNA was observed in comparison to those without PTLD ($p < 0.05$ for each; Fig.1). Seven of these gene products are known (3 capsid proteins, tegument protein, putative fusion protein, helicase and uracil-DNA-glycosidase); 6 are of unknown function but conserved across herpes viruses.

Alterations in PLHV-1 mRNA transcription occurred before the onset of clinical disease. Studies are underway to characterize additional homologous gene products between PLHV-1 and EBV as potential biologic targets for the diagnosis and treatment of PTLD. This model provides insights into the pathogenesis of PTLD and, by extension, potential diagnostic and therapeutic tools for human EBV-associated PTLD.

Publications

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Prof. Philip F. Halloran, Principal Investigator

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Mechanisms of Renal Allograft Rejection

The molecular mechanisms of rejection are important for diagnosis, drug development, and tolerance initiatives. We propose to study the effector mechanisms of kidney rejection by analyzing the key pathologic lesion of human renal rejection, tubulitis. The project was undertaken to describe the pathologic changes in mouse kidney transplants, and in particular establish the relationship between the transcriptome and the pathologic changes.

Our key strategy is to study the mechanisms in a model system that recreates the human Banff lesions that are the key features in human rejection; then to solve the transcriptome in humans and mice, using the mechanistic studies in mouse as the Rosetta stone for assigning transcripts to pathologic processes.

We have developed a model of vascularized renal allograft rejection which develops progressive tubulitis but remains viable because one host kidney is left in place. Interstitial infiltration peaks and stabilizes, while tubulitis develops slowly and persists. In all, approximately 500 mouse kidney transplants have been performed by our microsurgery team led by Dr. Lin-Fu Zhu. These have resulted in a number of publications since 2003[1–10]. However, it was this project that served as the basis for our expansion to a program funded by Genome Canada. This has allowed us to examine mouse kidney transplants, establish the pathogenesis-based transcript sets, and take this information to human kidney transplants.

The Pathogenesis of Tubulitis

We began the project with the belief that tubulitis represented an attack by effector T cells on the epithelium, probably mediated by cytotoxicity via CD103 engagement of E-cadherin. However, we found that tubulitis is unchanged in perforin-deficient mice, immunoglobulin-deficient mice, granzyme A and B double knockout (KO) mice, and CD103 KO mice. Using CD103 KO hosts we determined the role of CD103 in tubulitis, other lesions of rejection, and rejection of other vascularized organs. We hypothesize that CD103 deficiency will selectively abrogate tubulitis and the loss of E-cadherin, without affecting other rejection lesions. Nevertheless, the CD103 ligand E-cadherin is severely reduced and redistributed in tubulitis lesions by day 21. Tubulitis is completely T cell dependent, and does not occur in nude or RAG

KO mice. We conclude that tubulitis represents a stage in epithelial deterioration mediated by a delayed type hypersensitivity process acting on the epithelium, culminating in epithelial-mesenchymal transformation (EMT). We propose that the loss of cadherins permits the entry of inflammatory cells. We have examined the possibility that P and GrAB are homeostatic regulators by studying apoptosis and cycling in stained sections and by flow cytometry. However, these results have been largely negative. Instead, we now believe that the perforin-granzyme system is truly cytotoxic but its activity is usually held in check by inhibitory signals from the tissues induced by IFN-γ probably acting through inhibitory NK receptors on the CTL. We explored the possibility that tubulitis alters the β-catenin pathway of gene regulation, but have not found evidence for this. However, tubulitis triggers loss of epithelial transcripts and at least in humans may lead to EMT, creating both nephron loss and interstitial fibrosis. We will look for evidence of epithelial changes suggesting synapses.

Microarrays

We have two initiatives to explore the relationship of microarrays to tubulitis lesions. We will perform analysis in Affymetrix® GeneChips in the service facility in the IBD at the University of Alberta; and in Stanford arrays in an ongoing collaboration with Dr. Minnie Sarwal. We aim to define and validate at least 5 major patterns: normal kidney, (ischemic or toxic) renal injury, IFN-γ inducible genes in kidney; early rejection (day 5, day 7); and day-21 rejection with severe tubulitis, with LCM dissection of the latter to confirm tubulitis.

Significance

These new insights into pathogenesis of tubulitis will significantly advance our understanding of the elusive molecular mechanism of renal rejection.

Perspective

These results led directly into the Genome Canada project described in the midterm review from the Genome Canada grant. The ROTRF grant provided the data that served as the preliminary results on which this grant was based. We regard this as an extraordinary success story that has allowed us now to recruit industrial investment and to create the Alberta Transplant Applied Genomics Centre. Thus an ROTRF grant led to a Genome Canada award and a new university centre. None of this would have been possible without the ROTRF award.

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Prof. Alastair D. Burt, Co-Investigator
Dr Helen Robertson, Research Associate

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Therapeutic Elimination of Intraepithelial T Cells: A Strategy to Reduce Epithelial-Mesenchymal Transition and Chronic Allograft Dysfunction

The objective of this project was to define the relationship between chronic inflammation and the induction of allograft-damaging epithelial to mesenchymal transition (EMT) in the transplanted human kidney. The main finding is that activated T cells penetrate the renal allograft epithelium where a proportion differentiates to express the αEβ7 (CD103) integrin allowing adhesive interaction with E-cadherin-expressing epithelial cells. Importantly, these T cells also express high levels of the immunoregulatory marker FOXP3 and are maintained through the inhibition of apoptosis by the IL-15 rich intraepithelial microenvironment. They can also present membrane-associated TGF-β to adjacent epithelial cells. These epithelial cells respond by activation of the Smad 2/3 signalling pathway which induces the epithelial cells to de-differentiate to form fibroblastic cells which acquire expression of the characteristic marker S100A4 and penetrate the basement membrane to enter the interstitial tissues; this provides a potential explanation of some features associated with chronic allograft nephropathy. Antibody-blockade of TGF-β prevented $CD103^+$ T cell-driven EMT. Parallel *in vitro* studies demonstrated an apparent reversibility of this EMT process through activation of the antagonistic Smad 1/5/8 signalling pathway with bone morphogenetic protein-7, leading to the reacquisition of an epithelial phenotype.

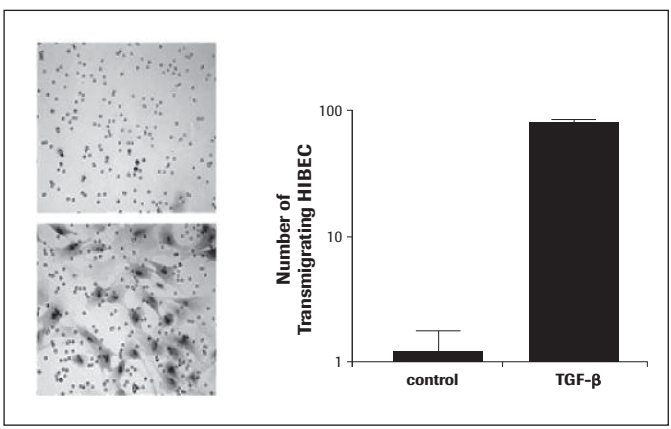

Figure 1. Invasion of Matrigel by TGF-β-stimulated HIBEC.

Following definition of this novel pathway, a series of studies was performed to define the potential of immune-driven EMT to damage transplanted human lung and liver. In both cases it was found that the presentation of TGF-β could also induce EMT, with intraepithelial T cells being spatially associated with the local induction of S100A4 in transforming epithelial cells. Figure 1 demonstrates how cultured human intrahepatic biliary epithelial cells (HIBEC) acquire the potential to invade an artificial basement membrane following activation of the EMT programme through treatment with TGF-β.

These studies suggest a novel mechanism by which TGF-β presenting immune cells can directly induce allograft dysfunction through chronic induction of EMT; the reversibility of this fibrogenic mechanism may lead to new strategies to prolong allograft survival.

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Towards a Human Hepatocyte-Based Bioartificial Liver

These studies analyze the molecular bases for liver repopulation via hepatocyte transplantation in a rat model developed in our laboratories, the retrorsine (RS)-based model[1]. We found that RS-treated rat livers over-express TGF-β and p27, two proteins which could be responsible for the long-lasting cell cycle block exerted by this natural agent. In addition, high levels of cyclin D1, CDK4 and cyclin D1-CDK4 complex were found in hepatocytes following RS treatment, in the absence of replicative activity. We have suggested that these cells are irreversibly blocked outside the G0 phase of the cell cycle in a state of "hypermitogenic arrest" described in cell culture systems. This condition can lead to cellular senescence and this could set the stage for the selective replacement of RS-damaged cells by normal transplanted hepatocytes[2].

Relevant to this point, we have recently found that the microenvironment of the aged rat liver is conducive to the selective expansion of normal transplanted hepatocytes; i.e. normal hepatocytes transplanted into the liver of old recipients are able to grow and give rise to large clusters[3]. This finding has several implications, both in the field of regenerative medicine and in the pathogenesis of age-associated neoplastic disease.

Finally, we are trying to exploit the potential of the RS model in a setting of xenotransplantation. We were able to reproduce the results of Wu et al.[4] and our next step will be to expose animals tolerised towards human cells to the RS-based protocol for liver repopulation.

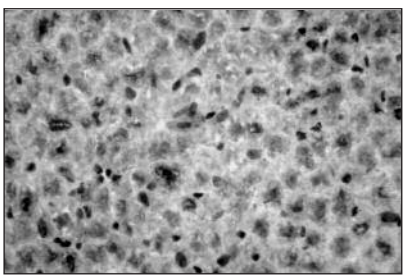

Figure 1. Human Hep-G2 cells (orange-rust) in the liver of a tolerized rat sacrificed 2 weeks after transplantation.

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The Role of Chemokines in Rejection and Bronchiolitis Obliterans Following Lung Transplantation

The specific aims of the proposal were to:

1. determine the unique roles of CXCL10 and CXCL9 in a model of lung transplantation, and to determine the molecular mechanisms regulating the differential expression of these chemokines;
2. establish a novel model of acute lung rejection;
3. define the chemokine profile of pathogenic T cells associated with bronchiolitis obliterans (BO).

We have now completed our goals in aim 1 and published our analysis of tracheal transplants in CXCR3$^{-/-}$, CXCL10$^{-/-}$ and CXCL9$^{-/-}$ mice[1]. These studies have demonstrated no differences in fibroproliferation or lymphocyte recruitment with deletion of CXCL10 or CXCL9, but profound differences in these measures with deletion of CXCR3 (Fig. 1A).

In addition, we demonstrate important differences in the regulation of CXCL10 and CXCL9 expression post-transplant. We have also completed our objectives for aim 2 and published our results using a novel model of acute lung rejection[2]. In these experiments, we have demonstrated that if activated OVA-specific CD8 lymphocytes are injected into CC10-OVA mice, the animals develop significant respiratory distress within 3 days with 100% mortality by day 7. Histology demonstrates profound acute inflammation of the airways that mimics the findings seen in acute rejection of lung transplants (Fig. 1B).

Finally we have published our findings from analysis of human BAL samples from patients s/p lung transplant[3] (Fig. 1C), thus fulfilling our objectives for aim 3.

This study has identified several chemokine receptors that seem to correlate with the development of BO.

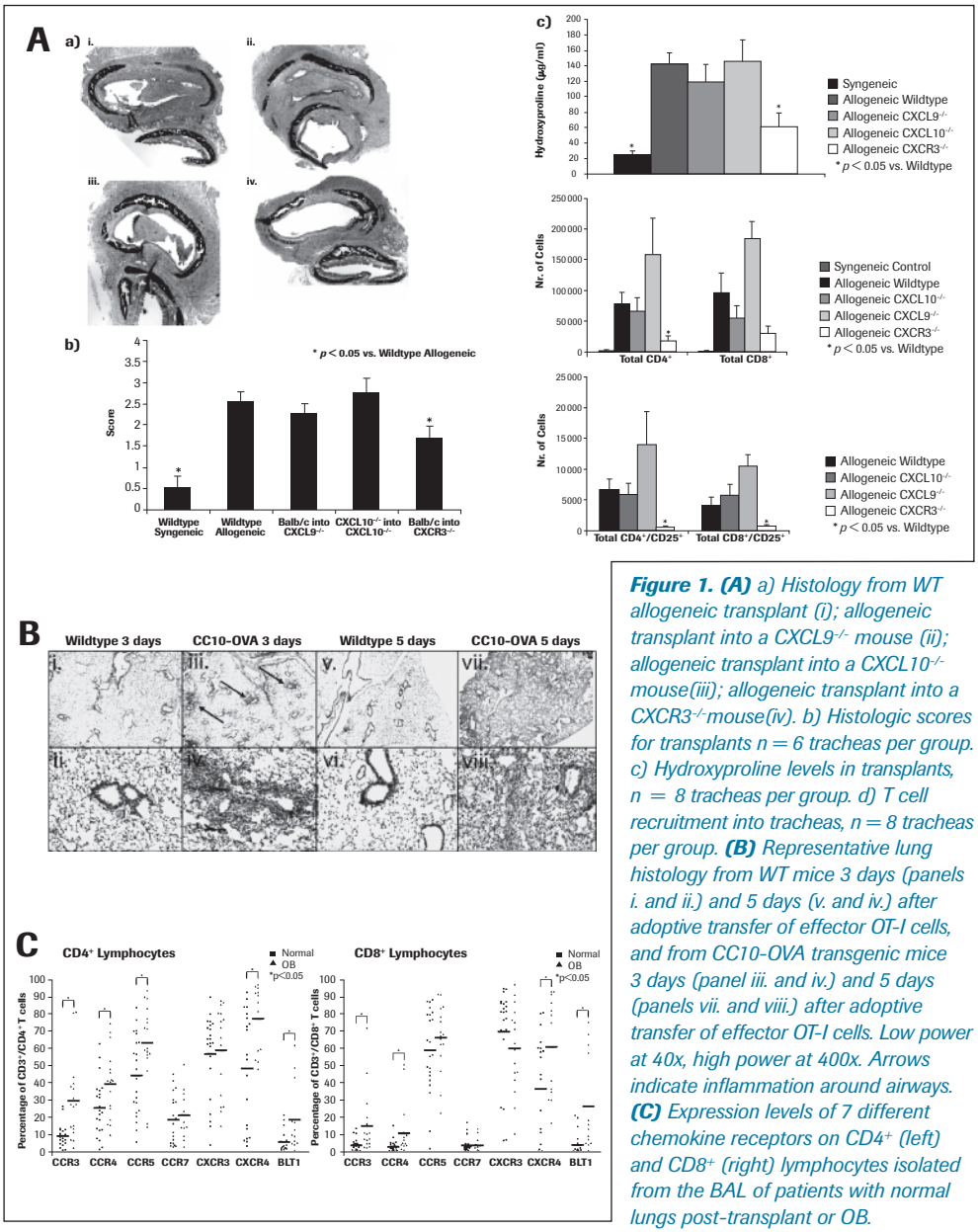

Figure 1. (A) *a) Histology from WT allogeneic transplant (i); allogeneic transplant into a CXCL9$^{-/-}$ mouse (ii); allogeneic transplant into a CXCL10$^{-/-}$ mouse(iii); allogeneic transplant into a CXCR3$^{-/-}$mouse(iv). b) Histologic scores for transplants n = 6 tracheas per group. c) Hydroxyproline levels in transplants, n = 8 tracheas per group. d) T cell recruitment into tracheas, n = 8 tracheas per group.* ***(B)*** *Representative lung histology from WT mice 3 days (panels i. and ii.) and 5 days (v. and iv.) after adoptive transfer of effector OT-I cells, and from CC10-OVA transgenic mice 3 days (panel iii. and iv.) and 5 days (panels vii. and viii.) after adoptive transfer of effector OT-I cells. Low power at 40x, high power at 400x. Arrows indicate inflammation around airways.* ***(C)*** *Expression levels of 7 different chemokine receptors on CD4^{+} (left) and CD8^{+} (right) lymphocytes isolated from the BAL of patients with normal lungs post-transplant or OB.*

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Role of Minor Histocompatibility Antigens in Solid Organ Transplant Rejection

Our laboratory is interested in studying minor histocompatibility antigens (mH-Ag)[1–6]. mH-Ag are polymorphic proteins that are capable of eliciting immune responses between HLA-matched donor and transplant recipient. Historically, mH-Ags have been defined as antigenic peptides that are capable of eliciting T cell-mediated immune responses. Recent studies, including those from our laboratory, have revealed that several full-length mH proteins possess novel cellular and immunological functions. One such murine mH-Ag that is proven to modulate immune responses is H60 and its molecular identity was defined through our earlier studies[1].

This antigen is one of the classical examples of full-length mH protein with interesting immunological functions. H60 protein belongs to the non-classical MHC class I family, which contains α1 and α2 domains but lacks α3 domain, thereby presumed not to bind to β2-microglobulin or to present antigenic peptides. An octameric peptide, LTFNYRNL is derived from H60 and presented on H2-K^b MHC[1, 2]. These LTFNYRNL/K^b complexes which are present in the BALB.B-derived grafts generate a strong T cell-mediated immune response in C57BL/6 strain. The complete absence of H60 mH protein in the C57BL/6 background is of significant immunological relevance due to the fact that it serves as the activating ligand for a natural killer (NK) cell receptor, NKG2D. In this context, expression of mH H60 protein in cells not only can activate peptide specific CD8+$\alpha\beta$ T cells, but also NK cells through the interaction of NKG2D and H60. Based on our studies, we are proposing a paradigm shift in the definition of mH antigens[7].

NKG2D is a major activation receptor for murine and human NK cells. Therefore, recognition of a full-length mH protein, such as H60 by NKG2D receptor has a direct impact on many clinical aspects. They include formulations of cellular anti-tumor immunotherapies, bone marrow and other hematopoietic cell-based transplantations, Graft-versus-leukemia and graft-versus-host disease.

Towards formulating NK cell-based therapeutic approaches, recently we addressed a series of questions. In our first set of experiments we asked questions on how the activating (NKG2D) and inhibitory (Ly49) signals balance each other inside the NK cells. Through these

studies we defined a novel phenomenon termed 'altered-balance' which explains that the effector functions of NK cells are not inhibited by the Ly49 receptors but rather are regulated[8].

'Induced-self' vs *'Missing-self'*: the true functional identity of H60 family

Murine NKG2D recognize inducible ligands such as H60, Rae-1 and Mult-1. H60 was identified and characterized as a minor histocompatibility antigen in our earlier work[1, 2]. One of the striking observations that we made was that not only the tumor cells are capable of expressing H60, but activated B, DCs and macrophages can also express this ligand[9].

This led us to ask a series of questions. What do NK cells do when a normal but activated B cell expresses H60? What is the immunological relevance of H60-NKG2D interaction under these circumstances?

Our findings indicate that NKG2D-mediated cytotoxicity depends on two critical aspects: ***a)*** target cells should express H60 and ***b)*** the target cells should down-regulate their MHC class I[8]. Thus, we describe that the *'induced-self'* is a missing component of the *'missing-self'*[10, 11]. This means trouble. What are the 'other' or 'true' immunological functions of H60 family members? What kind of effector functions are performed by NK cells during the interaction between H60 and NKG2D? H60 is a very important ligand that is expressed on B, DCs, and macrophages during viral or bacterial infections. Our working ***hypothesis*** is that H60 is a key molecular communicator between these cell types and NK cells. Towards addressing this, we have developed an influenza (PR8)-based animal model. We are also in the process of setting up an *E.coli*-based (DH5-α) infection model, which we hope will be usable in the near future. Both *in vivo* and *in vitro* studies will be employed to define the immunological relevance of H60-NKG2D interactions.

'Cis' interactions between MHC class I and the Ly49 receptors

One of the hallmarks of NK cell function is the recognition of *'self'* from *'missing-self'*. Inhibitory Ly49 receptors regulate the NK cell functions including the ones through the NKG2D receptor. How NK cells *'calibrate'* to 'self' is a mystery. Theories and explanations abound. *'Licensing'* and *'arming'* of NK cells are all possible. All these theories are based on only *'trans'* interaction of Ly49 receptor on the NK cells to MHC class I on target cells. One of the unusual yet novel findings is that Ly49 can *'cis'* interact with the respective MHC on the plane of NK cell membrane. Our results[12] indicate that Ly49C can *'cis'* interact with H2-K^b.

The exciting aspect in this story is: at what sub-cellular location does the *'cis'* interaction start? Does this occur only on the cell membrane or indeed it starts early during the biosynthesis of Ly49 and MHC in the endoplasmic reticulum? The answer to this question will

tremendously help us to provide a molecular definition for *'calibration'* and the theory of *'missing-self'*. We are also trying to address the effect of 'cis' interaction on NKG2D-mediated NK cell activation.

NK cell ontogeny and the signaling events responsible for it

NK cells develop in the bone marrow. Although, there has been recent progress in the understanding of NK cell development much of it is still unknown. Using *PLCγ2* gene knockout mice, we determined the differential and non-redundant roles of *PLCγ1* and *PLCγ2* during the development of NK cells[13].

NK could develop in the absence of *PLCγ2* albeit they could not acquire Ly49 receptors and thereby terminally mature. These NK cells failed to mediate cytotoxicity or cytokine generations. More importantly, over-expression of *PLCγ1* in *PLCγ2*-deficient NK cells could only rescue the expression of Ly49 receptors and not the effector functions. *PLCγ2* could successfully rescue both Ly49 expression and the effector functions. Since these two isoforms are only 50% similar in their amino acid homology, this model would help us to determine their distinct downstream effector molecules and their pathways through domain-swapping and reconstitution experiments. We are using this model (both the *PLCγ2*-deficient mice and the retroviral rescue system) to dissect the signaling requirements for NK cell development.

Unique signaling requirements for distinct NKG2D-mediated effector functions

NKG2D receptor mediates a multitude of effector functions including cytotoxicity and cytokine generation in NK cells. However, specific signaling events that are responsible for the divergence of distinct effector functions have yet to be determined. Our recent findings show that lack of caspase recruitment domain-containing protein Bcl10 exclusively affected the NKG2D-mediated cytokine and chemokine generation but not the cytotoxicity against tumor cells representing *'missing-self'* or *'induced-self'*[14].

Enumeration of the release of perforin granules further confirmed the normal cytotoxic potential of Bcl10-deficient NK cells. Commitment, development and terminal maturation of NK cells were largely unaffected in the absence of Bcl10. Thus, the Carma1-Bcl10-Malt1 signaling axis is critical for NKG2D-mediated cytokine and chemokine generation, although it is dispensable for cytotoxic granule release.

These results indicate that Bcl10 represents an exclusive *'molecular switch'* that links the upstream NKG2D-mediated signaling to cytokine and chemokine generations. Our present and future goals are to perform a series of biochemical analyses to determine other unique signaling molecules that are responsible for either cytotoxicity or cytokine secretions. To

further dissect the unique signaling requirements, we have used mice that are deficient for the PI3K-p85α subunit. In contrast to what has been shown in the literature, NK cells generated from these mice are not only defect in their ability to mediate cytotoxicity but also in generating cytokines[15]. Other knockout mice for signaling molecules such as PI3K-p110δ, CARMA-1, small GTPases-Rap1a and Rap1b are being used to further understand the signaling pathways downstream of NKG2D receptor.

In Summary

Our goal is to understand the immunobiology of NKG2D receptor and thereby the NK cells. Our studies will lead to successful immunotherapeutic approaches for both graft acceptance and tumor clearance.

In this period we have published two original peer-reviewed manuscripts and two review articles[8, 13]. We are also in the process of submitting three more manuscripts based on our work during this time with the financial support of ROTRF. Funding from ROTRF was timely and helped us to recruit excellent young postdoctoral fellows and to conduct cutting edge research as demonstrated by our publications.

We are also highly indebted to ROTRF because the preliminary work that we could perform with its support led to obtaining three major NIH grants in the past two years. Among these, one of the significant achievements is the five year NIH-RO1 grant awarded to Dr Malarkannan (July 2006-June 2011). Now his team stands as a well-funded research laboratory, which is in part due to the scientific recognition and whole-hearted financial support extended by ROTRF. We will continue to work harder in the exciting area of immunological research that is directly relevant to transplantation and tumor clearance.

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Vascularization of Engineered Human Skin Equivalents

Tissue engineering offers a potential solution to the shortage of organs available for human transplantation, but providing adequate perfusion has been a major obstacle for survival of tissue-engineered replacement organs. The goal of this project, initiated by Dr. Jeffrey Schechner, was to test if incorporation of human endothelial cells (EC) would result in earlier and more extensive perfusion of human synthetic skin substitutes.

Research supported by this grant demonstrated that:

1. human umbilical vein endothelial cells (HUVEC) can be successfully incorporated into synthetic skin substitutes formed from decellularized human dermis and human foreskin keratinocytes, and that incorporated HUVEC spontaneously generated human EC-lined microvessels when synthetic skin substitutes were orthotopically transplanted on to immunodeficient mice[1];
2. transduction of HUVEC with the survival gene bcl-2 enhanced the capacity of HUVEC to form mature functional vessels when implanted subcutaneously into immunodeficient mice[2];
3. human EC differentiated *in vitro* from circulating neonatal or adult endothelial progenitor cells, like HUVEC, formed human EC-lined vessels in synthetic skin substitutes[3].

Interestingly, stem cell-derived EC, unlike HUVEC, did not depend on Bcl-2 expression to promote vessel formation. More significantly, incorporated human EC from all of these sources still successfully formed blood vessels when the mice were treated with rapamycin, an agent that blocks angiogenesis in the host[3]. These findings establish proof-of-concept that human EC can be used to enhance perfusion of tissue-engineered substitutes and do so in a manner that is independent of host angiogenesis.

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T Cell Costimulation through Complement Receptors in Kidney Transplantation

We proposed that complement synthesised, activated and deposited at the site of tissue injury promotes T cell activation in graft rejection. Data showed that complement can mediate a form of stimulation to T cells in the absence of costimulation through usual pathways, such as CD28. Cell signalling, IFNγ synthesis, IL-4 synthesis and T cell proliferation were all enhanced in an *in vitro* test system if the cells were co-incubated with antibodies to complement receptors 1/2, found to be expressed on a subset of CD4 T cells. Interestingly, IL-2 synthesis was not regulated through this system. The project further developed a small interfering RNA (siRNA) strategy for the therapeutic inhibition of C3 synthesis by transfected cells in order to explore a clinical potential for an adjunct strategy to reduce C3 activity in human transplants. The transfection of siRNA was enhanced by the development of cationic co-polypeptides developed by a collaborating laboratory in Washington, USA. This was further tested in an *in vivo* model and has demonstrated early success in the treatment of donor kidney to reduce local C3 synthesis in transplantation. To further develop the clinical potential of this work, we developed a method for the co-detection of complement components C1q & C9, and C3b & C4b, in human renal transplant biopsies at ultrastructural level by scanning electron microscope. We hope to develop this method further to differentiate between donor and recipient sources of complement components in rejecting biopsies.

Work supported, or partly contributed to, by this award has been presented at the 2006 British Transplantation Society Annual Congress in Edinburgh and was nominated for the Medawar Medal[1]. Furthermore, the work has been accepted for presentation at the 2006 World Transplant Congress, for which Dr Pratt received a Young Investigator Award[2]. Dr Parker is the Research Fellow who was supported on the grant, and two manuscripts have so far been submitted to which Dr Parker has contributed. Dr Parker has also given academic assistance to our project on ischaemic epigenetics and is a co-author on this work as well.

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Plasmacytoid Dendritic Cells and Liver Transplant Tolerance

Bone marrow-derived precursor (pre)-plasmacytoid dendritic cells (pre-pDC) constitute a comparatively high proportion of DC in the murine liver compared with DC in secondary lymphoid tissues. DC precursors were propagated from C57BL/10 (B10; $H2^b$) mouse bone marrow in the presence of the DC poietin fms-like tyrosine kinase 3 ligand. Co-signaling molecule (B7-1/B7-2 and B7-H1) expression and stimulatory capacity of pre-pDC ($CD11c^+B220^+CD11b^-CD19^-$) and classic myeloid DC (MDC) for allogeneic (C3H; $H2^k$) T cells were compared. Unstimulated pre-pDC exhibited very low levels of surface MHC class II and classic costimulatory molecules (B7-1/ B7-2), whereas a minor population expressed B7-H1 at levels higher than on MDC. The pre-pDC were ineffective T cell stimulators and induced non-specific hyporesponsiveness to re-challenge with donor alloantigens *in vitro* and *in vivo*. Following stimulation with the TLR ligand CpG, B7 molecule expression was up-regulated on pre-pDC, however, the ratio between coinhibitory (B7-H1) and costimulatory (B7-1/ B7-2) signals was much higher (5–6 fold) on pre-pDC than on MDC (Table 1).

Molecule/Ratio	Mean Fluorescence Intensity	
	MDC (fold higher)	pDC (fold higher)
MHC class II	595.1 ± 72.4* (7.7)	77.1 ± 29.4
CD80 (B7-1)	377.2 ± 65.0* (9.5)	39.8 ± 22.6
CD86 (B7-2)	513.9 ± 89.0* (8.2)	62.3 ± 32.4
B7-H1(PD-L1)	270.0 ± 54.4 (1.6)	166.6 ± 62.6
B7-H1/ CD80 ratio	0.71 ± 0.02	4.30 ± 0.69* (6.1)
B7-H1/ CD86 ratio	0.52 ± 0.02	2.86 ± 0.55* (5.5)

Table 1. Expression of MHC class II, and B7 family molecules (B7-1, B7-2 and B7-H1) on CpG-ODN-stimulated MDC and pDC. *BM-derived immature MDC and pre-pDC were cultured with CpG-ODN (2 μg/ml) then stained with PE-conjugated mAb. Data are shown as means ± 1SD of mean fluorescence intensities (n = 3). Symbols indicate significantly higher values compared with the corresponding CpG-stimulated MDC or pDC population. *$p < 0.05$.*

Blockade of B7-H1 expression on pDC increased their T cell allostimulatory capacity significantly, implicating B7-H1 in the regulatory function of pDC. A single pre-operative infusion of C3H hosts with donor-derived pre-pDC prolonged B10 heart graft survival significantly but non-specifically compared with untreated mice (median graft survival times [MST] 22 versus 9 days, respectively), indicating that pre-pDC of donor origin have the potential to regulate T cell responses to alloantigens and to prolong organ graft survival. When infused systemically before heart transplantation, donor-derived pre-pDC in combination with anti-CD40 ligand (CD154) mAb markedly prolonged heart allograft survival (from MST 10 to 63 days), with 50% of the grafts surviving >100 days. Thus, pDC may contribute to the inherent tolerogenicity of liver allografts and hold promise as potential 'negative cellular vaccines' for therapy of allograft rejection.

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11. Tokita D, Abe M, Raimondi G, et al. Liver and spleen plasmacytoid dendritic cell allostimulatory functions are differentially modulated in response to TLR ligation. Poster presentation, *Am J Transplant* 2006; 6:1023; *(Abstract)*.

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Survival Mechanisms of Dendritic Cells as a Tool to Direct Immune Responses

Dendritic cells (DC) serve as an essential link between innate and adaptive immune responses. They can induce both primary and secondary immune responses and play a key role in immuno-stimulatory as well as immuno-suppressive responses. This dual function has made them potential targets in vaccine development for the treatment and prevention of cancer or allograft rejection respectively. The longevity of DC is a critical factor influencing the outcome of immune responses.

In this study we further explored our recent finding that the immunosuppressive drug rapamycin specifically induces apoptosis in human monocyte-derived DC. We found that dendritic cell apoptosis could not be inhibited by caspase inhibitors and no biochemical signs of caspase activation were observed (Fig. 1A, B). Using RNAse protection we confirmed that mcl-1 was the major target, although additional changes in expression of bcl-xL and Bim were observed.

For transplantation purposes it is important to establish the survival mechanism of tolerogenic DC. We generated human regulatory DC by generating DC in the presence of dexamethasone. Analysis of survival requirements showed that Dex-DC were less sensitive for rapamycin-induced apoptosis and did not require GM-CSF as a survival factor (Fig. 1C). To make a translation to transplantation models, we also analysed the survival of murine and rat DC. Using DC generated from bone-marrow mononuclear cells, we found that both populations showed a differential sensitive for rapamycin. Whereas murine DC were less sensitive for rapamycin-induced apoptosis, rat-DC underwent strong apoptosis after rapamycin exposure.

These experiments show that different DC subsets display a marked heterogeneity in sensitivity for immune modulating agents and more importantly that different DC subsets are dependent on different survival mechanisms. This information provides novel tools to interfere with immune responses through interference with longevity of specific DC subsets.

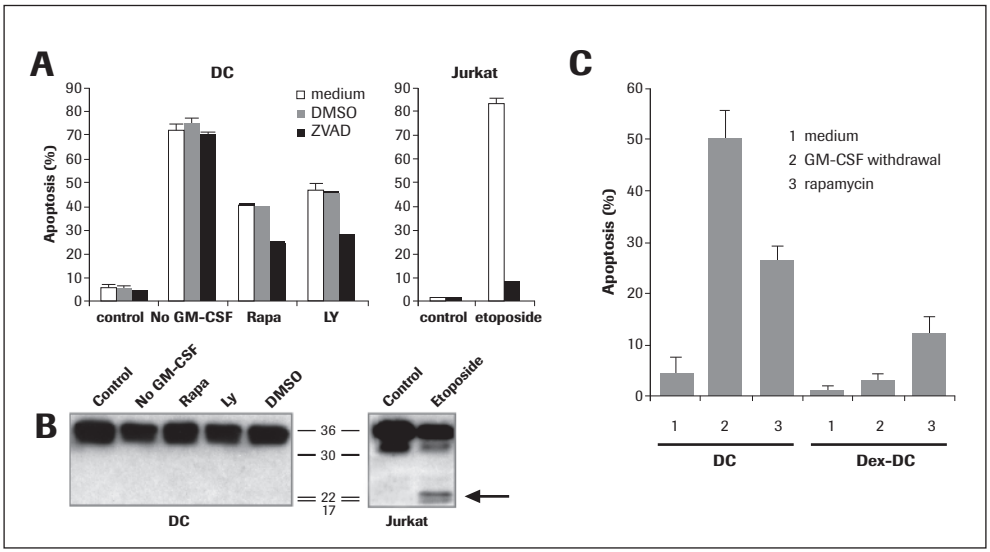

Figure 1. Survival mechanisms of human DC. *Human monocyte-derived DC undergo apoptosis when exposed to rapamycin, to the PI3K inhibitor LY, or after GM-CSF withdrawal. This specific apoptosis is not inhibited by the pan-caspase inhibitor ZVAD* ***(A)****, and is not associated with caspase activation* ***(B)****. Etoposide-induced apoptosis of Jurkat cells is used as a positive control.* ***(C)*** *Tolerogenic human DC generated in the presence of dexamethasone (dex-DC) were less sensitive for rapamycin-induced apoptosis and were independent of GM-CSF for survival.*

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