R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

October 2005

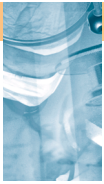
The Roche Organ Transplantation Research Foundation

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.

Table of Contents



1. Preface

On behalf of the Board of Trustees of the Roche Organ Transplantation Research Foundation (ROTRF), I am very pleased to announce that, in Cycle XIV, 2.1 million CHF has been awarded to eight conventional and two clinical research projects. The funding provided by the ROTRF continues to support the research of scientists working in the area of organ transplantation in many parts of the world.

With reference to the activities of the ROTRF, in summer 2004 a new clinical initiative was launched, aimed at supporting research projects using human clinical material and patients. This initiative supports projects which address new issues in organ preservation, human transplant pathology and other human transplant problems, and which promise to have a significant impact in the clinical setting in the near future. As had been the case in the previous cycle (XIII), the response by investigators to this new initiative was very positive, with almost 40% of the total applications submitted in Cycle XIV being applications for clinical research grants.

Furthermore, this year the ROTRF and the Juvenile Diabetes Research Foundation International (JDRF) have joined forces. A new joint grant award competition has been added to the grant award competitions already listed in the agenda of both foundations. This award will support research projects in immunology and biology research relevant to pancreatic islet transplantation, and transplantation solutions for human type 1 diabetes. The first submission deadline was 1 April 2006 and 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 were strongly encouraged to apply. We are pleased with the great attention that this additional grant award competition has received from investigators and hope that this will continue in the future.

During ROTRF symposia at transplantation congresses in Europe and USA, some of the excellent work carried out with ROTRF grants has been presented by the investigators. The quality of the research is further demonstrated by the quality of the Biannual Reports, and the papers published in peer-reviewed journals.

The ROTRF is deeply grateful to F. Hoffmann-La Roche Ltd, and wishes to thank them for their generous support throughout all the years since 1998.

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.

Finally, the ROTRF wishes to thank the Juvenile Diabetes Research Foundation International for their input and enthusiasm in joining with the ROTRF in the new grant award competition.

On behalf of the Board of Trustees

Philip F. Halloran, MD, PhD, OC

2. Facts and Figures

Funding Cycle XIV – Letters of Intent Submission in April 2005

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that in Cycle XIV, ten grants have been awarded to *conventional* and *clinical research applications.*

Eight conventional research and two clinical research applications were each awarded a grant to a combined total of 2.1 million Swiss francs (CHF). The Board of Trustees and the Scientific Advisory Committee of the ROTRF were once again very pleased with the high quality and innovation demonstrated in the applications received.

The awarded grants will support research that aims to advance the science of solid organ transplantation, thereby improving the care of thousands of patients undergoing transplantation every year. The funded investigators will focus their research on the understanding of clinical and scientific aspects of transplantation, such as processes involved in chronic allograft arteriopathy and glomerulopathy, the onset of bronchiolitis obliterans syndrome (BOS), aspects related to immunotolerance, transplant arteriosclerosis resulting in chronic rejection, islets transplant rejection and post-transplant lymphoproliferative disorder (PTLD).

The ROTRF received 127 Letters of Intent in funding Cycle XIV up to the submission deadline (April 2005), from scientists around the world. Of the applications, >38.6% were received from Europe, the major countries being the UK (6.3%), Germany (5.5%), and France (5.5%), 53.8% of applications were received from North America, the United States (48.3%) and Canada (5.5%). Australia/New Zealand (3.9%), South America (<1%), Asia (2.4%) and Africa (<1%) accounted for the remaining 7.6% of the applications. Based on the reviews of the Scientific Advisory Committee, the Board of Trustees invited 25 applicants to prepare full paper applications. After a thorough review of the full paper applications, grants were awarded to ten projects. The abstracts of these newly awarded grants are published in the first pages of this Biannual Report and on the ROTRF homepage.

Statistics on Applications to the ROTRF

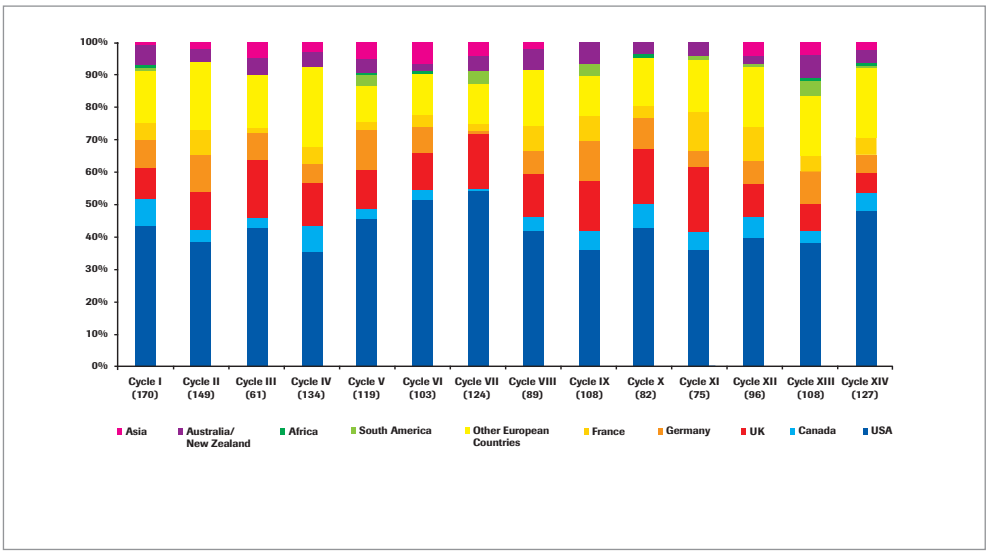


Figure 1. *Geographical distribution of the applicants who submitted Letters of Intent (LOI) during the first fourteen ROTRF funding cycles. The total number of LOIs submitted per cycle is shown in brackets.*

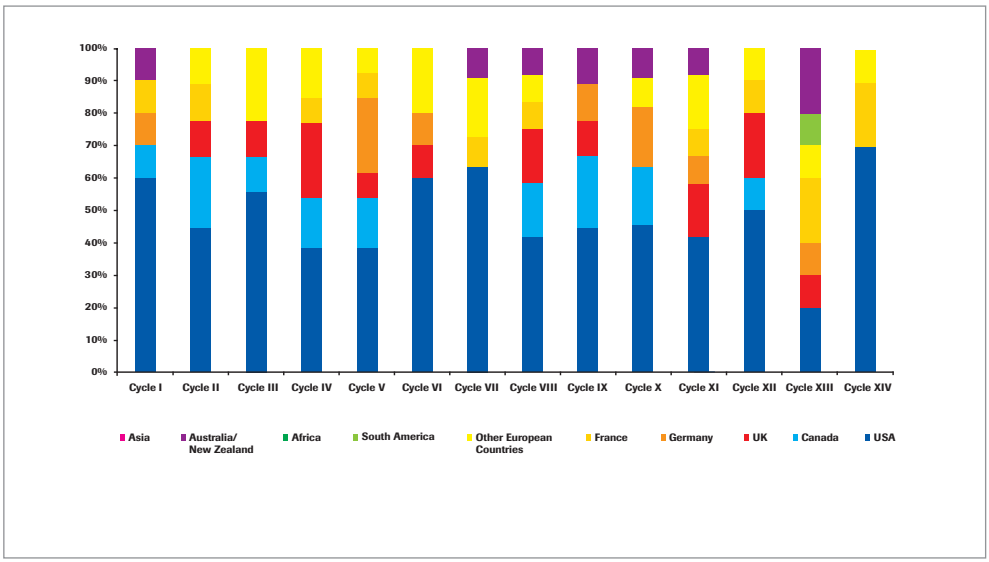
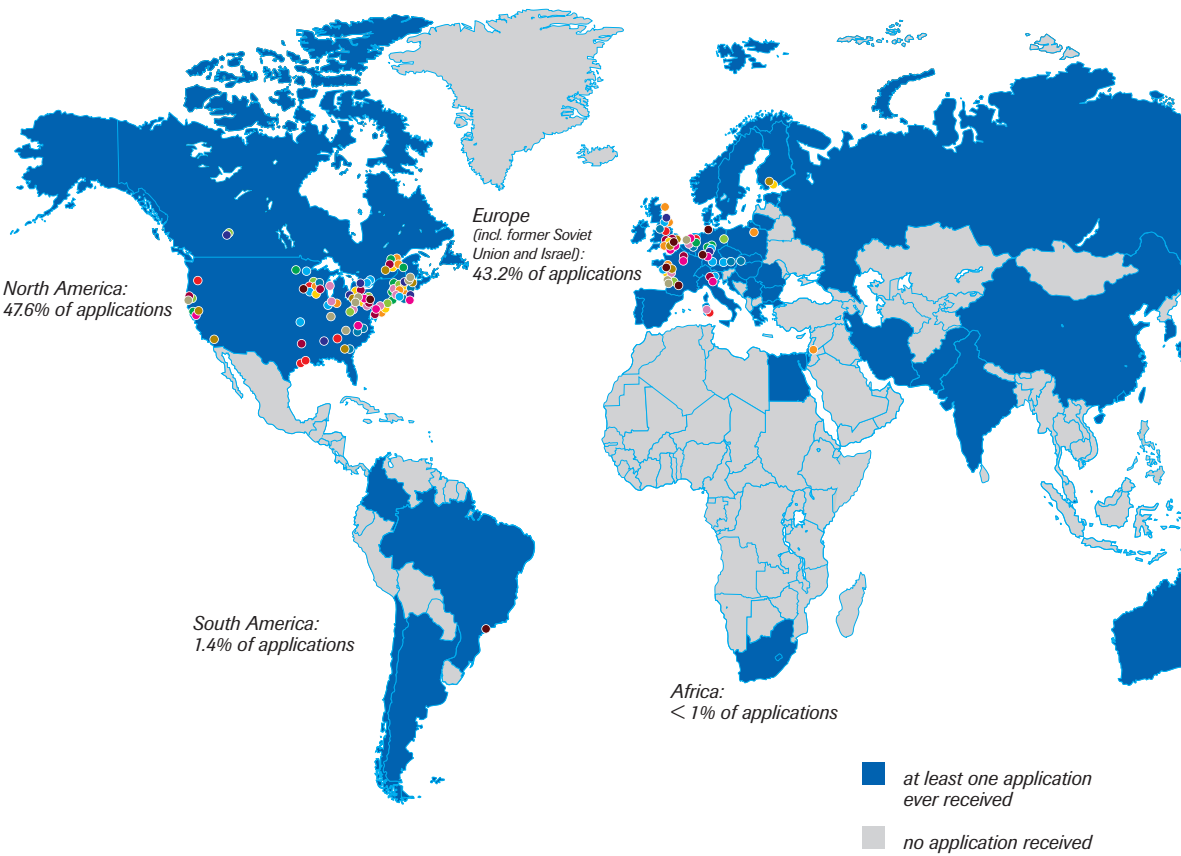


Figure 2. *Geographical distribution of the applicants who were awarded ROTRF grants during the first fourteen ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF Applications Worldwide



Grant Awards in:

Asia and The Middle East: 2.7% of applications

Australia and New Zealand: 4.7% of applications

Cycle I

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

Cycle II

Boston, USA
Helsinki, Finland
London, Canada
Madison, USA
Montreal, Canada
Nantes, France
New York, USA
Oxford, UK
Pittsburgh, USA

Cycle III

Atlanta, USA
Birmingham, UK
Cagliari, Italy
Houston, USA
Houston, USA
Madison, USA
Nijmegen, The Netherlands
Portland, USA
Winnipeg, Canada

Cycle IV

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

Cycle V

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

Cycle VI

Augusta, USA
Boston, USA
Boston, USA
Brussels, Belgium
Chapel Hill, USA
Durham, USA
Madison, USA
Manchester, UK
Regensburg, Germany
Vienna, Austria

Cycle VII

Boston, USA
Boston, USA
Brussels, Belgium
Cagliari, Italy
Chicago, USA
Columbus, USA
Milwaukee, USA
Melbourne, Australia
Nantes, France
New York, USA
Pittsburgh, USA

Cycle VIII

Baltimore, USA
Bergamo, Italy
College Station, USA
Heidelberg, Australia
London, Canada
London, UK
Madison, USA
Montreal, Canada
Oxford, UK
Paris, France
Pittsburgh, USA
San Francisco, USA

Cycle IX

Birmingham, USA
Boston, USA
Columbus, USA
Edmonton, Canada
Heidelberg, Germany
London, Canada
Newcastle-upon-Tyne, UK
New Haven, USA
Sydney, Australia

Cycle X

Boston, USA
Brussels, Belgium
Charlestown, USA
Heidelberg, Germany
Heidelberg, Germany
Herston, Australia
Iowa City, USA
Montreal, Canada
New Haven, USA
Sainte-Foy, Canada
Stanford, USA

Cycle XI

Baltimore, USA
Bergamo, Italy
Boston, USA
Chapel Hill, USA
Giessen, Germany
Leiden, The Netherlands
London, UK
London, UK
Paris, France
Pittsburgh, USA
Stanford, USA
Sydney, Australia

Cycle XII

Augusta, USA
Cambridge, UK
Cleveland, USA
Helsinki, Finland
Laval, Canada
London, UK
Los Angeles, USA
Nantes, France
New Haven, USA
Stanford, USA

Cycle XIII

Cambridge, UK
Fitzroy, Australia
Heidelberg, Germany
Leiden, The Netherlands
Madison, USA
Nantes, France
Pittsburgh, USA
São Paulo, Brazil
Sidney, Australia
Toulouse, France

Cycle XIV

Atlanta, USA
Boston, USA
Boston, USA
Cleveland, USA
Columbus, USA
Nantes, France
Nantes, France
San Francisco, USA
St. Luis, USA
Utrecht, The Netherlands

3. Grant Awards in Cycle XIV

3.1 Conventional Research Grant Awards

Dr. Ignacio Anegon, Principal Investigator
Dr. Christine Chaveau, Post-doctoral Fellow
Dr. Severine Remy, Technician
Mr. Régis Brion, Technician
Mr. Laurent Tesson, Technician
Dr. Marc Gregoire, Research Associate
Dr. Maria Grazia Roncarolo, Research Associate
Dr. Katja Kotsh, Research Associate
Dr. Georges Kollias, Research Associate

INSERM U643, ITERT, Nantes, France

Heme Oxygenase-1 promotes Tolerogenic Dendritic Cells: Analysis of Mechanisms and *in vivo* Applications

Despite enormous progress in controlling acute rejection and prolonging graft survival, continuous immunosuppression after transplantation is associated with side effects such as opportunistic infections and cancer, as well as progressive graft failure due to chronic rejection. The attainment of specific inhibition of immune responses directed against the donor antigens while preserving immune responses against infectious agents or cancer cells (*i.e.* tolerance) would allow for a reduction or elimination of immunosuppressive drug treatment.

Heme oxygenase-1 (HO-1) is an enzyme that has anti-inflammatory and graft protective actions. Dendritic cells (DCs) are not only initiators of pro-inflammatory but also of tolerogenic immune responses. It has recently been shown that HO-1 inhibits the pro-inflammatory properties of DCs while promoting their tolerogenic potential.

This project aims to more precisely define the mechanisms by which HO-1 promotes the tolerogenic potential of DCs. Particular attention will be paid to the effects of HO-1 on the capacity of DCs to generate different subpopulations of T lymphocytes with tolerogenic activities. The intracellular signaling pathways as well as the production of extracellular mediators of DC activity will also be analysed. DCs expressing HO-1 will be used to induce tolerance in rodent models of organ transplantation.

Such basic knowledge of the mechanisms whereby HO-1 acts on DCs and the use of HO-1-overexpressing DCs in transplantation may make it possible in the future to induce donor-specific tolerance and thus have a major impact on transplantation.

Dr. Robert Colvin, Principal Investigator
Dr. Paul S. Russell, Co-Investigator
Dr. Shuichiro Uehara, Research Associate
Ms. Catharine Chase, Laboratory Supervisor
Ms. Patricia DellaPelle, Senior Technologist
Dr. Dennis Sgroi, Collaborator
Dr. Michael Carroll, Collaborator

Massachusetts General Hospital, Boston, USA

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

Late graft loss remains a substantial obstacle to long-term success in organ transplantation. Prevention and treatment requires identification of the cause and understanding of the mechanisms of late graft injury. Recent studies in humans indicate that a substantial fraction of late graft failure is associated with formation of antibodies to the donor, deposition of complement (C4d) in graft vessels and chronic arterial and glomerular damage. Direct proof that antibodies cause these lesions is lacking, as well as the mechanism by which they might do so.

The goal of this project is to prove which lesions considered "chronic rejection" can be caused by antibodies and to determine the relevant molecular mechanisms by which they arise. Here we will develop a suitable experimental model, using well-characterized mouse models of heart and kidney transplantation in genetically defined strains to determine which of the chronic lesions can be initiated by antibody. We will analyze the cellular components and the evolution of the lesions and determine whether the endothelial cells express molecules that might serve to resist the injurious effects of antibody and complement. We will determine whether chronic tissue injury by antibody requires complement fixation, binding of antibody to cells (Fc receptors) or certain other mediators such as interferon-γ. Systematic study of gene expression promoted by antibody to donor endothelial cells *in vivo* will be done using isolated tissue obtained by laser capture microdissection and DNA microarrays. The results should provide clear insights into the possible role of antibodies in graft rejection in humans and may help identify new strategies of intervention.

Dr. Christiane Ferran, Principal Investigator
Dr. Mark Fisher, Surgical Resident/Research Fellow
Ms Sowmya Sennani, Research Associate

Beth Israel Deaconess Medical Center, Boston, USA

Role of A20 upon Homing of EC and SMC Progenitors to Lesions of Transplant Arteriosclerosis

Effective immunosuppressive regimens used in transplantation have drastically reduced the failure of vascularized grafts due to acute rejection (<10%). Chronic rejection is the major obstacle to long-term transplant function. Chronic rejection is characterized by the development of an accelerated arteriosclerosis, termed transplant arteriosclerosis (TA). TA is particularly dramatic in cardiac transplant recipients and has become the principle cause of late death and graft dysfunction. Mechanisms of chronic rejection and TA are poorly understood and likely multifactorial (immunological and non-immunological causes). Recently, homing of progenitor endothelial and smooth-muscle cells (EC; SMC) have also been implicated in modulation of TA lesions.

Numerous approaches (including increased immunosupression, anti-hypertensive and lipid lowering agents) to treating TA have been largely unsuccessful and carry additional toxicity. We propose a novel anti-TA approach aimed at shielding the blood vessel from immune and non-immune effectors of TA. Our approach is based on our finding that the fate of the graft does not solely depend upon the host immune and non-immune effectors, but also on its ability to protect itself from injury. Our data suggest that this might be safely achieved by genetic engineering of the vessel wall with a single gene: A20. A20 is a physiologic protective response to injury in EC and SMC, preventing TA regardless of the precipitating offender. A20 is anti-inflammatory and anti-apoptotic in EC. A20 is anti-inflammatory and anti-proliferative in SMC and sensitizes abnormal SMC to death. In addition, A20 may promote homing of progenitor EC, which accelerates healing, while inhibiting recruitment, differentiation and survival of the deleterious progenitor SMC. We aim to provide direct *in vivo* proof of the protective effect of A20 against TA mainly regarding its role upon homing of EC and SMC progenitors. This could provide novel therapeutic leads to protect from TA and other vascular diseases.

Dr. Peter Heeger, Principal Investigator
Dr. Shuguong Yuan, Post-doctoral Fellow/Microsurgeon
Ms. Earla Biekert, Technician

The Cleveland Clinic, Cleveland, USA

Complement Proteins regulate the Immune Responses that cause Transplant Rejection

Transplantation is the preferred therapy for end-stage organ failure, but despite significant improvements, long-term graft survival is limited, largely due to chronic immune-mediated injury to the transplanted organ. The goal of the present study is to better understand the basic mechanisms of graft rejection so as to be able to use this information to design novel therapies that will prolong graft survival. T cells and antibodies reactive to the "foreign" transplanted organ are the key mediators of the graft rejection but we presently do not fully understand how these cellular and molecular immune mediators function to destroy an organ.

Our group recently discovered that soluble proteins comprising the complement cascade and cell-surface proteins that control the activation of complement surprisingly influence the strength of T and B cell immune responses directed at the graft. This important link between elements of the innate (complement) and adaptive (T cells and B cells) immune systems has not been well studied in transplantation. We therefore propose to use a mouse model of heart transplantation, using animals with and without targeted deficiencies or overexpression of complement/complement regulatory proteins, to assess how complement and the decay accelerating factor influence T cell alloimmunity, alloantibody production, and transplant survival. In addition to providing new information that could be exploited for prevention of graft rejection, the fundamental insights derived from this work could be applied to a variety of immune-mediated processes, including the development of approaches to enhance vaccine efficacy.

Dr. Régis Josien, Principal Investigator
Dr. Dominique Chabannes, Research Associate
Mr. Asmahan Ouabed, Research Associate
Dr. Frederic Lavainne, Research Associate
Mr. Michele Heslan, Research Associate

INSERM U643, ITERT, Nantes, France

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

The induction of donor-specific transplantation tolerance, i.e. the indefinite acceptation of an allogeneic organ in the absence of immunosuppressive drugs and of allograft injury, remains the ultimate goal of research in the field of allograft tolerance. Among different possibilities is the use of so-called regulatory or suppressor T cells that have been extensively studied during the last decade. Naturally occurring $CD4^+CD25^+$ regulatory T cells (Tregs) play a major role in peripheral tolerance of T cells. The idea is to use donor antigen-presenting cells to expand large numbers of recipient regulatory T cells that could be re-injected to the recipient for preventing allograft rejection. However, the nature of the best antigen-presenting cells for promoting T regulatory cells expansion is not well defined. We have found that, among the different subsets of professional antigen-presenting cells or dendritic cells, only the subset of plasmacytoid dendritic cells (pDC) could induce strong proliferation of allogeneic regulatory $CD4^+$ T cells endowed with potent suppressive activity. Interestingly, the adoptive transfer on the day of transplantation of low numbers of donor pDC-expanded regulatory T cells to non-irradiated syngeneic hosts induced significant prolongation of heart allograft survival. In this project, we will further study the role of pDC in regulatory T cell expansion and function by: 1) determining the optimal conditions for using donor pDC-expanded Tregs *in vivo* to control allograft rejection, 2) determining the molecular mechanisms involved in the capacity of pDC to stimulate Tregs *in vitro* and *in vivo* and to analyze *in vivo* the interaction between pDC as well as other DC subsets and Tregs, and 3) reproducing our *in vitro* data in primates and in human. These data will be important for designing in the future a preclinical model for using donor pDC expanded Tregs to promote allograft tolerance in primates.

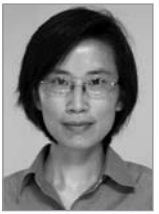
Dr. Daniel Kreisel, Principal Investigator
Dr. Laurence Turka, Collaborator

Washington University in St. Louis, St. Louis, USA

The Role of Vascular Endothelium in Regulating Immune Responses

Acute and chronic rejection remains a major obstacle to the success of transplantation. The main strategy to prevent the recipient's immune system from rejecting the graft is the administration of immunosuppressive drugs. As these drugs weaken the recipient's immune system, they can be predisposed to infections and certain forms of cancer. Therefore, the goal in transplantation remains the development of strategies to induce specific immune tolerance, so that the immune system does not reject the graft but otherwise maintains normal function. There is increasing evidence that regulatory T cells can control alloimmune responses and could therefore play an important role in the induction and maintenance of such specific immune tolerance to allografts. Vascular endothelial cells, which line the blood vessels of grafted organs, have been historically thought to have no immunological function. However, recent studies have suggested that they do play an active role in regulating immune responses in allograft rejection. We have previously demonstrated that allograft vascular endothelial cells can activate a subset of the recipient's T lymphocytes ($CD8^+$), induce their expansion and trigger allograft rejection. Vascular endothelial cells don't induce an expansion in the other subset of T lymphocytes ($CD4^+$). Interestingly, recent studies in our laboratory have shown that vascular endothelium can induce a population of regulatory $CD4^+$ T cells, which can suppress alloimmune responses. In this project we will examine the mechanism how vascular endothelium induces regulatory $CD4^+$ T cells. We will analyze which costimulatory pathways are important in this induction. We will also test whether these vascular endothelium-induced regulatory T cells can suppress alloimmune responses *in vivo* and prevent the rejection of heart allografts. The proposed experiments will further dissect our novel observation, provide insight into how the allograft itself can regulate alloimmune responses and could lead to the development of novel strategies to induce tolerance to allografts.

Dr. Qizhi Tang, Principal Investigator
Mr. Mingying Bi, Research Associate



UCSF Diabetes Center, San Francisco, USA

Visualizing Regulatory T Cell Control of Islet Transplant Rejection

One of the fundamental properties of the immune system is the ability to distinguish healthy self-tissue from infected, cancerous or foreign tissues. Thus, when tissue from one individual is transplanted onto another, the immune system mounts a vigorous response to eliminate the transplant, a process referred to as alloimmunity. The situation is further complicated in patients with type-I diabetes, whose immune system fails to refrain from attacking normal tissues, destroying the insulin-producing β cells in the pancreatic islets leading to the loss of blood glucose control. Such immune-mediated self destruction is referred to as autoimmunity. One promising approach to restore β cell function in these individuals is islet transplantation. However, the newly transplanted islets become targets for both alloimmune and autoimmune rejection. Generalized immunosuppression can prolong the graft survival, but such treatments are associated with high toxicity and make patients more susceptible to infections and cancer. Thus, we need to develop a novel therapy to specifically fend off the alloimmune and autoimmune attacks while preserving other immune functions. A small population of white blood cells called regulatory T cells (Tregs) has been critical for preventing autoimmunity. However, the normal level of Tregs is not sufficient to protect against potent alloimmune responses. We propose that increasing Treg numbers will control both the allo- and autoimmune responses in diabetic islet graft recipients. The goal of the research proposed in this grant application is to develop such treatments in mouse models, and to investigate the mechanisms of Treg control of allo- and autoimmunity. We will monitor both types of the immune responses from its initiation in the lymph nodes to graft rejection *in vivo* and determine which processes are controlled by Tregs. Results from these studies will help us to design better treatments by harnessing the body's powerful self-control mechanism.

Dr. Anne M. VanBuskirk, Principal Investigator

Ohio State University, Columbus, USA

Cytokine Interactions in Post-Transplant Lymphoproliferative Disorder (PTLD)

Post-transplant lymphoproliferative disorder (PTLD) is a life-threatening complication often associated with Epstein-Barr virus (EBV). In healthy individuals, EBV-reactive memory responses prevent clinical symptoms of the life-long EBV infection. In PTLD, EBV-reactive memory responses, particularly $CD8^+$ cytotoxic T lymphocyte (CTL) responses, are unable to control EBV lymphoproliferation and tumor formation.

Based on our work and that of others, we hypothesize that IFN-γ/TGF-β interactions are important in determining if PTLD develops. It is well established that IFN-γ and TGF-β act antagonistically in immune responses and angiogenesis. We know that lymphoproliferative disease in hu-PBL SCID mice, an established model of PTLD, is associated with the low producer genotype for IFN-γ (A/A at position +874). An A allele at position +874 is associated with the ability of TGFβ to inhibit re-stimulation of EBV-reactive CTL *in vitro*. New data indicate that TGF-β inhibits memory CTL restimulation via antigen-presenting cells (APC) and IFN-γ can overcome the inhibition, suggesting that APC are important regulators of memory CTL restimulation. Neutralization of TGF-β in hu PBL-SCID mice allows expansion of human $CD8^+$ cells and reduced tumor incidence. While neutralizing TGF-β protects hu PBL-SCID mice from LPD, the mechanism is unknown. We hypothesize that TGF-β neutralization alters the cytokine balance to favor IFN-γ, which counteracts TGF-β, allowing effective CTL restimulation and reducing tumor angiogenesis. There are several predictions that follow from our hypothesis. First, IFN-γ and inflammatory chemokines will be increased in the sera and tumors of anti-TGF-β treated mice compared to controls. Second, tumors from anti-TGF-β treated mice will have altered adhesion molecule profiles and reduced angiogenesis. Thirdly, T cells and APC from anti-TGF-β treated mice will show hallmarks of increased IFN-γ. Testing these predictions using *in vitro* and *in vivo* approaches will help define the mechanism by which TGF-β neutralization prevents LPD in hu PBL-SCID mice. Understanding lymphomagenesis and its prevention will provide insights into how to prevent and treat clinical PTLD.

3.2 Clinical Research Grant Awards

Dr. Henderikus G. Otten, Principal Investigator
Ph.D, MD Ed A.van de Graaf, Co-Investigator
Ph.D Ken I. Welsh, Advisor
Ph.D, MD Jules M. van den Bosch, Advisor
Ph.D Frits G.J. Gmelig Meyling, Advisor

University Medical Centre Utrecht, Utrecht, The Netherlands

Prediction of Chronic Rejection which is the Main Cause of Long-Term Mortality after Lung Transplantation

The bronchiolitis obliterans syndrome (BOS) occurring after lung transplantation (LTx) is the main cause of long-term morbidity and mortality after LTx. Although the detailed etiology and pathogenesis of BOS are not clear, it has become evident that both the humoral and the cellular allogeneic immune response against airway epithelial cells (AEC), contributes significantly to the pathogenesis of BOS. Recently, it was demonstrated that the presence of patient antibodies reacting with non-HLA antigens expressed on AEC may precede BOS development, suggesting that such non-HLA antigenic systems may play a role in chronic lung allograft rejection. These data are in line with results obtained in kidney transplantation, in which it was demonstrated that endothelial cell-reactive non-HLA antibodies can be found in sera of patients which have suffered from hyperacute or acute kidney allograft rejection. Patient antibodies reactive with non-HLA antigens are at present not recognized due to the lack of suitable routine screening procedures.

The main purpose of this study is to identify non-HLA target antigens on donor lungs recognized by patient antibodies. Identification of these antigens may provide a routine screening technique which can predict the occurrence of BOS, by monitoring the development of humoral or T cell immunity against these antigens. Target antigens expressed on AEC recognized by patient sera can be identified by SEREX (serological analysis of antigens by recombinant expression cloning); an immunoscreening technique which is an established method for defining immunogenic antigens in a variety of disorders including malignancies and autoimmunities. This technique allows a high-resolution and sensitive search for antibody responses against proteins expressed by donor AEC. After identification of the non-HLA antigens recognized, it will be analysed which antibody or concomitant T cell responses are predictive for BOS development. If so, this can be used to guide immunosuppressive therapy after LTx.

Dr. Allan Ramirez, Principal Investigator
Dr. Jesse Roman, Co-Investigator

Emory University, Atlanta, USA

Peroxisome Proliferator-Activated Receptor Gamma in Obliterative Bronchiolitis

The major limitation to long-term survival in lung transplantation is chronic rejection, which manifests as a progressive, fibrotic disorder of small airways called obliterative bronchiolitis (OB). The factors that initiate and perpetuate this process are poorly understood, though it was widely believed that alloimmune mechanisms were solely responsible. However, strategies to further suppress immune responses in OB have been largely unsuccessful, suggesting that other processes belie OB. It appears that the profibrotic cytokine, transforming growth factor-β (TGFβ), may play a critical role in this disease, although its regulation in lung transplantation has not been clearly defined. We have found that the effects of TGFβ in OB are mediated by the second messenger, Smad3. Through Smad3, TGFβ induces a phenotypic change in fibroblasts, enabling them to produce excess amounts of connective tissue matrices that are deposited in the airways of transplanted lungs with OB. In fact, using an animal model of experimental OB, our studies show that mice lacking Smad3 developed far less airway fibrosis than their wild-type littermates. Our search for mediators to counteract the activity of Smad3 has led us to the study of the peroxisome proliferator-activated receptor γ (PPARγ). These nuclear hormone receptors are better known for their effects on insulin sensitivity and adipocyte differentiation, but we have observed *in vitro* that they can prevent the expression of extracellular matrix genes in cultured fibroblasts, all through the inhibition of Smad3. The focus of this research is twofold: i) study the expression and biology of PPARγ in human lung transplantation and ii) test the effects of PPARγ agonists in the animal model of OB. Of particular interest is the fact that PPARγ activators are already commercially available as treatments for diabetes such that their adaptation into the field of transplantation can rapidly be applied.

4. Progress Reports of ROTRF Grantees

Dr. Maria-Cristina Cuturi, Principal Investigator
Dr. Régis Josien, Collaborator
Dr. Elise Chiffoleau, Research Associate
Dr. Jean-Marie Heslan, Research Associate
Mr. Thomas Condamine, Master's Student

INSERM, Nantes, France

Characterization of Tolerance-Related and Induced Transcript, a New Member of the CD20/FcεRIβ family, Over-Expressed in a Model of Allograft Tolerance

We identified TORID (tolerance-related and induced transcript), a new member of the CD20/FcεRIβ family over-expressed in tolerated allografts induced by donor-specific blood transfusion (DST). We showed that TORID is strongly expressed in macrophages and dendritic cells (DCs) and localizes to the nuclear envelope. Moreover, its expression decreases following maturation/activation and over-expression of TORID in DCs through viral transduction inducing the development of immature cells refractory to further stimulation. The ROTRF project aims at improving our understanding of the regulation of the expression of TORID in different cell types and different models of allograft tolerance, and to characterize its function in immune responses, particularly in transplantation through the generation of gene expression systems that over-express or inhibit TORID.

ROTRF first year report

During this first year of ROTRF funding, we aimed at generating different tools to analyze the expression and function of TORID and have been able to publish and present some of the results[1,2].

We first analyzed TORID expression in different models of allograft tolerance.

Long-term allografts (>day 100) induced by DST treatment express high levels of TORID mRNA. However, in this model, allografts display some signs of chronic rejection. We therefore assessed TORID expression in different models of partial or true tolerance (allografts with no signs of chronic rejection). In a model of true tolerance, we observed high TORID mRNA expression in long-term allografts. On the contrary, very low amounts of TORID were detected in models displaying strong signs of chronic rejection. In addition, we observed a significant increase of TORID expression in long-term kidney allografts with no signs of chronic rejection. These results show that TORID expression is not associated with chronic rejection and suggest that it could be involved in tolerance processes common to distinct models.

We have generated transgenic rats for TORID (ROTRF funding does not include the generation of transgenic rats but includes their analysis).

Transgenic rats for TORID were generated under an ubiquitous promoter (CAG). Three transgenic founders were obtained. One does not transmit the transgene to F1 rats. The second one generated about 50% of transgenic F1 rats. In this line, TORID transgene expression is found in a lot of organs and PBMC but at a relatively low level. We didn't observe differences of secondary response of transgenic rats immunized with KLH in footpad compared to that of non-transgenic rats. These results suggest that TORID over-expression in these rats could be too weak to enable the study of TORID function. We will further continue the analysis of F2 rats who could express TORID stronger (homozygous rats). Interestingly, the third founder gave birth to 15% of F1 transgenic rats that died at 4 weeks of age. These rats gained little weight after birth (Fig. 1A,B). In these rats we observed a strong expression of the transgenic TORID especially in lung, kidney, heart, liver and intestine (Fig. 1C). Histology did not reveal abnormalities in organ genesis or organisation. Further analysis will be performed to determine the cause of death. To prevent death that could be due to non-hematopoietic origin, we did generate chimeras by injecting bone marrow cells in lethally irradiated recipients. These chimeras are now 8 weeks of age and will be analysed.

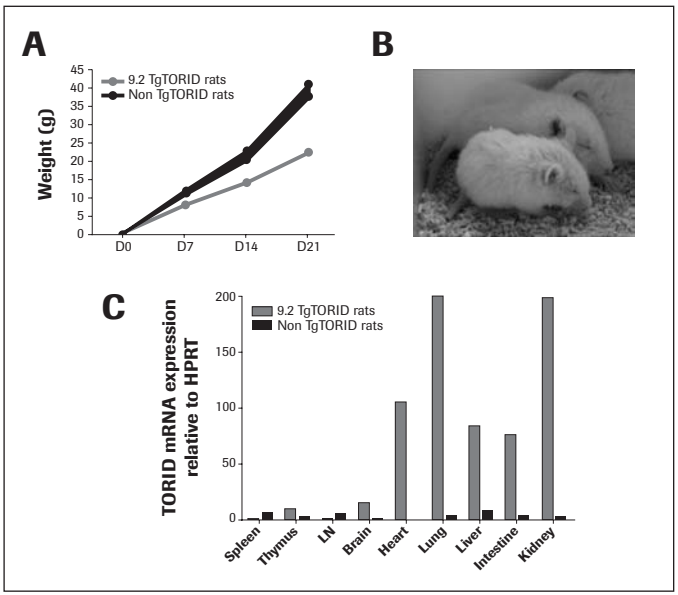


Figure 1. TORID transgenic rats exhibit weight loss and early lethality. A) *Body weight,* ***B)*** *Representative photographs of control and TORID transgenic rats at 3 weeks of age,* ***C)*** *Expression of TORID mRNA in organs of transgenic TORID or non-transgenic rats assayed by quantitative RT/PCR.*

Generation of a monoclonal antibody

Mice have been immunized with purified TORID protein directly in the spleen, and a fusion has been performed. Unfortunately, no anti-TORID hybridoma have been obtained. We are now trying a new method of generation of monoclonal antibody by injecting directly the TORID-coding lentivirus plus oligonucleotides CpG in the footpads of mice.

Generation of a TORID/GFP lentivirus

We have generated a TORID/GFP lentivirus but that was not very efficient at transducing cells. We are therefore now generating another TORID/GFP lentivirus by using a new vector. This lentivirus will allow us to analyze TORID function in isolated cells and to generate chimeras and transgenic rats.

Generation of KO mouse

The "Mouse clinical institute", Strasbourg, is generating a TORID KO mouse. The chimeras, generated by two different recombinant clones are in breeding. We will analyze the phenotype of these mice and function of immune cells.

This first year will permit the generation of different tools that will help to determine the role of TORID in the immune system.

Publications

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2. Condamine T, Chiffoleau E, Louvet C, et al. Identification of a new member of the CD20/FcεRIβ family, TORID overexpressed in tolerated allografts and involved in differentiation/maturation of dendritic cells. *9th Basic Science Symposium* 2005; *(Oral presentation)*.

Dr. Claude Daniel, Principal Investigator

INRS-Institut Armand-Frappier, Laval, Canada

The Role of Histocompatibility Antigens in the Regulation of Allograft Rejection

We have established a TCR-transgenic model that allows us to study the contribution of direct and indirect pathways of allorecognition in graft rejection whilst using the same $CD4^+$ T cell clonotype. Importantly, we have shown that direct alloresponses can be initiated within the allograft itself, whereas indirect responses are primed in secondary lymphoid organs. Hence, it could be hypothesized that priming of $CD4^+$ alloreactive T cells in different physiological contexts, depending on the alloreactivity pathway involved, would have important biological consequences on the secondary effectors of allograft rejection regulated by these T cells.

The purpose of this research project is to delineate further the relative contribution of the direct and indirect pathways of allorecognition on the regulation of immune effectors mediating allograft rejection. The specific aims proposed were

1. To evaluate the contribution of $CD4^+$ T cell help for the maturation of naïve and memory $CD8^+$ cytotoxic T cells, and

2. To visualize and elucidate the mechanisms of $CD4^+$ T cell help for the production of alloantibodies.

How direct or indirect alloreactivity pathways contribute to the maturation of alloreactive $CD8^+$ cytotoxic T cells has not been demonstrated yet. We have established a skin allograft rejection model which uses as recipients of skin allografts $\alpha\beta$T cell-deficient mice adoptively reconstituted with $CD4^+$ transgenic T cells. Isogeneic, purified naïve $CD8^+$ T cells were co-transferred without or with transgenic $CD4^+$ T cells at different ratios. Recipients were grafted with skin from donors which can prime the $CD4^+$ T cell response by direct and/or indirect pathways. The primary $CD8^+$ T cell response following allograft rejection was characterized by flow cytometry and ELISPOT at 15 days post-transplant. Because of homeostatic proliferation of transferred $CD8^+$ T cells, it was difficult from the flow cytometry analyses to draw any conclusion on the contribution of $CD4^+$ T cells to $CD8^+$ effector T cells. However, IFN-γ ELISPOT analyses clearly show that maturation of $CD8^+$ effector T cells require help from $CD4^+$ cells, and individual direct or indirect pathways are sufficient to

provide such help (Fig. 1). Furthermore, graft rejection kinetics demonstrated that $CD4^+$ T cells were essential for complete allograft rejection in these conditions. Similar analyses will be performed in the next months to characterize the $CD8^+$ T cell memory response at 60 days post-transplant. Furthermore, skin allograft recipients will be re-challenged with allografts in order to characterize secondary $CD8^+$ T cell responses.

We have previously shown, using the adoptive transfer model described above, that the indirect pathway of allorecognition was absolutely required for antibody production. In this model, the production of IgG alloantibodies is not supported following direct alloreactivity. We now want to characterize further the physiology of antigen presentation and alloreactive T and B cell interactions required for alloantibody production. These interactions will be visualized and characterized using confocal microscopy, following the adoptive transfer of cells labeled *ex vivo* with different fluorescent dyes. To perform these studies we have been working in the last 6 months on the development of a new adoptive transfer model using rag1-deficient mice as recipients.

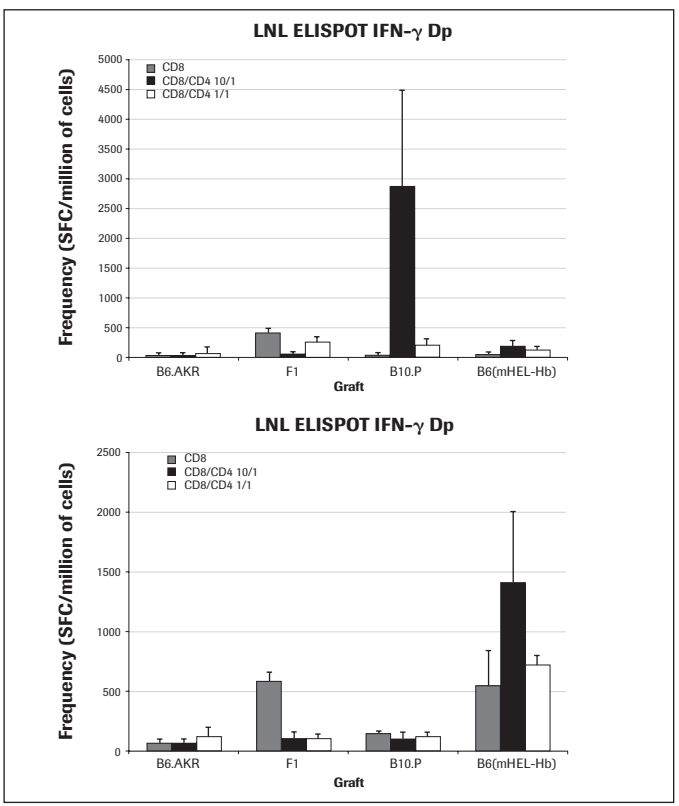

Figure 1. The maturation of alloreactive $CD8^+$ CTL is dependent on $CD4^+$ T cell help provided by the direct or indirect pathway.
The frequency (spot-forming cells (SFC)/millions of cells) of H-2Dp- or H-2Db-specific alloreactive $CD8^+$ T cells in the graft draining lymph nodes was determined directly ex vivo by IFN-γ ELISPOT. Draining lymph nodes were recovered 15 days post-transplant from recipient that received an isogeneic graft (B6.AKR), a direct pathway (B10.P), an indirect pathway (B6(mHELHb)), or F1 (B10.P x B6(mHEL-Hb)) allografts.

Dr. Thomas J. Dengler, Principal Investigator
Prof. Stefan Meuer, Research Associate
Prof. Jordan Pober, Research Associate
Dr. Vijay Shankar, Post-doctoral Fellow
Mrs. Natascha Sommer, Research Technician
Mr. Berouz Kherat, Dissertation Student

Medizinische Universitätsklinik, Heidelberg, Germany

Immunomodulation and Protection against Transplant Vasculopathy by Endothelial Precursors

Endothelial progenitor cells (EPCs) home to sites of angiogenesis and therefore have potential implications in allogenic transplantation settings and in various vascular diseases. The current project investigates the antigen presenting and immunomodulatory capacity of autologous EPCs and tests if exogenous application of (genetically modified) EPC can achieve cellular chimerism in the endothelium.

EPCs have been isolated from peripheral blood mononuclear cells (PBMCs) by selective adhesion to fibronectin-coated plates and by culturing in endothelial specific media for 5 days. Our results demonstrate that PBMC-derived EPCs display predominantly monocytic cell markers, including T cell costimulatory antigens. EPCs when compared with HUVECs for the surface expression of T cell costimulators, expressed relatively higher amounts of CD30L and SLAM (Fig. 1A). As determined by quantitative RT-PCR, EPCs upon stimulation with LPS and IL-1 upregulated TNF-α similar to monocytes, but failed to show EC-typical regulation of E-selectin or ICAM-1. EPCs were, however, incorporated into existing vascular networks of HUVECs on matrigel *in vitro*, confirming their angiogenic potential.

EPCs were compared with HUVECs and monocytes for induction of allogenic $CD4^+$ T cell proliferation. $CD4^+$ T cells were activated by PHA, SEA/B and allogeneic co-culture alone (MLR-like). With all experimentally tested modes of T cell activation, $CD4^+$ T cells co-cultured with EPC consistently showed intermediate proliferation rates compared to co-culture with monocytes or IFNγ stimulated HUVECs (Fig. 1B), which displayed stronger (monocytes) and markedly weaker (HUVEC) proliferation responses, respectively. These data support the monocytic characteristics of adhesion-derived EPC and confirm considerable immune-activating and antigen-presenting potential of this cell type, which will have to be expected from PBMC-derived EPCs also when used therapeutically[1]. The immunomodulatory antigen ILT3 was inducibly expressed on HUVECs after stimulation with IL-10, subsequently suppressing proliferation of allogenic T cells[2]. Therefore, retro- /adenoviral vectors expressing ILT-3 have

been constructed for the transfection of HUVEC or EPC – preliminary data suggest a pronounced immunosuppressive effect of such transgenic cells.

Two hybrid vascular models for determination of endothelial chimerism [implantation of EPC-containing gels in SCID mice (data not shown), aortic interposition of human artery segments in SCID mice] have been established. EPCs overexpressing the anti-apoptotic protein BCl-2 after adenoviral transduction demonstrated increased survival in *in vitro* culture on matrigel and upon implantation *in vivo* (data not shown). In parallel experiments, administration of the immunosuppressant FTY720 – also a major inducer of EPC-derived angiogenesis - suppressed the development of atherosclerosis in ApoE-deficient mice[3], further supporting the concept of EPC-mediated vascular protection. In addition, a mouse hind-limb ischemia model has been established to determine the therapeutic potential of *ex vivo* vector-transduced EPCs (bcl-2, ILT-3; data not shown).

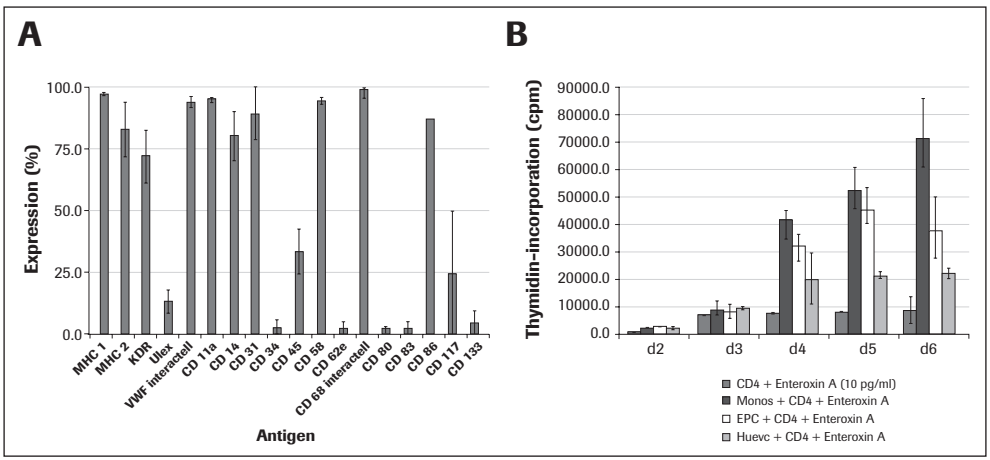

Figure 1. A) *Expression of surface antigens in EPCs (% positive cells are shown),* ***B)*** *$CD4^+$T cell costimulatory potential of EPCs, HUVECs and monocytes: SEA/B-activated $CD4^+$ T cells were co-cultured with EPCs, HUVECs and monocytes. EPCs showed intermediate $CD4^+$ proliferation potential in contrast to monocytes and HUVECs, which showed stronger and markedly weaker costimulation, respectively.*

Currently ongoing research: Detailed analysis of allogenic $CD4^+$ and $CD8^+$ T cell activating potential of EPCs, including naïve versus memory phenotypes. Induction of T cell activation / proliferation by autologous EPCs ($CD4^+$ T cells after pulsing with tetanus toxoid, $CD8^+$ T cells by ovalbumin cross priming or intracellular overexpression of CMV IE2 protein).

Immunomodulatory function of EPCs transgenic for ILT-3.

Generation of angioblasts and derived endothelial cells using CD34 or CD133 isolation from leukapheresis products.

Publications

1. Sivanandam V, Rämer P, Haemmerling S, et al. EPC have antigen presenting capacity and stimulate allogenic T cells. *70th Annual Meeting of the German Cardiac Society* 2006; *(Accepted abstract)*.
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3. Klingenberg R, Bea F, Blessing E, et al. The immunomodulator FTY720 inhibits early atherosclerosis in ApoE-/- mice; *(Manuscript submitted)*.

Dr. Robert L. Fairchild, Principal Investigator

Cleveland Clinic Foundation, Cleveland, USA

Short-Term Therapies Directed at Inflammation and T Cell Activation

Our studies follow clinical observations that allograft outcome is worse in cadaver- versus living- graft donors and that increased ischemia times are an additional negative factor in graft outcome. We have shown that MHC-mismatched heart allografts have long-term survival (>130 days) in mice when recipients are treated for 2 days at transplant to inhibit neutrophil activation in the allograft and to delay the activation of donor-reactive T cells. These results led us to propose the hypothesis that attenuation of early graft inflammation (e.g. inhibition of neutrophil-mediated allograft damage) provides a window in which low-dose immunosuppression or costimulatory blockade will abrogate the rejection of complete MHC-mismatched heart allografts. During the first 9 months of this study we have concentrated on experiments proposed in specific aims 2 and 3.

First, we tested whether this strategy resulted in donor-specific tolerance. Groups of heart allograft recipients were treated with neutrophil-depleting antibodies and a short course of anti-CD154 mAb at the time of transplant. Consistent with our previous observations, approximately 90% of the allografts survived long-term and at day 100 received skin allografts from the heart allograft donor strain or skin isografts. The skin allografts were rejected within 14 days and this was followed about 10 days later by rejection of the heart allografts (that had survived beyond day 100 post-transplant up to that point). In contrast, skin isografts did not provoke the rejection of the heart allografts. We are now testing third-party donor skin allografts for rejection of the heart allografts. We will also perform a thorough analysis of the activation and function of the donor reactive T cells in these skin allograft recipients and histological analysis of all grafts.

Second, we tested the ability of donor-primed T cells to reject the heart allografts when transferred to the recipients at day 30-40 post-transplant. This transfer does not provoke rejection of the heart allograft whereas the transfer accelerates the rejection of heart allografts in recipients treated with anti-CD154 mAb alone when the transfer is performed at day 18-20 post-transplant (the grafts in these animals normally reject about day 30 post-transplant). As proposed in the application we are now repeating these experiments with CFSE-labeled T cells in order to track their recruitment (or the absence of recruitment) into the heart allografts in these two sets of recipients. We have recently established a reliable method to

induce ischemic injury to the long-term surviving heart allografts and will test the effect of this injury on the trafficking of the transferred T cells into heart allografts that are normally maintained long-term. We will transfer donor-reactive polyclonal and TCR-transgenic T cells in these experiments. In the coming year we will also test the activation of donor-reactive T cells in recipients treated with this novel therapy. We expect these results to provide important mechanistic insights into the inability of the treated recipients to reject the heart allografts and that these insights will be useful to design strategies to attenuate neutrophil-mediated allograft damage.

Prof. Andrew J.T. George, Principal Investigator
Dr. Giovanna Lombardi, Co-Investigator
Mr. Frank Larkin, Co-Investigator
Prof. Robert Lechler, Collaborator

Imperial College London, London, UK

Modification of Dendritic Cells and DNA Vaccination for the Induction of Tolerance

Introduction

Stimulation of allogeneic T cells requires not only antigen-specific signals but also costimulatory signals. The most important of these interactions are between CD80/86 on the antigen-presenting cell and CD28 on the T cell. T cell activation is also controlled by secretion of cytokines and expression of the tryptophan-catabolising enzyme indoleamine 2,3 dioxygenase (IDO). Depletion of this essential amino acid, and the production of metabolites of tryptophan, blocks T cell proliferation.

In previous work we have developed a strategy for blocking expression of CD80/86 by expressing a fusion protein; CTLA4-KDEL. The CTLA4 portion of the molecule binds to CD80/86, while the KDEL sequence retains the complex within the endoplasmic reticulum. In the human system we showed that this prevented CD80/86 expression on dendritic cells (DCs), and that T cells interacting with CTLA4-KDEL DCs adopted an anergic/regulatory function. Transfection of human DCs with the gene encoding for IDO also induced non-responsiveness and apoptosis in T cells.

Aims of the project

The original aims were to determine whether CTLA4-KDEL could block graft rejection in animal models *in vivo* and to compare CTLA4-KDEL DCs with dexamethasone-treated DCs. Given recent data obtained with IDO we will also compare IDO-expressing DCs.

Progress

In the first year we have developed the expression systems and *in vitro* assays. In our original proposal we proposed using a non-viral method of gene transduction that works well with human DCs. However, with murine DCs it produced variable results. Therefore, we adopted the backup outlined in our original application of using lentiviruses.

We cloned the IDO and CTLA4-KDEL (the latter had been done prior to the study, but not fully characterised) into equine infectious anaemia virus (EIAV) under control of the CMV promoter. We removed from the EIAV constructs the GFP reporter gene that is normally used to titrate

virus numbers, because of its potential immunogenic properties. We have therefore developed a PCR system that allows us to rapidly determine viral titre following transduction of a reporter cell line. We are preparing a methodology paper describing this approach.

The viruses are capable of transducing murine bone marrow-derived DCs, as determined by real-time RT-PCR (IDO) and Western blotting. They are functionally active. In the case of IDO this was done by measuring L-kynurenine (the breakdown product of tryptophan) in the supernatant of transduced cells. For CTLA4-KDEL we used flow cytometry to show lack of CD80/86 expression (and normal expression of other markers).

Using *in vitro* assays with allogenic combinations of DCs and T cells (a 1:3 or 1:5 DC:T cell ratio) we have shown that both IDO and CTLA4-KDEL-expressing DCs (as well as dexamethasone treated DCs) cannot sustain proliferation by allogeneic T cells.
We have initiated a collaboration with Prof. K. Wood's laboratory in Oxford in which we are testing the ability of modified DCs to prevent allorecognition of vessel grafts.

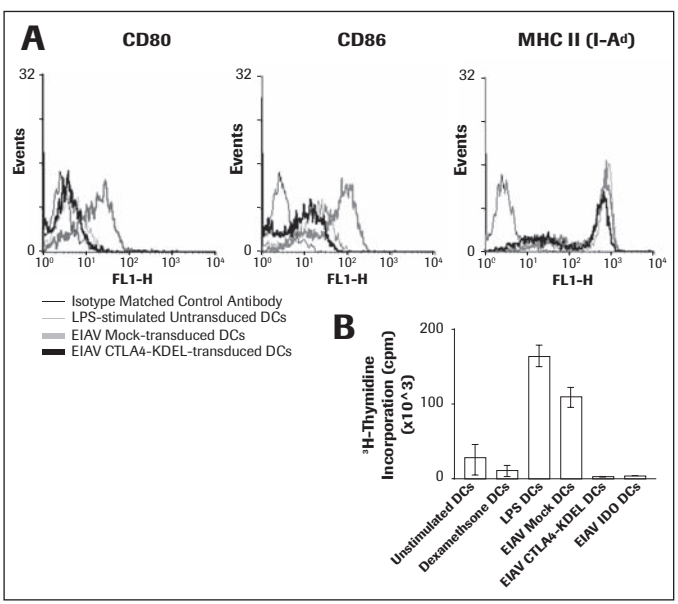

Figure 1. Transduction of murine DCs with EIAV CTLA4-KDEL and IDO lentivirus. *Murine bone-marrow-derived dendritic cells were differentiated with mGM-CSF and were transduced on day 4 with EIAV CTLA4-KDEL, EIAV IDO and EIAV Mock (Irrelevant protein), or left untransduced until day 7 when the DCs were either stimulated with lipopolysaccharide, treated with dexamethasone, or left unstimulated.* ***A)*** *On day 8, the expression of CD80, CD86 and MHC II was analysed using flow cytometry.* ***B)*** *In a separate experiment, DCs were tested for their ability to stimulate an MLR by allogeneic T cells. The results are expressed as mean ± SD of triplicate wells.*

Objectives for the next year

We have successfully shown that the constructs we have generated function appropriately *in vitro*. We are going on, as outlined in the original application, to determine if they can induce anergy *in vitro*, and whether the suppression of proliferation can be reversed. We will also be moving into the animal models outlined in our application to compare the effectiveness of these protocols *in vivo*.

Dr. David A. Gerber, Principle Investigator
Dr. Jian Wang, Research Associate
Dr. Lujun Song, Technician

University of North Carolina, Chapel Hill, USA

The Immunologic Basis of Hepatic Progenitor Cell Transplantation

Experiments and Results

1. Analysis of the proliferative ability of hepatic progenitor cells to determine their potential in cell therapy

Our initial procedure for isolating hepatic progenitor cells (HPC) from the adult liver generated a mixed cellular population (e.g. hepatic progenitor population, kupffer cells, stellate cells, etc.). This heterogeneity presented a challenge for analyzing specific characteristics of the HPC. During the past year we continued our efforts to enrich the HPC population from the surrounding non-parenchymal cells. Using flow cytometry we demonstrated that ~28.5% of the cells in our supernatant fraction were positive for a cell-surface marker, Sca-1. Additional analysis demonstrated that this marker was uniquely expressed on our previously characterized HPC population. Subsequent cell sorting was performed using magnetic activated cell sorting according to the manufacturer (Miltenyi, Biotec. Inc.). Purified Sca-1^+ HPC obtained by MACS have subsequently been established under standard culture conditions.

2. Analysis of the *in vitro* HPC proliferation

In the next experiments we utilized an MTT assay to analyze *in vitro* HPC proliferation. Figure 1 shows HPC proliferation under standard conditions throughout 96 hours of primary culture. A period of HPC doubling during the initial 24 hours of culture is followed by a static period and subsequent decline in cell number at 96 hours of culture. These results suggest that the HPC are rapidly passing through the cell cycle but after this initial burst of cell division the cells become quiescent under our primary conditions.

3. The effect of epidermal growth factor on the HPC

We studied the effect of epidermal growth factor (10 ng/ml EGF), a known mitogen for mature hepatocytes, on the HPC. Surprisingly the HPC lost their initial proliferative phase during the first 24 hours of culture. This period of cellular quiescence was followed by marked cellular proliferation between 68 and 96 hours of culture; evidenced by a tripling of the HPC number (Fig. 1).

4. Data Interpretation

These findings suggest that under standard culture conditions the HPC pass through the cell cycle and undergo mitosis within 24 hours (more rapidly than mature hepatocytes) but a known hepatic mitogen (e.g. EGF) has a paradoxical effect on this unique cellular population. Additional experiments will be planned accordingly.

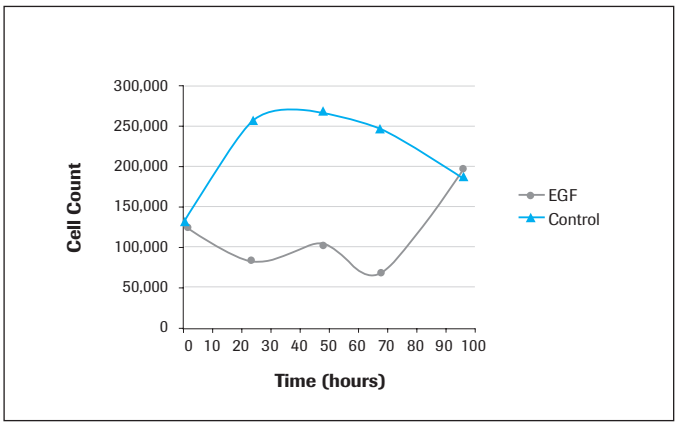

Figure 1. Analysis of the proliferation of the hepatic progenitor cells.

Future Experiments

1. To analyze alloimmune responses in HPC transplantation

We are beginning *in vitro* experiments to determine the ability of the Sca-1^{+} HPC to initiate an alloimmune response using a CFSE assay. The results of this experiment will provide insight into how the host will respond after cellular transplantation with the HPC population.

2. To establish a model for cellular engraftment

We have begun developing a model for 3D encapsulation of the HPC using alginate. This process is important because preliminary experiments with syngeneic HPC transplantation demonstrated a low yield of engraftment and a surprisingly high incidence of HPC pulmonary emboli. Initial results have demonstrated that the HPC:- can be encapsulated in alginate beads with a 200 micron diameter, - remain viable after encapsulation and - demonstrate functions consistent with differentiated hepatocytes (glycogen synthesis).

Dr. Elaine Holmes, Principal Applicant
Dr. Gerrard Murphy, Co-Investigator
Dr. Hector Vilca-Melendez, Co-Investigator

Imperial College, London, UK

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

A comprehensive assignment of the endogenous metabolites present in liver biopsies obtained from donor livers prior to and post-transplant has been carried out using ^{1}H magic angle spinning nuclear magnetic resonance (MAS-NMR) spectroscopy of intact tissue. Inter-person differences were found in the profile and content of lipids, indicating that NMR may provide a powerful rapid method for assessing liver fat status, considered to be an important criterion of graft viability. Changes in other metabolites, particularly in phospholipid components, were detected for each liver over the transplantation process. For example, glycerophosphocholine was found to decrease from the first (pre-transplant) to the third (post-transplant) biopsy of all livers, except one, which was confirmed histologically as having high lipid content. This reflects differences in physico-chemical and metabolic properties of the organ at different stages of the transplantation process and may confer information regarding graft survival. Diffusion edited and T_1 and T_2 weighted pulse sequences were adapted to the ^{1}H MAS-NMR analysis to focus on the mobility and compartmentalization of lipids and other metabolites. Proton T_1 and T_2 relaxation times indicated higher mobility of triglycerides in samples from sub-optimal grafts.

Additionally, both NMR and HPLC-MS based methods have been optimised for profiling bile acids and other biochemical components of hepatic bile. The identification of individual bile acids is particularly interesting as these major bile components are known to affect biliary lipid secretion and bile flow. ^{1}H-NMR spectral profiles obtained from sequential samples of bile at 10-minute intervals after transplantation demonstrated major changes in bile composition over time. The time course changes of the recipient bile, which included UW solution washout (preservative), bile acid secretion and lactate decrease, are under further investigation in order to establish if their excretion rate correlates with the liver function recovery. Although the donor bile samples show several qualitative and quantitative differences, most of the samples contain high amounts of phospholipids and bile acids. In one sample, which was particularly low in these compounds, the graft subsequently developed primary graft dysfunction, ultimately leading to recipient's death. A progressive increase in the amount of phospholipids in bile over the 40 minutes of collection time was also observed in this patient, together with relatively higher levels of glucuronic acid and a low concentration of aromatic

molecules. Other differences between donor bile samples include the relative concentrations of some organic acids, namely 3-hydroxybutyrate and acetate, and in some carbohydrates. As the number of samples in the database increases (currently 15 donor-recipient pairs, plus additional non-paired samples), novel chemometric methods for deconvolving spectral data and integrating spectral and clinical data, developed in-house, will be applied to the data to increase the sensitivity of detection of biomarkers of good or adverse graft function. Preliminary analyses already show promise. The correlation of experimental findings with clinical and histopathological data is currently ongoing in order to achieve the ultimate goal of this project: building a method based on ^{1}H NMR and pattern recognition that can have diagnostic significance in assessing the viability of donor grafts.

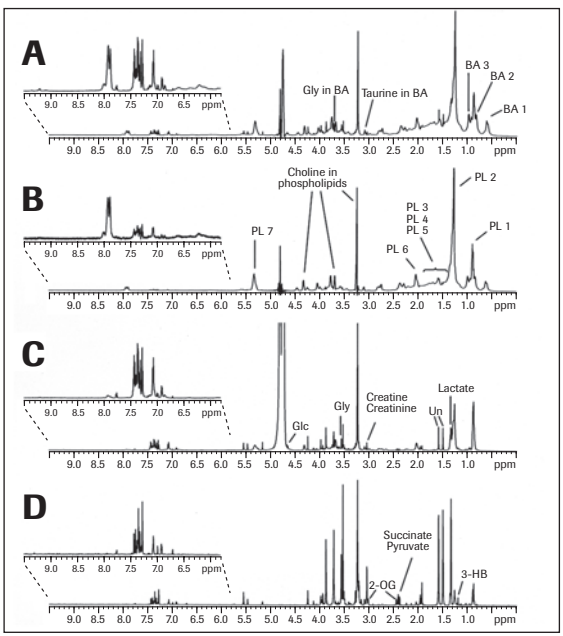

Figure 1. 600 MHz ^{1}H NMR spectra of human hepatic bile. A) *single pulse spectrum,* ***B)*** *diffusion-edited spectrum,* ***C)*** *spin-echo (cpmg) spectrum,* ***D)*** *J-res f_2 projection.*
Assignment: PL1, phospholipid $C\underline{H}_3$; PL2, phospholipid $(C\underline{H}_2)_n$; PL3, phospholipid $C\underline{H}_2CH_2CO$; PL4, phospholipid $C\underline{H}_2$-CH=CH; PL5, phospholipid $C\underline{H}_2CO$; PL6, phospholipid CH=CH-$C\underline{H}_2$-CH=CH; PL7, phospholipid $C\underline{H}$=$C\underline{H}$; BA1, bile acids CH_3 at C18; BA2, bile acids CH_3 at C19; BA3, bile acids CH_3 at C21; Glc, glucose; Gly, glycine; 3-HB, 3-hydroxybutyrate; 2-OG, 2-oxoglutarate; Un, unknown.

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4. Legido-Quigley C, Stella C, Ordovas J, et al. The effects of sample preparation for UPLC-MS(TOF) metabonomic studies of human urine; *(Manuscript in preparation).*
5. Legido-Quigley C, Vilca-Melendez H, Heaton N, et al. Combined LC-cryoprobe-NMR-MS and UPLC MS for characterization of human bile; *(Manuscript in preparation).*
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Dr. Anatolij Horuzsko, Principle Investigator

Medical College of Georgia, Augusta, USA

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

In vivo models of immune suppression are the most useful models for studies in transplantation because of their relevance to clinical transplantation in humans. The newly developed HLA-G transgenic mouse is an excellent model system for such studies. HLA-G is a human MHC 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.

During last year we performed a number of experiments that focused on the goals described in specific aims 1, 2, and 3.

Specific Aims

Specific Aim 1: We will test the hypothesis that (1) dendritic cells from HLA-G transgenic mice, and (2) HLA-G tetrameric complexes are able to induce hyporeactivity to allogeneic skin grafts.

We will use an *in vivo* model of adoptive transfer of dendritic cells into recipients followed by allogeneic skin grafts. We will compare the effect of wild-type dendritic cells, dendritic cells from HLA-G transgenic mice, and dendritic cells from HLA-G mice matured *ex vivo* on graft rejection and cytotoxic T cell development. We will use an *in vivo* model to deliver tetramers into recipient mice followed by allogeneic skin grafts. We will compare the effect of HLA-G tetrameric complexes and control tetramers on allograft rejection.

Specific Aim 2: We propose to determine the mechanisms responsible for allograft hyporeactivity in HLA-G transgenic mice.

The first mechanism to be tested is that allograft hyporeactivity is caused by a defect in cytokine secretion by immature HLA-G$^+$ dendritic cells. The second mechanism to be tested is whether allograft hyporeactivity is mediated by the induction of T regulatory cells with suppressive ability.

Specific Aim 3: We will test the hypothesis that the effect of HLA-G is mediated by binding to the PIR-B receptor, causing altered myelomonocytic cell development in HLA-G transgenic mice.

We will examine at the molecular and cellular levels of the HLA-G and PIR-B receptor interaction and their effects on the function of dendritic cells. The results gained in this application will provide a basis for a trial of HLA-G–modified dendritic cell therapy in patients receiving organ transplants.

Studies and Results

For the past year our major goal has been to determine the mechanism of the modulation of murine dendritic cells by HLA-G. This mechanism involves an interaction between HLA-G and PIR-B, an inhibitory receptor.

We demonstrate that HLA-G tetrameric complexes inhibit maturation of murine dendritic cells *in vitro*, 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. Analysis of the expression of PIR-B and its phosphorylation status in transgenic mice showed that the PIR-B receptor is highly phosphorylated on dendritic cells from HLA-G transgenic mice. Furthermore, HLA-G tetrameric complexes bind to PIR-B receptors on transfected cell lines and induce tyrosine phosphorylation of PIR-B on cells expressing receptors. Confocal microscopy analysis confirmed the association of PIR-B and HLA-G on dendritic cells. In addition, triggering of PIR receptors with anti-PIR mAb or HLA-G tetramer-coated microspheres resulted in increases in allogeneic skin graft survival. To facilitate *in vivo* studies of interaction between HLA-G and PIR-B we generated 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, are currently breeding, and expression experiments are in progress.

Significance

Our findings provide support that HLA-G is an important tolerogenic molecule for the acceptance of a semiallogeneic fetus and transplanted tissue/organ. In addition, our findings provide support on an important inhibitory function of PIR-B in dendritic cells *in vivo* and opens strategies for the targeting of dendritic cells for prevention of graft rejection.

Publications

1. Horuzsko A, Ristich V, Liang S, et al. Regulation of dendritic cell function by HLA-G. Dendritic Cells at the Center of Innate and Adaptive Immunity. *Keystone Symposia* 2005; *(Abstract)*.
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4. Horuzsko A. Murine models of HLA-G-based tolerance to allografts. *4th International Conference on HLA-G* 2006; *(Abstract)*.

Prof. John A. Kirby, Principal Investigator
Prof. Alastair Burt, Co-Investigator
Dr. Helen Robertson, Research Associate

University of Newcastle, Newcastle-upon-Tyne, UK

Therapeutic Elimination of Intraepithelial T Cells: a Strategy to Reduce Epithelial to Mesenchymal Transition in Chronic Allograft Dysfunction

Current objectives

We continue to define the relationship between inflammation and the induction of graft damaging epithelial-mesenchymal transition (EMT) in kidney and, more recently, in liver and lung tissues. We are also initiating further study of the differentiation of intraepithelial T cells in order to define more fully their functions and mechanisms which regulate their survival.

Progress to date

Dual labelling of renal tissue sections to detect E-cadherin and S100A4 in transplant renal tissue sections has revealed that the epithelial marker E-cadherin is at least partially lost at adherens junctions in tubular epithelial cells that also express the mesenchymal (transition) marker S100A4.

Studies of phosphorylated Smad 2 and Smad 3 (pSmad 2/3) proteins both *in situ* in transplant renal tissue sections and *in vitro* in primary human renal epithelial cells (HREC) have confirmed that TGFβ is a key regulator of EMT in humans. In addition, we have shown in tissue sections that phosphorylation of the Smad 1, 5 and 8 transcription factors by BMP-7 is reduced during acute rejection and is absent in tissue with chronic allograft nephropathy. *In vitro* we have shown that nuclear pSmad 2/3 is diminished and that there are high levels of nuclear pSmad 1/5/8 in HREC when both TGFβ and BMP-7 are added to the medium. We have also shown that BMP-7 can reduce S100A4 expression by TGFβ-treated HREC. Significantly we have now extended this work to include studies which show the potential of inflammation-induced EMT also to damage lung and liver tissue following transplantation.

Our results indicate that the TGFβ-antagonist BMP-7 has the potential to facilitate tubular repair and decrease interstitial fibrosis in injured human kidney. Therefore, exogenous BMP-7 could delay chronic renal allograft dysfunction. It is possible that other members of the BMP family could exhibit similar functions in damaged lung and liver tissue.

We have demonstrated that CD103+ve MOLT-16 T cells have a 'regulatory' anti-proliferative activity when added to mitogen-activated peripheral blood T cells in culture. MOLT-16 cells

have also been shown to express TGFβ, whilst HREC co-cultured in the presence of MOLT-16 cells upregulate S100A4. Importantly, T cells from mixed leukocyte reactions (MLR) stimulated with TGFβ upregulate CD103 and cause upregulation of S100A4 and nuclear localisation pSmad2/3 when co-cultured with HREC.

We have also shown that allospecific CD103+ T cells co-express the FOXP3 transcription factor (quantitative PCR shows that TGFβ increases both CD103 and FOXP3 expression by activated human T cells); these data suggest that the CD103 +ve T cells observed within renal tubules might have a regulatory phenotype. Preliminary immunocytochemistry with a polyclonal FOXP3 antibody has reinforced this conclusion but we are currently confirming this work with a new range of monoclonal antibodies specific for human FOXP3.

Work from our group has shown that activated T cells are susceptible to specific induction of apoptosis. Whilst apoptotic deletion of intratubular T cells might delay epithelial-mesenchymal transition, we are keen to fully explore the functions of these cells as a regulatory activity might also have a beneficial effect on acute inflammation.

Post-transplant liver sections have shown that S100A4 is induced in small intrahepatic bile ducts in a biopsy with a diagnosis of cholestasis taken 24 days after transplant; as with kidney, EMT was associated with a T cell infiltrate. There was subsequently recurrent primary biliary cirrhosis in this liver – 9 months post transplant – when S100A4 was still present in bile ductules and this coincided with expression of vimentin. Both S100A4 and vimentin were also present in fibroblast-like cells surrounding the bile ductules at this stage. Nuclear pSmad 2/3 was markedly stronger in recurrent PBC and pSmad 1/5/8 was very low when compared to normal tissue. In a preliminary study of human intra-hepatic bile duct epithelial cells cultured in the presence of TGFβ these cells attained a fibroblast-like morphology and expressed high levels of S100A4. Control, untreated HIBEC retained their cobblestone appearance in culture and did not express S100A4.

In explants from chronic cholestatic liver diseases (PBC and primary sclerosing cholangitis) and alcoholic liver disease we have found high expression of S100A4 and MMP2 in small and intermediate bile ducts. In addition there is a correlation of expression of S100A4, vimentin and MMP-2 in ductules within the ductular reaction. S100A4 has been shown to colocalise with cytokeratin 19 and with nuclear pSmad 2/3 in the ductular reaction. Nuclear pSmad 2/3 is generally very high in biliary tracts in these explants and pSmad 1/5/8 has all but disappeared.

The results in liver point to a role for TGFβ-regulated EMT in fibrogenesis in liver.

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Dr. Andrew D. Luster, Principal Investigator
Dr. Leo Ginns, Co-Investigator
Dr. John Wain, Co-Investigator
Dr. Benjamin Medoff, Research Associate

Massachusetts General Hospital, Charlestown, USA

The Role of White Blood Cell Attractants in the Development of Rejection and Organ Failure after Lung Transplantation

Specific Aims

The specific aims of the project have not been modified from the original aims. We have generated new data in support of all three aims in the original proposal.

Results

Aim 1: To determine the unique roles of the lymphocyte-specific chemokines CXCL10/IP-10 and CXCL9/Mig in a model of lung transplantation, and to determine the molecular mechanisms regulating the differential expression of these chemokines

In the preliminary data for this proposal we demonstrated increased expression of the lymphocyte-specific chemokines CXCL10/IP-10 and CXCL9/Mig in allogeneic tracheal transplant. We have now completed our analysis of tracheal transplants in CXCR3$^{-/-}$, IP-10$^{-/-}$ and Mig$^{-/-}$ mice using similar analysis. These studies have demonstrated no differences in fibroproliferation, obliteration or lymphocyte recruitment with deletion of IP-10 or Mig, but profound differences in these measures with deletion of CXCR3 (Fig. 1).

In separate experiments we have begun to investigate the mechanisms that lead to early upregulation of IP-10 in tracheal allografts. We now have data demonstrating that very early IP-10 expression is from donor tissue and dependent on NF-κB while expression at 24 hours after transplant is recipient-derived and dependent on the IFNγ and the transcription factor STAT1 (Fig. 2). These data are currently being submitted for publication.

Aim 2: To establish a novel model of T cell-mediated airway rejection and BO

In the preliminary data of the original proposal we demonstrated the establishment of two founder populations of transgenic mice that contain a construct made up of a modified gene for chicken egg albumin (OVA) under control of a lung-specific promoter (CC10). In subsequent experiments, we have demonstrated that the OVA gene is specifically expressed in the lung of these mice. The mice do not develop any abnormal pathology. However, if activated OVA-specific CD8 lymphocytes from a TCR-transgenic mouse (OT-I) are injected into these mice, the animals develop significant respiratory distress within 3 days with 100% mortality by

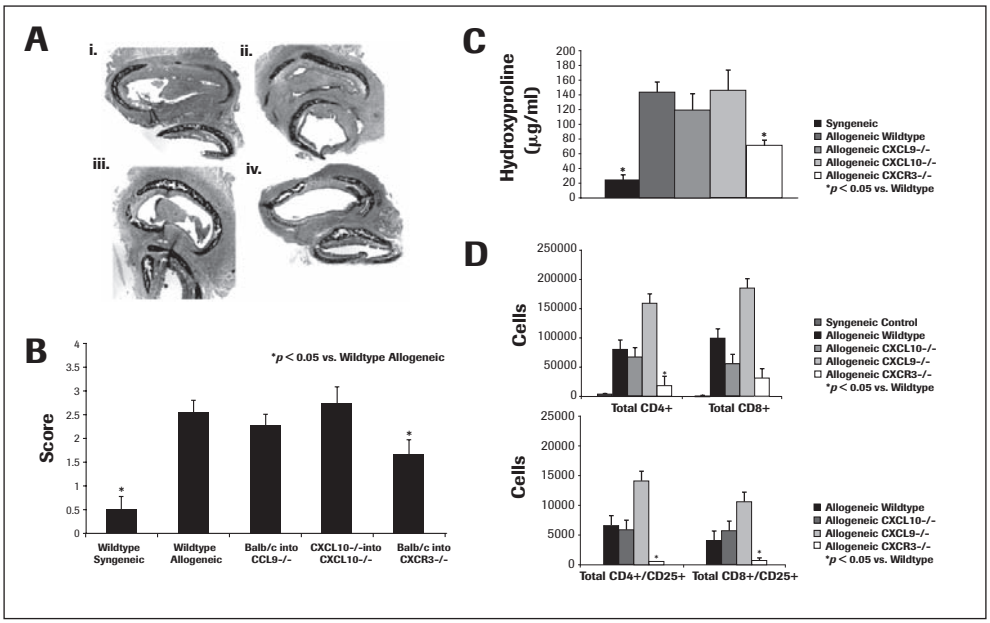

Figure 1. A) *Histology from **i.** Wild-type allogeneic transplant; **ii.** allogeneic transplant into a CXCL9$^{-/-}$ mouse; **iii.** Allogeneic transplant into a CXCL10$^{-/-}$ mouse; **iv.** Allogeneic transplant into a CXCR3$^{-/-}$ mouse.* ***B)*** *Histologic scores for transplants n=6 tracheas per group.* ***C)*** *Hydroxyproline levels in transplants, n=8 tracheas per group.* ***D)*** *T cell recruitment into tracheas, n=8 tracheas per group.*

day 7. Histology demonstrates profound acute inflammation of the airways that mimics the findings seen in acute rejection of lung transplants. In recent experiments, we have shown that injection of CXCR3$^{-/-}$ OVA-specific CD8 T cells leads to a significant reduction in mortality with less than 10% mortality in the CC10-OVA recipients compared to nearly 100% mortality in the CC10-OVA mice that received wild-type CD8 cells (Fig. 3). Co-transfer of equal numbers of wild-type and CXCR3$^{-/-}$ OVA-specific CD8 T cells demonstrates a significant reduction in recruitment of the CXCR3$^{-/-}$OVA-specific CD8 T cells into the lung and airways (Fig. 4).

We have also bred the CC10-OVA mice with Mig$^{-/-}$ mice to generate Mig$^{-/-}$/CC10-OVA mice. When these mice receive OVA-specific CD8 cells they have 100% mortality with similar lymphocyte recruitment to Mig$^{+/+}$/CC10-OVA mice. These data combined with the data in aim 1 suggests a cooperative role for IP-10 and Mig in the development of acute rejection of the lung whereby expression of either chemokine is sufficient to generate rejection of allografts.

Aim 3: To comprehensively define the chemokine profile and phenotype of pathogenic T cells associated with AR and BO

We analyzed BAL fluid taken from surveillance bronchoscopies of 40 lung transplant recipients.

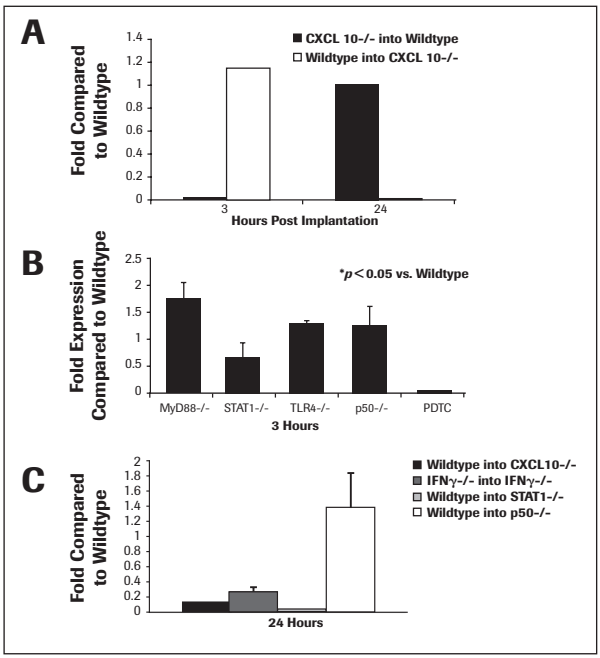

Figure 2. A) *IP-10 RNA levels in tracheas removed 3 and 24 hours after transplant. At 3 hours there is no detectable IP-10 when the donor is IP-10$^{-/-}$. At 24 hours there is no detectable IP-10 when the recipient is IP-10$^{-/-}$.* ***B)*** *IP-10 RNA levels in tracheas transplanted into different knockout animals and removed after 3 hours. Treatment with the NF-κβ inhibitor PDTC results in complete inhibition of IP-10 expression.* ***C)*** *IP-10 RNA levels in tracheas transplanted into different knockout animals and removed after 24 hours. Deletion of STAT1 or IFNγ eliminated IP-10 expression.*

These patients were at least 45 days out from their transplants and were not acutely ill. Patients were classified by clinicians blinded to the analysis as normal after transplant, acute rejection (AR), obliterative bronchiolitis (OB), or infected based on accepted clinical criteria. Patients diagnosed with acute rejection or active infection were excluded from analysis (8 patients). Of the remaining 32, 18 were classified as normal and 14 were diagnosed with BO. There were no significant differences in the time since transplant, the amount of fluid recovered, the number of cells recovered, or total lymphocyte number recovered between these two groups. However, there was significantly greater expression of several chemokine receptors on both $CD3^+/CD4^+$ and $CD3^+/CD8^+$ lymphocytes isolated from patients with BO compared to normal patients (data not shown). A manuscript from these data has been submitted for publication.

Significance

During the second year of this proposal we have continued to define the molecular signals that control T cell recruitment into tracheal allografts. We have worked to establish a new murine model of lung transplantation that allows a functional assessment of the allografts. The CC10-OVA transgenic mouse has the potential to be very useful in modeling acute rejection as it faithfully mimics acute rejection in human transplants. Our data demonstrate that Mig and IP-10 are differentially regulated following transplantation and that each contributes to the development of acute rejection and airway fibroproliferation through their interaction with CXCR3.

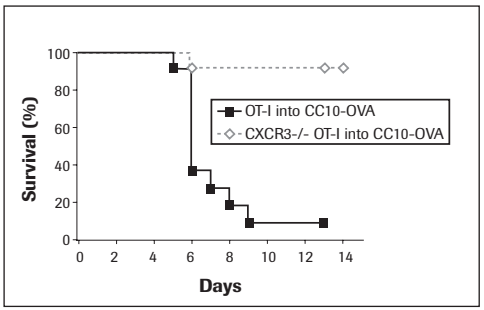

Figure 3. Mortality for CC10-OVA mice injected with 0.5x10^6 activated OT-I or CXCR3$^{-/-}$ OT-I cells (n = 11 mice per group).

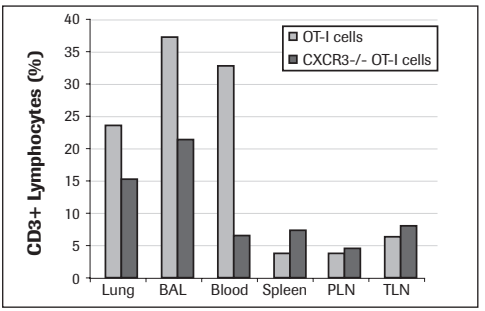

Figure 4. Co-transfer of activated wild-type OT-I and CXCR3$^{-/-}$OT-I cells into CC10-OVA mice.
*Comparison of the percentage of CD3$^+$ cells that are wild-type (Thy1.1$^+$) or CXCR3$^{-/-}$ (Thy1.2$^+$) OT-I recruited into various tissues of CC10-OVA mice 4 days after transfer (n=4). * = $p \leq 0.05$*

The early expression of IP-10 following transplantation may be a significant factor in the development of ischemia injury of the airways. Our preliminary experiments suggest that ischemia-reperfusion may mediate the expression of IP-10 either through an NF-κβ dependent mechanism.

Our study of human transplant recipients has identified several chemokine receptors that seem to correlate with the development of BO. The pattern of receptor expression is consistent with a Th2-type inflammatory response, which has been associated with fibroproliferation. These data suggest that a switch to Th2-like inflammation may promote the scarring seen in BO.

Plans

There are no significant modifications to the plans proposed. Aim 1: We have completed the proposed studies and are preparing a manuscript. Aim 2: We will continue to characterize the CC10-OVA mouse in terms of the inflammation that is induced with adoptive transfer of various lymphocyte subsets. We have also begun to breed the CC10-OVA mouse with IP-10 KO mice. Further experiments with the CXCR3$^{-/-}$ OT-I T cell transgenic mice are also being planned. This should allow a detailed assessment of these chemokines and their receptor in a whole lung model of acute rejection. Aim 3: We are working to develop a predictive model of BO based on the chemokine receptor profile of lymphocytes isolated from lung transplant patients. We will then study normal patients who develop the characteristic pattern of chemokine receptor expression to see if these patients are more likely to develop BO.

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Prof. Subramaniam Malarkannan, Principal Investigator
Dr. Jeyarani Regunathan, Research Associate

Medical College of Wisconsin, Milwaukee, USA

Role of Minor Histocompatibility Antigens in Solid Organ Transplant Rejection

My laboratory is interested in studying minor histocompatibility antigens (mH-Ag). Minor histocompatibility antigens (mH-Ags) 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[1]. Recent studies, which include studies 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[2].

To better understand the clinical applications, we have recently identified and characterized many cDNAs that encode murine mH-Ags[1-3]. Recently, with the financial support of ROTRF, we defined the molecular identity of murine mH-Ags, H4[1] and H7[4]. Both these mH-Ag peptides differ from their allelic counter parts by one amino acid variation. Excitingly, our recent study reveals that one of the allelic peptides from H4 mH-Ag is degraded and failed to be presented as an antigenic epitope[5]. The H4 mH-Ag provides us with a novel model to study specific aspects of proteasomal degradation of antigenic peptides.

Another mH-Ag that we have identified through our earlier studies is H60[2]. This antigen is one of the classical examples of a full-length mH protein (Fig. 1) with interesting immunological functions[6]. H60 protein belongs to the non-classical MHC class-I family, which contains α1 and α2 domains and lacks the 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[2-3]. 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 the 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.

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

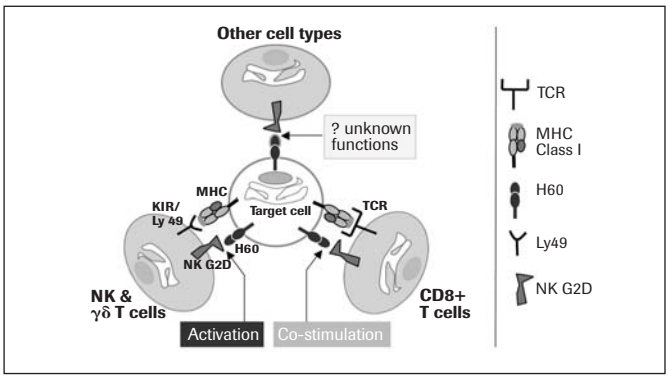

Figure 1. H60 protein, a non-classical MHC class I family member.

graft-versus-host disease[7-9]. Recently we addressed a series of questions towards formulating NK cell-based therapeutic approaches. 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[10]. 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 regulated. In our next set of experiments we extended our studies to define specific signaling events that are involved in implementing the 'altered-balance' phenomenon. One of the important findings we made was that the PLC-γ2 is the major positive signal transducer in the NK cells. Further, we show that the presence of functional PLC-γ2 is critical for the development and terminal maturation of NK cells[11]. Our current studies extend our focus on characterizing, and understanding the immunobiology of mH-Ags.

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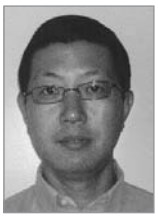
Dr. Yuan Zhai, Principal Investigator
Dr. Jerzy Kupiec-Weglinski, Collaborator
Dr. Genhong Cheng, Collaborator
Dr. Xiuda Shen, Research Associate
Dr. Bo Qiao, Research Associate



University of California, Los Angeles, USA

The Toll-Like-Receptor 4 and Liver Ischemia Reperfusion Injury

Introduction

In this year's progress report, we addressed the question of whether lipopolysaccharides (LPS) were involved in the initiation of hepatic ischemia reperfusion injury (IRI). Firstly, as proposed in the grant application, we have provided evidence *in vivo* that the development of liver IRI was not inhibited by LPS neutralization with an endotoxin inhibitor, recombinant bactericidal/permeability-increasing protein (rBPI21), administered prior to the onset of IR. Secondly, we demonstrated that LPS-independent, heat-sensitive materials were present in liver perfusates and able to activate macrophages via TLR4 by using macrophages from TLR4 or TLR2 KO mice stimulated with liver perfusates generated *ex vivo*. These data, hopefully, will convince that further study to biochemically identify TLR4 endogenous ligands generated during hepatic IRI is appropriate, and that dissecting the specific signaling pathway and effect mechanism downstream of TLR4 activation leading to liver damage will be important for us to better understand the pathophysiology of hepatic IRI which may help in identifying novel therapeutic targets for clinical application.

Materials and methods

Animals. Male wild-type (WT) C57BL/6 mice (8-12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the UCLA animal facility under specific pathogen-free conditions, and received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985).

Mouse warm hepatic IRI model. We have developed a warm hepatic IRI model in mice, as described[1,2]. Sham WT controls underwent the same procedure, but without vascular occlusion. For endotoxin neutralization experiments, rBPI21 was administered at 5 mg/kg (in PBS), i.v. either one dose at 1 hour prior to the operation or two doses at 1 hour prior to and 2 hours after the operation. For LPS-induced liver inflammation, rBPI21 (5 mg/kg, i.v.) was administered 1 hour prior to LPS injection (200 ng in PBS, i.v.), and livers and spleens were harvested at 3 hours post LPS injection. Control mice were injected with PBS.

Model of *ex vivo* liver reperfusion (Rat). We will use an isolated perfusion rat liver apparatus, which has been developed and successfully used in our laboratory[3-5].

Mouse macrophage cultures. Murine bone marrow macrophages (BMM) were differentiated from marrow from 6-10-week old C57B/[6] mice as described previously[6]. LPS or liver perfusate stimulation and rBPI inhibition experiments were performed in a 12-well plate seeded with the cells at $8x10^5$/well 24 hours prior to the start of the experiment. LPS was added to the culture medium at 10 ng/ml, and cells were incubated with LPS for 4 hours before being harvested for RNA preparations. To test the effect of rBPI *in vitro*, rBPI was added to cell cultures (5 μg/ml) 30 minutes prior to the addition of LPS. No obvious cytotoxic effects were observed in cell cultures with either LPS (1–1000 ng/ml), rBPI (5-100 μg/ml), or both at the concentrations used in our experiments. Liver perfusates were diluted with culture medium at 1:20. Heat-inactived perfusates were prepared by incubating diluted perfusates in a boiling water bath for 5 min. After a brief spin, supernatants were further diluted prior to use in macrophage stimulation experiments.

Quantitative RT-PCR. Five μg of RNA was reverse-transcribed into cDNA using oligo (dT) primers with OmniscriptTM reverse transcriptase (Qiagen, Valencia, CA). Quantitative PCR was performed. The primers used to amplify a specific 100-200 bp fragment of mouse IP-10, TNF-α and HPRT transcripts are the following:

IP-10 s-GCTGCCGTCATTTTCTGC, as-TCTCACTGGCCCGTCATC
TNF-α s-GTAGCCCACGTCGTAGCAA, as-TTGAGATCCATGCCGTTG
HPRT s-TCAACGGGGGACATAAAAGT, as-TGCATTGTTTTACCAGTGTCAA

Statistical analysis. All values are expressed as mean±SD. Data were analyzed with an unpaired t test with Welch's correction. $p < 0.05$ was considered to be statistically significant.

Please refer to Dr. Zhai for detailed methods.

Results

The rBPI effectively inhibited LPS-induced inflammation *in vitro* and *in vivo.*

The efficacy of rBPI in blocking LPS activity was first tested *in vitro* in a mouse macrophage cell line. As shown in Fig.1A, 10 ng/ml LPS induced TNF-α expression in the cells by more than 30 fold, as measured by quantitative RT-PCR. The addition of rBPI (5 μg/ml) into cell culture media 30 minutes prior to the addition of LPS effectively inhibited the LPS activity. The induction of TNF-α gene expression was suppressed by more than 90%. The rBPI could even block existing LPS activity added 30 minutes after cells were incubated with LPS (Fig.1A). To further evaluate the efficacy of rBPI in blocking LPS activity, increasing doses of LPS were

added to the cell cultures with a fixed concentration of rBPI at 5 μg/ml. The induction of TNF-α remained suppressed by 50% when LPS concentration increased to 100 ng/ml (Fig.1B).

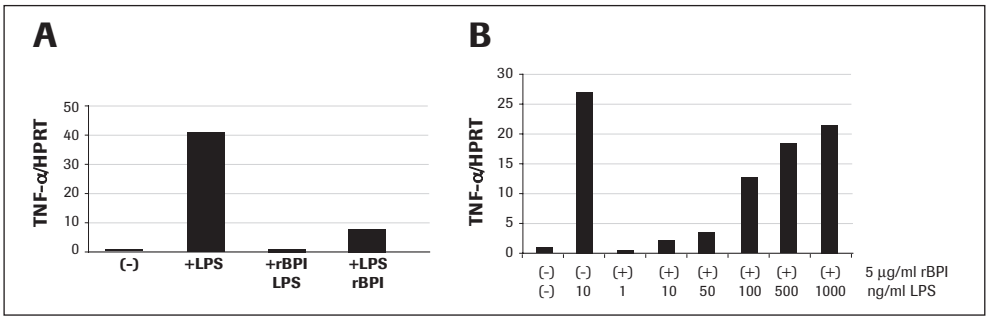


Figure 1. Efficacy of rBPI in blocking LPS-induced inflammation in vitro.

To test the *in vivo* efficacy of rBPI in blocking LPS activity, we utilized a model of LPS-induced intrahepatic inflammation. Intraperitoneal injection of 40 ng of LPS (0.2 ml of 200 ng/ml in PBS) in B6 mice induced a rapid inflammation (3 hours post-injection) in livers as measured by intrahepatic IP-10 and TNF-α gene upregulation. No obvious liver pathology was observed with this amount of LPS. Administration of rBPI at 5 mg/kg i.v. 30 minutes prior to the LPS injection suppressed both gene induction by approximately 50% (Fig. 2A). Thus, *in vivo* inhibition of LPS-induced inflammatory gene expression by rBPI was not as complete as it was in *in vitro* cell cultures. However, significant suppressive effect was detectable.

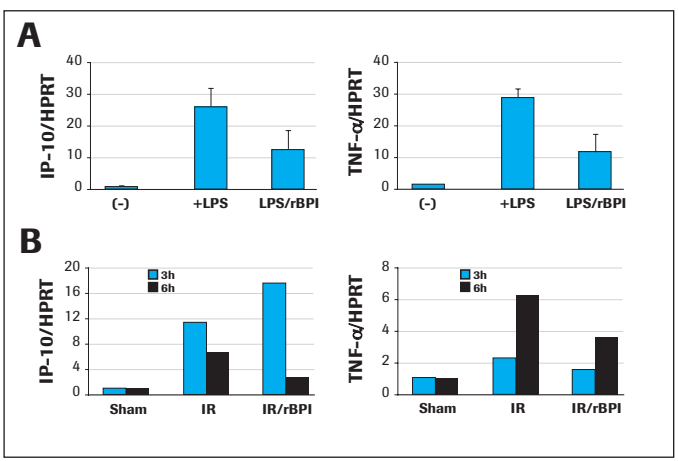


Figure 2. Efficacy of rBPI in blocking LPS-induced inflammation in vivo.

The rBPI failed to block the development of liver warm IRI.

To test the potential involvement of LPS in the triggering of hepatic IRI, rBPI was administrated i.v. 30 minutes prior to the start of ischemia, and a second dose was given after one hour of reperfusion in one group of animals to ensure its biological activities *in vivo* (*in vivo* half life= 6 hours). Livers were harvested at either 3 or 6 hours of reperfusion for gene analysis. This is a well established warm hepatic IRI model, in which liver damage developed after intrahepatic inflammation. Gross liver pathology and increases in serum liver functional enzyme (sALT) was observed after 6 hours, but not 3 hours of reperfusion. However, inflammation was detected early at 3 hours and persisted onto 6 hours. sALT levels from either one or two doses ($p = 0.06$) of BPI treated liver IRI mice were not significantly different from untreated liver IRI ones, and were all significantly higher than those of sham controls (Fig. 3). Liver pathology from H/E stained sections of IRI lobes confirmed similar hepatocellular damages in all three groups (data not shown). To further examine the effect of BPI in liver IRI, intrahepatic inflammation induced by IR was evaluated in the presence BPI treatment. As we determined previously, intrahepatic inductions of IP-10 and TNF-α by liver IR were correlated with the development of liver damages, we analyzed IRI livers from both 3 and 6 hours of reperfusion. Sham operated livers were used as controls and their IP-10/HPRT or TNF-α/HPRT ratios were calibrated as the baseline. The folds of IP-10 and TNF-α increase in IRI livers were plotted in figure 2B. Clearly, BPI treatment failed to suppress IP-10 or TNF-α induction early (3 hours) after reperfusion, but did so later at 6 hours. This is consistent with our hypothesis that LPS is not responsible for triggering intrahepatic inflammation, but may contribute to sustain the response, as a consequence of liver damages.

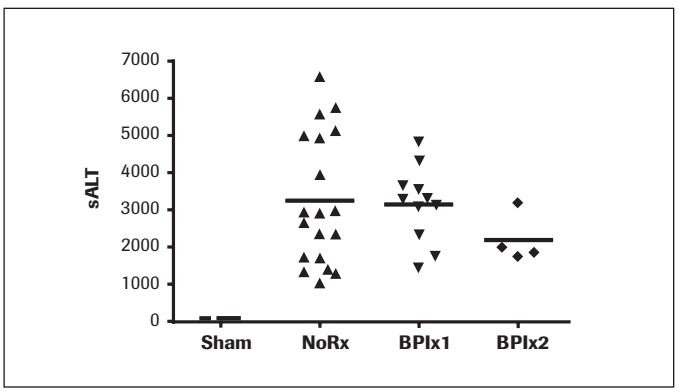


Figure 3. Failure of rBPI to block the development of liver warm IRI.

The presence of LPS-independent, heat-sensitive materials in liver perfusates able to activate macrophages via TLR4

To further establish the presence of endogenous TLR4 ligands during liver IRI, we tested liver perfusates (LP) directly *in vitro* of their ability to activate macrophage cells. Liver perfusates

were generated from *ex vivo* perfusion of an isolated LEW rat liver after no or 24 hours of cold ischemia in UW solution. As shown in figure 4A, liver perfusates were very potent in activating macrophage cells; induction of TNF-α gene expression remained significant with a dilution rate of 1:1500. Heat-inactivation of liver perfusates (1:200 dilution) eliminated their ability to activate macrophages significantly (Fig. 4B). The inclusion of rBPI in macrophage cultures did not have any additional effects in further eliminating liver perfusate activities (Fig. 4B).

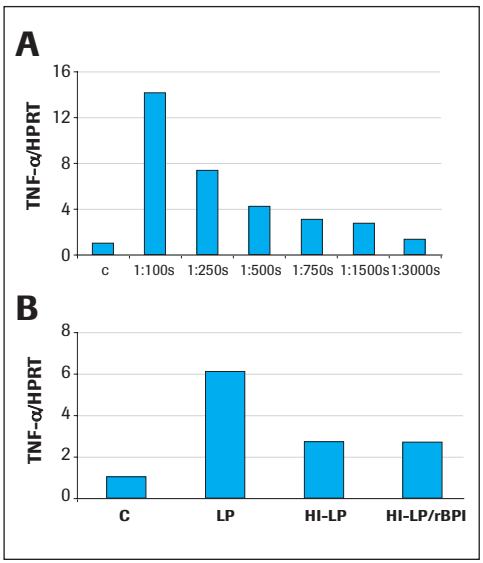


Figure 4. Activation of macrophage cells by liver perfusates.

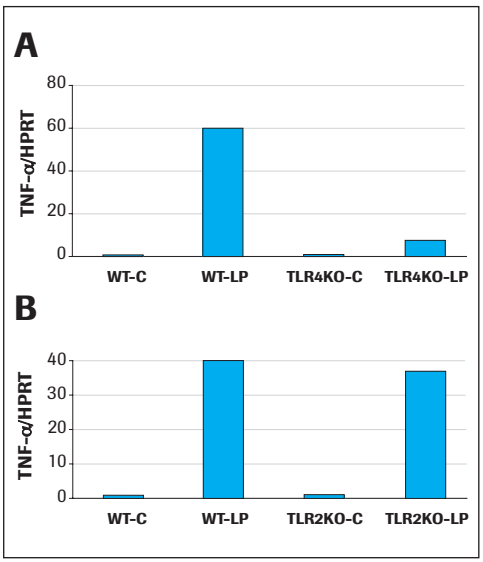


Figure 5. Receptors mediating liver perfusate activities.

To determine receptors mediating liver perfusate activities, we utilized primary macrophages from either WT, or TLR4 KO or TLR2 KO mice. Liver perfusates activated WT primary macrophages with TNF-α induction rates much higher than those with macrophage lines. Clearly, liver perfusates failed to activate TLR4 KO macrophages at similar degrees (59 fold in WT, vs. 7.8 fold in KO) (Fig. 5 A). In contrary, TLR2 KO macrophages were the same as their WT controls in their TNF-α induction by liver perfusates (40 fold in WT, vs. 36 fold in KO) (Fig. 5 B). Thus, endogenous TLR4 ligands, which are heat-sensitive and independent of LPS, are present in liver perfusates and able to activate macrophages via TLR4.

Publications

1. Shen XD, Ke B, Zhai Y, et al. CD154-CD40 T-cell costimulation pathway is required in the mechanism of hepatic ischemia/reperfusion injury, and its blockade facilitates and depends on heme oxygenase-1-mediated cytoprotection. *Transplantation* 2002; 74:315.
2. Shen XD, Ke B, Zhai Y, et al. Stat4 and Stat6 signaling in hepatic ischemia/reperfusion injury in mice: HO-1 dependence of Stat4 disruption-mediated cytoprotection. *Hepatology* 2003; 37:296.
3. Amersi F, Buelow R, Kato H, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest* 1999; 104:1631.
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5. Amersi F, Shen XD, Anselmo D, et al. *Ex vivo* exposure to carbon monoxide prevents hepatic ischemia/reperfusion injury through p38 MAP kinase pathway. *Hepatology* 2002; 35:815.
6. Chin AI, Dempsey PW, Bruhn K, et al. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* 2002; 416:190.

5. Final Reports of ROTRF Grantees

Dr. Tausif Alam, Principal Investigator
Dr. Michael MacDonald, Consultant
Dr. James Malter, Consultant
Dr. John Young, Consultant

University of Wisconsin Medical School, Madison, USA

Glucose-Regulated Improved Insulin Production from Hepatocytes

Towards the goal of correcting diabetic-hyperglycemia by insulin gene-therapy, we generated insulin gene-constructs and tested them *ex vivo* and *in vivo*. The insulin construct SATEM (details in figure legend) in adenovirus yielded a substantial amount of insulin from isolated hepatocytes in a glucose-dependent manner and corrected hyperglycemia *in vivo* after a 9-12 hour fast among streptozotocin (STZ)-induced diabetic rats (previous progress report to ROTRF).

Because of increasing concerns over inherent safety risks of the initially proposed use of viral-vectors, we investigated alternatives that circumvent their use. Thus, the gene constructs SATEM and SAM were cloned in a plasmid containing the minimum necessary bacterial sequences and delivered *in vivo* by the hydrodynamics-based method. The results of naked plasmid injection-based insulin gene-therapy in STZ-diabetic mice compared favorably with our previous results using adenovirus in diabetic rats; SATEM performed significantly better than SAM in correcting diabetic-hyperglycemia and unlike the adenovirus treatment, the glycemic correction lasted longer (Fig. 1). Presence of human insulin mRNA in liver by RT-PCR, presence of human insulin and virtual absence of mouse C-peptide in serum of STZ-diabetic mice by RIA confirmed that glycemic correction was caused by insulin plasmid injections.

A new series of insulin gene-constructs containing modifications involving an enhancer and an intron in 5'-UTR and a new 3'-UTR have recently been generated. Preliminary *ex vivo* comparative results show that one new construct is capable of producing more than a 2-fold larger amount of insulin than SATEM and is, therefore, anticipated to correct postprandial hyperglycemia more effectively.

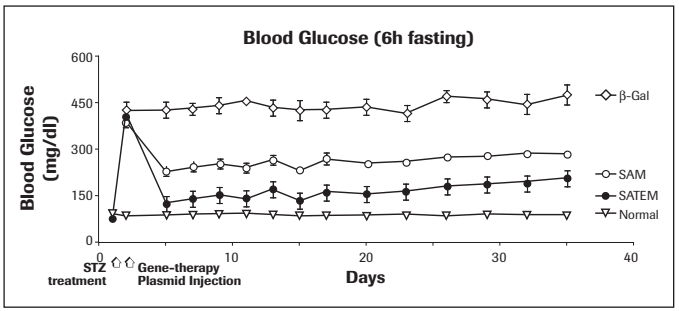

Figure 1. Reduction in hyperglycemia caused by intravenous injections of insulin gene construct containing plasmids in diabetic mice. *Normal mice (20-24 g) were treated with 200 mg/kg STZ to induce diabetes. Each group consisted of 6 STZ-diabetic mice; each mouse was injected with an equal amount of plasmid DNA containing SAM* or SATEM**, as indicated, in Ringer Lactate equivalent to 10% of the body weight. The control group shown was injected with an equal amount of an unrelated reporter plasmid containing ß-galactosidase (β-Gal). Blood glucose levels of all groups of experimental mice and a group of normal healthy controls, were recorded at three time points: fed ad libitum, after a 3-hour fast, and after a 6-hour fast. The blood glucose levels of β-Gal control group were statistically indistinguishable from the untreated diabetic group and the diabetic group injected with an equal amount of only Ringer Lactate. Diabetic mice treated with SATEM consistently showed lower blood glucose levels than mice treated with SAM; the difference was statistically significant after a 6-hour fast. During the period of study, diabetic mice in three control groups appeared unhealthy and progressively lost weight, as expected. Although the insulin gene-treated diabetic mice did not gain significant weight, they were either able to maintain their body weight or lost significantly less weight than untreated diabetic mice and generally appeared more active and in better health. Diabetic mice treated with SATEM lost less weight than mice treated with SAM but the difference in weight between the two groups of mice was not statistically significant. [*The insulin construct SAM contained 3 units of "glucose-inducible regulatory elements" (GIREs) derived from the promoter of S14, the albumin promoter, and the human insulin cDNA modified for compatibility for processing by furin. ** In addition to the components of SAM, the construct SATEM also included a translational enhancer (TE) derived from VEGF.]*

Publications

1. Alam T, Sollinger HW. Glucose-regulated insulin production in hepatocytes. *Transplantation* 2002; 74:1781.
2. Nett PC, Sollinger HW, Alam T. Hepatic insulin gene therapy in insulin-dependent diabetes mellitus. *Am J Transplant* 2003; 3:1197.
3. Ludwig S, Sollinger HW, Alam T. Can gene therapy make pancreas and islet transplantation obsolete? *Curr Opin Org Transplant; (In press)*.
4. Nett PC, Sollinger HW, Alam T. Systemic plasmid DNA gene transfer into diabetic mice causes glucose-dependent hepatic insulin expression; *(In preparation)*.
5. Alam T. Reduction in diabetic hyperglycemia by glucose-regulated insulin release from transduced hepatocytes. *Fifth International Conference on New Trends in Immunosuppression* 2002; *(Abstract)*.
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8. Alam T, Nett PC, Ludwig S, Sollinger HW. Systemic plasmid DNA gene transfer into diabetic mice causes glucose-dependent hepatic insulin expression and reduces hyperglycemia. Oral presentation, *Am J Transpl* 2004; 8:293; *(Abstract)*.
9. Nett PC, Sollinger HW, Alam T. Translational enhancement improves glucose-dependent hepatic insulin expression and reduces hyperglycemia in STZ-induced diabetic rats. Poster presentation, *Am J Transpl* 2004; 8:466; *(Abstract)*.
10. Alam T, Nett PC, Ludwig S, Sollinger HW. Systemic plasmid DNA gene transfer into diabetic mice causes glucose-dependent hepatic insulin expression. *Transplantation* 2004; 78;109; *(Abstract)*.

Dr. Daniel R. Goldstein, Principal Investigator
Dr. Ruslan Medzhitov, Collaborator

Yale University, New Haven, USA

Role of Toll-Like Receptor Signaling in Neonatal Transplantation Tolerance

Introduction

This 1-year award has provided the opportunity to generate preliminary data to clarify the role of the innate immune system in neonatal transplantation tolerance. Neonates manifest immunological defects characterized by susceptibility to infection, inability to respond to vaccination, and skewing towards Th2 vs. Th1 T cell responses. Conversely, the neonate appears to have a privileged response for transplantation tolerance: in a murine skin transplantation model, BALB/C mice injected with CAF1/J spleen cells as neonates are subsequently able to tolerate CAF1/J skin grafts as adults. The basis of this altered immune function in the neonate, and especially the contribution of the innate immune system, has not been fully characterized.

In our proposal, we hypothesized that neonates manifest impaired TLR-dependent innate immune responses. TLR signaling is critical, under certain conditions, in inducing DC (dendritic cell) maturation, and immature DCs have been shown to possess tolerogenic stimuli. Therefore, we proposed that defective innate immune function contributes to transplantation tolerance in the neonate. Our goal for this project was to examine whether neonatal DCs manifest impaired TLR dependent immunity.

Results

Composition of the neonatal vs. adult spleen

As a preliminary investigation, we assessed the cell contents of neonatal and adult spleens. We found that the neonatal spleen contains a high proportion of FSC^{hi} SSC^{hi} cells, resembling a monocyte profile, in contrast to their adult counterparts (data not shown). These cells are predominantly $CD11b^{+}$, consistent with a monocyte phenotype. In keeping with this observation, $CD11b^{+}$ cells are more prevalent in the neonatal vs. adult spleen, and their proportion gradually decreases as the neonate ages, from day 5 to day 18. $CD11c^{+}$ DCs are less prevalent in the neonatal vs. adult spleen, and the proportion of $CD11c^{hi}$ cells is markedly lower.

The proportion of $B220^+$ cells (containing B and T cells) increases slightly with age, but nevertheless, this represents the most common cell type in the neonatal spleen (32% at day 5 vs. 64% in the adult). The predominant cell type within the $B220^+$ fraction is the B cell; $B220^+$ $CD19^+$ cells increase from 24% in the day-2 neonate to 46% in the adult. There is a much lower percentage of $CD4^+$ and $CD8^+$ T cells in the neonatal vs. adult spleen and the proportion of $CD4^+$ $CD25^+$ cells (regulatory T cells) within the $CD4^+$ population increases slightly with age (data not shown).

In summary, there are substantial changes in the cell content of the spleen during development. These differences in the cell content of neonatal vs. adult spleens might have implications for immune function. For example, as DCs are the most potent cell in the TLR-dependent innate immune response, their lower frequency in the neonate might lead to a reduced innate immune response. In a similar fashion, the lower numbers of $CD4^+$ and $CD8^+$ T cells might lead to a reduced adaptive immune response.

Proinflammatory cytokine secretion

The production of proinflammatory cytokines (IL-6 and TNFα) is an important component of TLR-dependent innate immunity. Initially, we examined inflammatory cytokine production by the entire antigen presenting-cell (APC) population (comprised of DCs, B cells and monocytes) of the neonatal and adult spleen, in response to a range of TLR agonists.

We isolated splenic APCs by depleting $CD90.2^+$ T cells via magnetic separation from BALB/C neonatal mice of various ages (5–18 days) and BALB/C adult mice (aged 6–8 weeks) and measured the proinflammatory responses (IL-6 and TNFα via ELISPOT) after overnight stimulation with specific TLR ligands (data not shown).

Contrary to our hypothesis, we found that neonatal APCs produced an enhanced number of IL-6 and TNFα secreting cells compared to their adult counterparts in response to LPS (an agonist for TLR4), peptidoglycan (TLR2), poly I:C (TLR3), CpG (TLR9), RN40 (TLR7) and flagellin (TLR5; with the possible exception of flagellin, TNFα). This response declined as the neonates aged. This occurred despite the lower prevalence of DCs (the most potent APC) in the neonate. Similar results were found when the B6 strain was used (data not shown).

Next, we tested the innate immune function of specific cell types, to investigate their responses on a cell-by-cell basis. We isolated splenic $CD11c^+$ DCs via magnetic separation from neonatal (aged 4 days) and adult (aged 6–8 weeks) BALB/C mice and once again measured the proinflammatory responses after overnight stimulation with specific TLR ligands (Fig. 1). With every agonist tested, neonatal DCs produced an enhanced number of cells secreting IL-6 and TNFα. These data demonstrate that neonatal DCs generate an enhanced proinflammatory cytokine secretion response to TLR agonists vs. their adult counterparts. This indicates that

an intrinsic impairment in the ability of neonatal DCs to undergo maturation is unlikely to explain the permissive tolerant state in the neonate.

We found similar results in B cells (purified via magnetic separation) and macrophages (isolated by a density centrifugation method; data not shown). Thus, the enhanced production of proinflammatory cytokines TNFα and IL-6 in response to TLR agonists seems to be conserved amongst the various innate APC populations.

IL-12 production

One of the defects reported in the neonatal immune system is a skewing towards a Th2 phenotype. It has been demonstrated that the bias towards a Th1 or Th2 $CD4^+$ T cell response is controlled via cytokine production by innate immune cells such as DCs, in response to TLR agonists. IL-12 is secreted by DCs and binds to IL-12 receptor on $CD4^+$ T cells; this represents the major pathway leading to Th1 commitment. Therefore, we sought to determine whether IL-12 production in response to TLR agonists is compromised in neonatal DCs, as this could lead to Th2 skewing of the neonatal response, and perhaps affect transplantation tolerance.

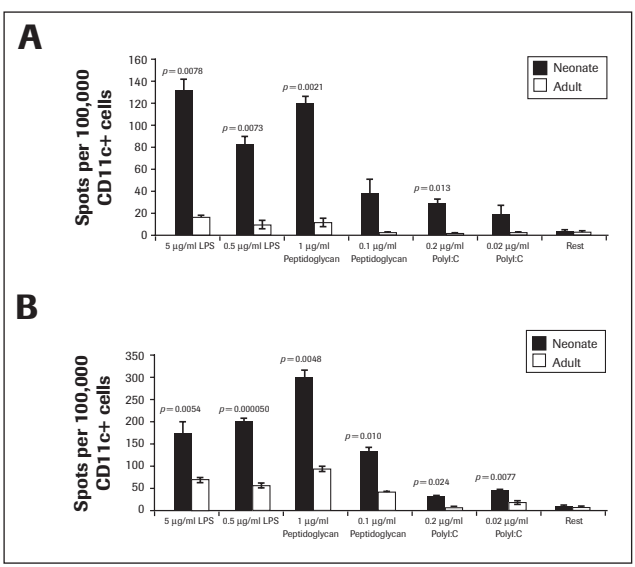

Figure 1. Neonatal DCs show enhanced A) IL-6 and B) TNFα secretion in response to TLR agonists vs. adult counterparts. *P values are shown where a 2-tailed T-test determined that the difference between neonate and adult was statistically significant. Graph shows mean +/- standard error.*

Towards this end, we purified CD11c+ DCs from neonatal (day 5) and adult (6-8 weeks) BALB/C mice as previously described, and cultured them for 24 hours with or without LPS. Subsequently we harvested the media and assessed the IL-12p40 content by ELISA (Fig. 2). We found that neonatal DCs produce a higher amount of IL-12 vs. their adult counterparts. Therefore, reduced IL-12 production by neonatal DCs is unlikely to explain the Th2 bias observed in the neonate.

Costimulatory molecule upregulation

Upregulation of costimulatory molecules is another important component of TLR-dependent innate immunity. Therefore, we assessed the ability of neonatal splenic CD11c$^+$ DCs to upregulate costimulatory molecules in response to TLR activation. Splenic CD11c$^+$ cells were isolated from neonatal and adult mice via magnetic separation. The cells were then cultured overnight in the presence or absence of LPS, harvested the next day and then stained with CD11c and CD40 monoclonal antibodies and analyzed by flow cytometry to assess the upregulation of costimulatory molecule CD40 on DCs (data not shown).

The results demonstrate that the upregulation of costimulatory molecules in response to LPS is enhanced in purified splenic neonatal DCs, compared to their adult counterparts (data not shown). Similar results were found with CD86 (data not shown). Interestingly, when whole spleen cells from neonatal or adult mice were cultured with TLR ligands, we noted an impaired ability of neonatal CD11c$^+$ cells to upregulate costimulatory molecules, and lower background levels in unstimulated cells (data not shown). The above two sets of data imply that there is a population within the neonatal spleens that inhibits the upregulation of costimulatory molecules.

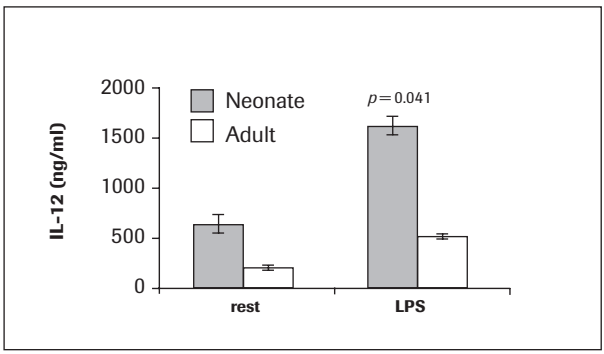

Figure 2. IL-12 production by CD11c$^+$ DCs. *P value is shown where a 2-tailed T-test determined that the difference between neonate and adult was statistically significant. Graph shows mean +/- standard error.*

Summary

In conclusion, contrary to our hypothesis, our data indicate that the neonate manifests enhanced TLR-dependent immunity. Our data also suggest that there may be a subpopulation within the neonatal spleen that possesses immunoregulatory properties. Our future goals are to investigate the nature of this subpopulation and whether it is important in neonatal transplantation tolerance.

Dr. Holger Hackstein, Principal Investigator
Bs. Christoph Steinschulte, Research Associate
Dr. Anette Bohnert, Research Associate
Dr. Thomas Stadlbauer, Collaborator
Prof. Angus Thomson, Consultant

Justus Liebig University Giessen, Giessen, Germany

Impact of Sanglifehrin A, A Novel Immunosuppressant on Dendritic Cell Function in Solid Organ Transplantation

ROTRF has funded our research project entitled: *"Impact of sanglifehrin A (SFA), a novel immunosuppressant on dendritic cell (DC) function in solid organ transplantation"* for a 1-year period. Within this relative short time period we have made remarkable progress and have generated a significant amount of novel data shedding light on the immunosuppressive action of SFA. Currently, we are in the process of preparing a second publication on the *in vivo* impact of SFA on DCs. Within the 1-year funding period we have made considerable progress on the following specific aims of our proposal:

1. To characterize the effects of SFA on DC function and maturation in different *in vivo* models

2. To investigate the effect of SFA-exposed DCs and parenteral SFA on immune responses and organ allograft survival

1. With respect to the first aim we have analysed the capacity of SFA to impact on proinflammatory (IL-12p70, TNFα) and immunomodulatory (IL-10) cytokine production and endocytosis of FLT-3 ligand (Flt3L)-expanded murine DCs *in vivo.*

Methods: Mice (C57BL/10, $H2K^b$) were injected intraperitoneally (*i.p.*) for 3 to 10 days with SFA (10 mg/kg/d) or drug vehicle to study drug effects under steady-state conditions. Additionally, to explore the effects of SFA specifically on DCs under dynamic conditions, we injected Flt3L (10 μg/d) and SFA (10 mg/kg/d). Subsequently, animals were stimulated with CpG DNA or LPS and IL-4 or injected with Fitc-Dextran or Fitc-Albumin *i.p.* for *in vivo* endocytosis. Four hours later, peripheral blood was drawn and splenic and bone marrow DC subsets were analysed by flow cytometry.

Results: Under dynamic conditions, a 10-day course of SFA blocked 95% of LPS/IL-4 induced IL-12p70 and 98% of CPG-induced IL-12p70 production of Flt3L-expanded DC *in vivo.* These effects are not due to suppressive effects of SFA on total DC numbers or DC subsets, as indicated by four colour flow cytometry. The production of TNF-α and IL-10 were only

moderately affected by SFA. Our *in vivo* endocytosis experiments show a highly significant decrease of the endocytotic activity of Flt3L-expanded DCs from 147.4 (MFI± 20.8) in control animals compared to 35.3 (MFI± 6.1) in animals treated with SFA. For receptor-mediated endocytosis we found a lower decrease (MFI) of about two times from 25.64 (SD± 7.5) in control animals compared to 12.84 (SD± 0.76) for animals treated with SFA.

2. With respect to the second aim, we have performed rat heart transplantation experiments (Wistar > Lewis) to analyse the impact of SFA injections on solid organ transplant survival.

The results indicate that SFA alone has no significant immunosuppressive action but in combination with low dose cyclosporine A is strongly synergistic (Fig. 1). Whereas low dose cyclosporine A prolonged mean heart allograft survival only to 23 days, the combination of low dose cyclosporine A with SFA prolonged mean heart allograft to 82 days ($p = 0.0007$; Fig. 1).

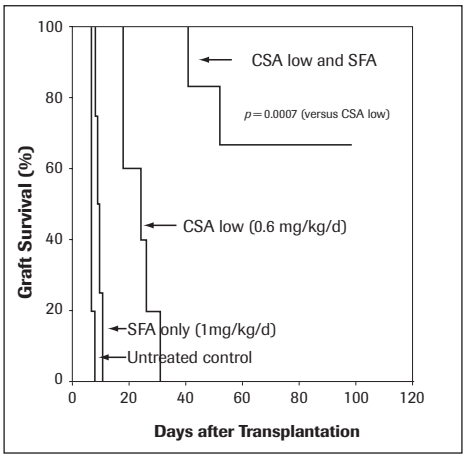

Figure 1. SFA and rat heart allograft survival.

Taken together, our *in vivo* data suggests that SFA represents functionally a novel class of immunophilin-binding immunosuppressants with a high selectivity and potency to abrogate production of the major proinflammatory and Th1-skewing cytokine IL-12p70 and a strong impairment on antigen uptake by DCs. SFA in combination with Flt3L has the potential to expand putative immunoregulatory DCs *in vivo*. Moreover, the organ transplant data indicate that SFA alone has no potent immunosuppressive effects but SFA in combination with cyclosporine A acts highly synergistic to prolong allograft survival.

We think that these results are clinically relevant and suggest potential for SFA to be used as a novel agent that potentiates the immunosuppressive effects of cyclosporine A. Based on these novel findings we have initiated further experiments on the molecular mechanism of action of SFA and have started additional transplant experiments in other models (rat kidney transplantation).

Dr. Sheri M. Krams, Principal Investigator
Dr. Hideaki Obara, Research Associate
Dr. Christine Hsieh, Research Associate

Stanford University, Stanford, USA

NK Cells in Transplantation

The original specific aims of this grant were: 1) analyze the role of NK cells post-transplant, 2) determine the expression of NK cell activation receptors after transplant, and 3) determine if signaling through NK cell receptors induces cytokine production and cytotoxicity.

We made important progress and have made significant findings in each of these aims. We have published one manuscript[1] and we anticipate completing the studies for two additional manuscripts.

Recipient-derived NK cells infiltrate allogeneic liver grafts.

In previous studies we determined that $CD8^+$ T cell depletion does not prolong the survival of liver allografts in a high-responder OLTx. We did, however, note a marked infiltration of NKR-$P1^+$ NK cells in these liver allografts. To expand upon this observation, NK cell infiltration was examined in a model where fully allogeneic donor DA ($RT1^a$) livers were transplanted into Lewis ($RT1^l$) recipients, and liver-infiltrating mononuclear cells (LIMC) were isolated according to our previously published procedures. LIMC were labeled with mAbs against NKR-P1, αβTCR, and $RT1A^{a,b}$ for analysis by two-color flow cytometry. Twenty-four hours after transplantation, 18.6 ± 3.0% ($n=3$) of the LIMC isolated from allografts were $\alpha\beta TCR^+$ T cells, while 56.4 ± 6.2% ($n=3$) were $\alpha\beta TCR^-$ NKR-$P1^+$ NK cells. To determine if the NK cells were of donor or recipient origin, LIMC were labeled with FITC-anti-$RT1A^{a,b}$ mAb-specific for donor MHC class-I ($RT1^a$) in combination with PE-anti-NKR-P1 mAb. At 24 hours post-transplant, 22.9 ± 6.1% ($n=3$) of the LIMC were NK cells of recipient origin ($RT1A^{a,b,-}$). We included only the NKR-$P1^{high}$-positive subsets to eliminate NKT cell and monocyte populations, since hepatic NKT cells and activated monocytes exist mainly in NKR-$P1^{dim}$-positive subsets in the rat.

Recipient-derived NK cells infiltrate liver grafts in the first few hours post-transplant.

As previously discussed, $\alpha\beta TCR^-$ NKR-$P1^+$ NK cells are a major component of the infiltrating cells in liver allografts. To evaluate the kinetics of NK cell infiltration after transplantation, LIMC isolated from allografts were analyzed for the proportion of NK cells at eight time points post-transplant. The proportion of NK in LIMC cells peaked at 12-24 hours post-transplant (56.4 ± 6.2%; $n=3$) and decreased to 35.6 ± 3.0% ($n=3$) by day 2 as the proportion of T cells increased in the allograft. NK cells of recipient origin (NKR-$P1^+$ $RT1A^{a,b-}$), infiltrated the grafts

very early post-transplant constituting over half of the NK cells at 6 hours post-transplant (Fig. 1A). The proportion of recipient-derived NK cells in allografts peaked at 12 hours post-transplant, decreased by day 3, then increased again at day 5 and comprised 29.5 ± 7.0% of the total number of infiltrating cells at day 7 post-transplant (Fig. 1B). Conversely, in syngeneic liver grafts the overall proportion of NK cells and T cells were 32.2 ± 2.1% and 31.2 ± 4.0 % respectively, prior to transplant. NK cells peaked at 12 hours post-transplant (NK cells, 49.7 ± 3.3% and T cells 19.9 ± 5.4%) and then decreased to pre-transplant levels by day 3 post-transplant (NK cells, 30.6 ± 3.3% and T cells 32.4 ± 3.4%) and remained constant (NK cells, 34.0 ± 4.3% and T cells 30.4 ± 4.4%). In syngeneic grafts it is not possible to separately quantitate the numbers of host and infiltrating NK and T cells thus these numbers reflect the total cell pool.

These data suggest that there is a bimodal infiltration of NK cells into liver allografts, early as a result of non-specific surgical stress, including ischemia/reperfusion injury and later at the time that effector cells are infiltrating the allograft and mediating rejection.

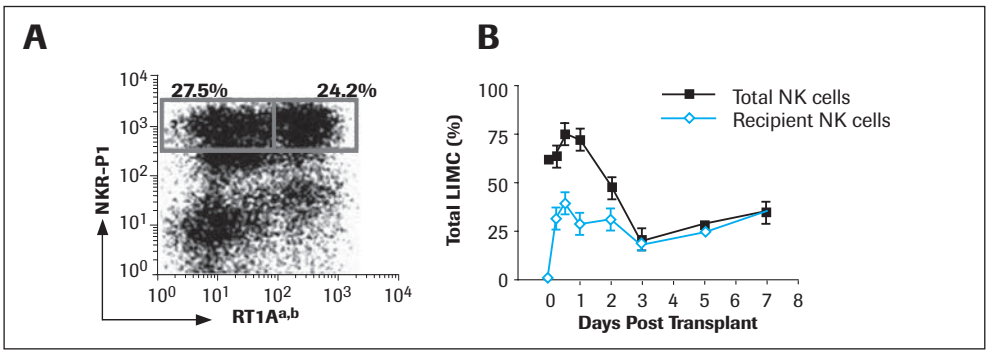

Figure 1. Host-derived NK cell infiltrate liver allografts by 6 h post-transplantation. A) *Representative flow cytometric analysis of LIMC from an allograft at 6 hours post-transplant. Over half of the NK cells are recipient-derived NK cells (RT1A$^{a,b-}$ NKR-P1^{+} cells: 27.5% and RT1A$^{a,b+}$ NKR-P1^{+} cells: 24.2%).* ***B)*** *The kinetics of total (NKR-P1^{+}, filled squares) and recipient-derived NK cells (RT1A$^{a,b-}$ NKR-P1^{+}, open diamonds) in allografts. LIMC isolated from allografts were analyzed for the proportion of recipient-derived NK cells among the total LIMC at serial time points post-transplant. Note that donor-derived NK cells are virtually gone by 3 days post-transplant. Data are presented as the mean percentage of three to four individual samples ± SD.*

Increased expression of chemokines is detected early after transplantation.

Because NK cells were recruited to liver allografts rapidly following transplantation and because chemokines are important in the trafficking of lymphoid cells to areas of inflammation, we determined the expression of chemokines in liver grafts. Total RNA was isolated from both syngeneic and allogeneic grafts at 0, 6, 12, and 24 hours and days 3 and 7 post-transplant and analyzed by ribonuclease protection assay (RPA; Fig. 2). The chemokines CCL2 and

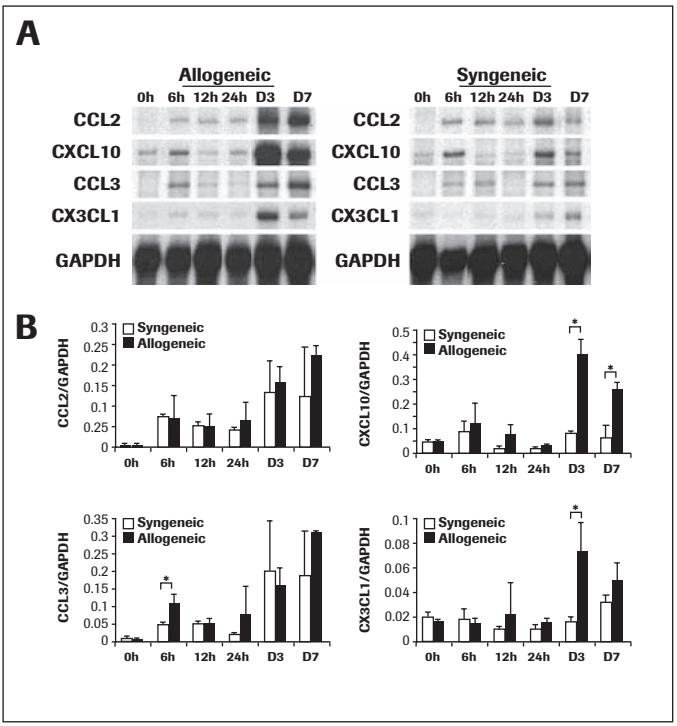

Figure 2. CCL2, CXCL10, CCL3 and CX3CL1 genes are expressed in liver grafts. *Total RNA was isolated from syngeneic and allogeneic grafts at 0, 6, 12, and 24 hours and days 3 and 7 post-transplant. Total RNA (10 μg) was hybridized with CCL2, CXCL10, CCL3, CX3CL1 and GAPDH riboprobes and analyzed by RPA. The day 0 sample is RNA isolated from non-transplanted Lewis livers for syngeneic grafts or DA livers for allogeneic grafts.* ***A)*** *Representative results from three individual grafts per group per time point are shown.* ***B)*** *The intensity of each chemokine band was measured and normalized to the GAPDH band intensity for each sample. Data are presented as the mean intensity of three individual samples ± SD. * $p < 0.02$ (syngeneic versus allogeneic grafts).*

CXCL10 were elevated as early as 6 hours post-transplant in both syngeneic and allogeneic grafts, suggesting that these chemokines were increased in liver tissue due to non-specific surgical stress and ischemia/reperfusion injury. Expression of CCL3 was detected by 6 hours post-transplant in both syngeneic and allogeneic grafts (Fig. 2A) but was significantly increased ($p=0.015$) in the allografts as compared to the syngeneic grafts (Fig. 2B, bottom left panel). CXCL10 and CX3CL1 were significantly upregulated ($p=0.014$ and 0.013, respectively) in the allografts at day 3 post-transplant as compared to the syngeneic grafts (Fig. 2A,B, top right panel and bottom right panel), suggesting a role for these chemokines in promoting the recruitment of effector cells to allogeneic tissue.

Depletion of NK cells prolongs graft survival.

To define the role of NK cells after liver transplantation we treated a group of rat liver allograft recipients ($n=7$) with a single dose (50 μl) of rabbit anti-asialo-GM1 antibodies (AGM1) or control rabbit serum (500 μg), on the day prior to transplantation. Using this protocol NK cells are quickly (by 24 hours) depleted from the periphery, and begin to reappear in the blood 7 days later (Fig. 3B). Depletion of NK cells was confirmed by flow cytometry in all graft recipients treated with AGM1. We determined, by flow cytometry, that AGM1 does bind to rat NK cells,

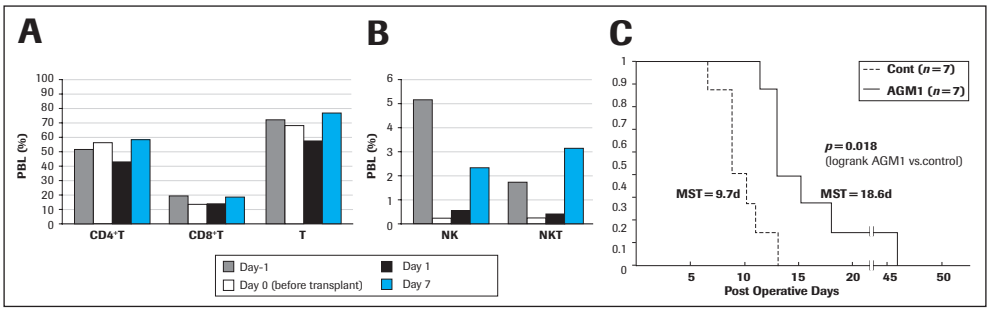

Figure 3. Depletion of NK cells prolongs allograft survival. A&B) *Lewis recipients of DA livers were treated with a single dose (50 μl) of rabbit anti-asialo GM1 antibodies and bled prior to treatment (day 1) and after treatment and transplantation (day 0,1,7) for analysis of* ***A)*** *CD4T, CD8T and T cells;* ***B)*** *NK and NKT cells.* ***C)*** *Lewis recipients of DA livers (n=7) have a MST of 9.7 days (dashed line) while depletion of NK cells by AGM1 treatment on the day before transplantation significantly (p=0.018) prolonged allograft survival to 18.6 days (solid lines) in another group of Lewis recipients of DA livers.*

NKT cells and the majority of T cells, however, NK cells are AGM1^{bright} as compared to T cells (data not shown). Interestingly, AGM1 treatment depletes virtually all of the NK cells and NKT cells, yet spares the majority of T cells (Fig. 3A). Allograft recipients depleted of NK cells had significantly prolonged survival ($p=0.018$) as compared to control allograft recipients ($n=7$; Fig. 3C). Note in this group of graft recipients all rats were killed by 50 days post-transplant. We have determined, using another group of liver allograft recipients, that there is long-term survival in 30% of the transplanted rats. These data suggest that NK cells have a role in the early events post-transplantation that contribute to liver allograft rejection.

NK cells are a major source of IFN-γ in liver allografts.

We and others have demonstrated that there is increased expression of inflammatory cytokines during rejection of liver allografts. To specifically analyze the IFN-γ levels post-transplantation, serum was obtained from recipients of liver grafts ($n=3$-5) at 0, 6, 12, 24 hours and 2, 3, 5, and 7 days after transplant. IFN-γ was generally below the level of detection in recipients of syngeneic grafts at all time points (Fig. 4A). In contrast, the levels of IFN-γ increased by day 1 post-transplantation in recipients of allogeneic liver grafts and peaked at 3 days post-transplantation.

To assess the role of NK cells in the production of IFN-γ we depleted NK cells, *in vivo*, by injecting AGM1 into a group ($n=3$) of allograft recipients. The levels of IFN-γ in the serum was significantly reduced ($p=0.001$) by >70%, 3 days after transplantation (the peak day for IFN-γ production), in NK cell-depleted graft recipients as compared to recipients of liver allografts treated with control rabbit serum (Fig. 4B).

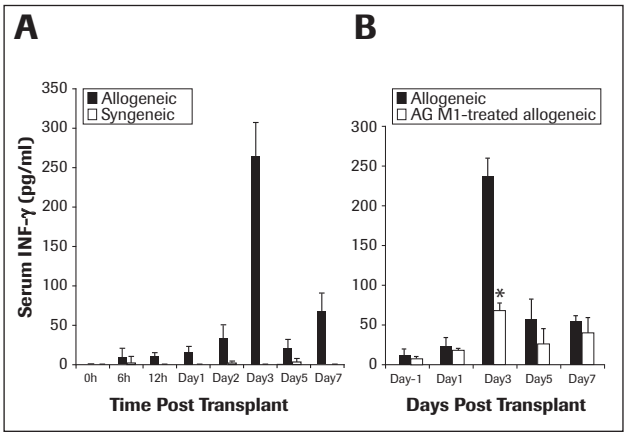

Figure 4. Serum levels of IFN-γ peak 3 days post-transplantation. *Serum from recipients of liver grafts were analyzed for IFN-γ levels by ELISA.* ***A)*** *Rats that received syngeneic grafts (n=3) did not produce IFN-γ before or after transplantation. In contrast, recipients of allogeneic grafts (n=3-5) had robust levels of IFN-γ by day 3 post-transplantation.* ***B)*** *The levels of IFN-γ were markedly diminished in rats depleted of NK cells (AGM1-treated allogeneic, n=3) prior to transplantation as compared to rats that received allogeneic liver grafts (n=3). All ELISA measurements were performed in triplicate. Data are presented as the mean ± SD of all rats in the group. * p=0.001 (AGM1-treated allogeneic versus allogeneic grafts).*

To further analyze the cellular production of IFN-γ in the allografts, LIMC were isolated from allografts, post-transplant and IFN-γ production was assessed in permeabilized cells and analyzed by flow cytometry. In a representative experiment, 2 days post-transplant, 18% of NK cells and 9% of T cells within the allograft produced IFN-γ (Fig. 5, top right and lower right panels, respectively), while 2% of NK cells and 0.4% of T cells in the non-transplanted Lewis rat liver were IFN-γ positive (data not shown). Furthermore, the proportion of IFN-γ^+ NKR-P1$^+$ cells was markedly greater (8.3%, middle top panel) than that of IFN-γ^+ $\alpha\beta$TCR$^+$ cells (3.4%, middle bottom panel) among the total LIMC. Similar results were observed at 12 hours post-transplant (absolute number=0.6 million IFN-γ^+ NK cells/g liver and 0.24 million IFN-γ^+ T cells/g liver), and 3 days post-transplant (0.55 million IFN-γ^+ NK cells/g liver and 0.2 million IFN-γ^+ T /g liver) as were observed on day two post-transplant (0.6 million IFN-γ^+ NK cells/g and 0.38 million IFN-γ^+ T cells/g). Specifically there are a greater number of NK cells expressing IFN-γ than T cells. In contrast, on day 7 post-transplant there are more T cells than NK cells producing IFN-γ (0.4 million IFN-γ^+ NK cells/g and 2.45 million IFN-γ^+ T cells/g). Donor NK cells are the source of 20-25% of the NK cell derived IFN-γ on day 2 post-transplant (data not shown). These data further indicate that NK cells are a major source of IFN-γ in liver allografts early after transplantation but not at the time of rejection.

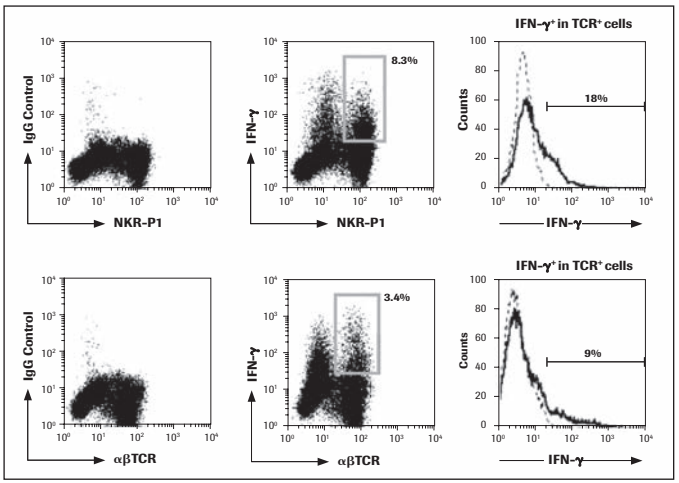

Figure 5. IFN-γ is expressed by infiltrating NK cells. *LIMC were isolated from an allograft at day 2 post-transplant and cultured with IL-2 for 24 hours. Cells were stained for cell surface antigens using FITC-anti-NKR-P1 or -anti-αβTCR mAb, fixed, permeabilized, and then stained with either PE-anti-IFN-γ mAb or PE-mouse IgG1 as an isotype control. Numbers in dot plots represent percentages of IFN-γ⁺NKR-P1⁺ cells (upper middle panel) and IFN-γ⁺ αβTCR⁺ cells (lower middle panel) among the total LIMC. Profiles of IFN-γ staining are expressed as histograms after gating on the NKR-P1⁺ cell population (upper panel) or the αβTCR cell population (lower panel). Cells stained with an anti-IFN-γ mAb are shown with a solid black line; cells stained with an isotype control are shown with a dotted line. Numbers in histograms represent percentages of IFN-γ-producing cells among indicated cell populations. Representative results from three individual grafts are shown.*

Publications

1. Obara H, Nagasaki K, Hsieh CL, et al. IFN-γ, produced by NK cells that infiltrate liver allografts early after transplantation, links the innate and adaptive immune responses. *Am J Transplant* 2005; 5: 2094.

Dr. Fadi G. Lakkis, Principal Investigator
Dr. Nancy Ruddle, Research Associate
Dr. Fady K. Baddoura, Research Associate

Yale University School of Medicine, New Haven, USA

Immunologic Ignorance of Transplanted Organs

We successfully completed both specific aims of the grant. With respect to specific aim 1, we demonstrated that ectopic production of lymphotoxin-α in the graft breaks immunological ignorance. Specifically, we found that skin allografts that express lymphotoxin-α transgenically undergo acute rejection in a recipient that lacks secondary lymphoid tissues. The same recipient ignores (does not reject) wild-type skin allografts. We also demonstrated that ectopic production of lymphotoxin-α in the allograft leads to the production of effector and memory T cells. We therefore conclude that tertiary lymphoid-like structures generated by ectopic production of lymphotoxins lead to a productive adaptive alloimmune response that breaks immunological ignorance. This work has been submitted for publication.

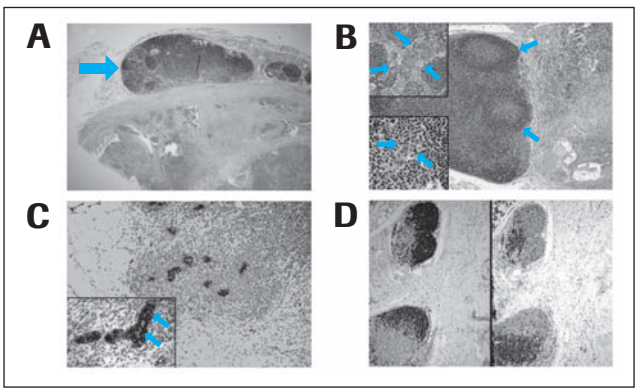

Figure 1. Tertiary lymphoid organs (TLO) in murine cardiac allografts undergoing combined acute and chronic rejection. A) *Lymph node-like structures (arrow) in the pericardium of a cardiac allograft rejected 174 days after transplantation (H&E, 100x).* ***B)*** *Intracardiac lymph-node like structure in an allograft rejected 185 days after transplantation (H&E, 100x). Main panel, arrows point to distinct lymphoid follicles with germinal centers and outer mantle zones. Top inset, pale staining sinuses (arrows) surrounding dense lymphocyte zones (H&E, 200x). Bottom inset, high endothelial venule (HEV)-like vessel (arrows) with plump endothelial lining that occupies the lumen (H&E, 400x).* ***C)*** *HEV-like vessels within TLO are intensely positive for PNAd in a cardiac allograft harvested 187 days after transplantation (MECA-79, 100x). Inset, high power view showing PNAd$^+$ HEV with the stronger staining more evident on the luminal side of the endothelium (arrows) (MECA-79, 400x).* ***D)*** *Distinct compartmentalization of B cells (left panel) and T cells (right panel) within TLO follicles in a cardiac allograft harvested 180 days after transplantation (B220/anti-CD3, 100x).*

With respect to specific aim 2, we found that intragraft inflammation is associated with lymphoid neogenesis within the graft. Specifically, we observed in a retrospective analysis of 319 murine cardiac allografts that lymphoid neogenesis [either classical tertiary lymphoid organs or peripheral node addressin (PNAd) expression on endothelial cells] is a common occurrence (78 allografts; Fig. 1). Importantly, lymphoid neogenesis was associated with chronic rejection. This finding indicates a link between chronic inflammation and lymphoid neogenesis in transplantation and suggests a potential cause-effect relationship between lymphoid neogenesis and chronic rejection. This work has already been published.

Publications

1. Baddoura FK, Nasr IW, Wrobel B, et al. Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection. *Am J Transplant* 2005; 5:510.
2. Nasr IW, Drayton DL, Reel M, et al. Intragraft tertiary lymphoid organs generate effector and memory immune responses that lead to rejection. *Am J Transplant* 2005; 5:157; *(Abstract)*.
3. Nasr IW, Wrobel B, Ruddle NH, et al. Formation of tertiary (ectopic) lymphoid organs in murine cardiac allografts undergoing chronic or delayed acute rejection. *Am J Transplant* 2004; 4:249; *(Abstract)*.

Dr. Karl Lemström, Principal Investigator
MD. Erkki Kallio, Co-Investigator
Dr. Petri Koskinen, Co-Investigator
MD. Jussi Tikkanen, Co-Investigator
MD. Antti Nykänen, Research Associate
MB. Mikko Keränen, Research Associate
MB. Sandelin Henrik, Research Associate
MSc. Rainer Krebs, Research Associate

Helsinki University Hospital, Helsinki, Finland

Role of Hypoxia-Inducible Factor-1 in Cardiac Allograft Arteriosclerosis

Preoperative graft cold ischemia time is a risk factor for cardiac allograft vasculopathy (CAV) but the underlying mechanisms are unknown. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor linking hypoxia to cellular adaptation. We characterized how graft preservation, ischemia reperfusion, and acute and chronic rejection regulate HIF-1 and its downstream genes in rat heterotopic heart transplants. In addition, we investigated the functional role of HIF-1 in the development of transplant coronary artery disease. Knowledge on the biology and function of HIF- 1 will open new therapeutic possibilities in the treatment of heart transplant recipients.

We found that prolongation of allograft preservation time results in progression of CAV in rat cardiac allografts (Fig. 1A). In non-transplanted hearts, graft cold ischemia time correlated with prominent HIF-1 protein stabilization in parenchymal and vascular cell nuclei (Fig. 1B). Further, warm ischemia significantly aggravated HIF-1 stabilization (Fig. 1B). Reperfusion of syngeneic grafts normalized HIF-1 protein expression to baseline level in vascular endothelial cells (Fig. 1C). While ischemia induced HIF-1 stabilization, acute rejection led to prominent HIF-1α mRNA expression. HIF-1 activity was localized to graft-infiltrating mononuclear inflammatory cells by HIF-1 target gene carbonic anhydrase IX (CAIX) expression (Fig. 1D-F). AAV-mediated gene transfer of stable HIF-1α resulted in increased inflammation and vascular permeability, but protected against the development of CAV (Fig. 1G-I). The results suggest that HIF-1 may play an important role during ischemia and acute rejection in cardiac allografts. Uncovering the biological effects of HIF- 1 after cardiac transplantation may open novel therapeutic strategies to prevent the development of CAV.

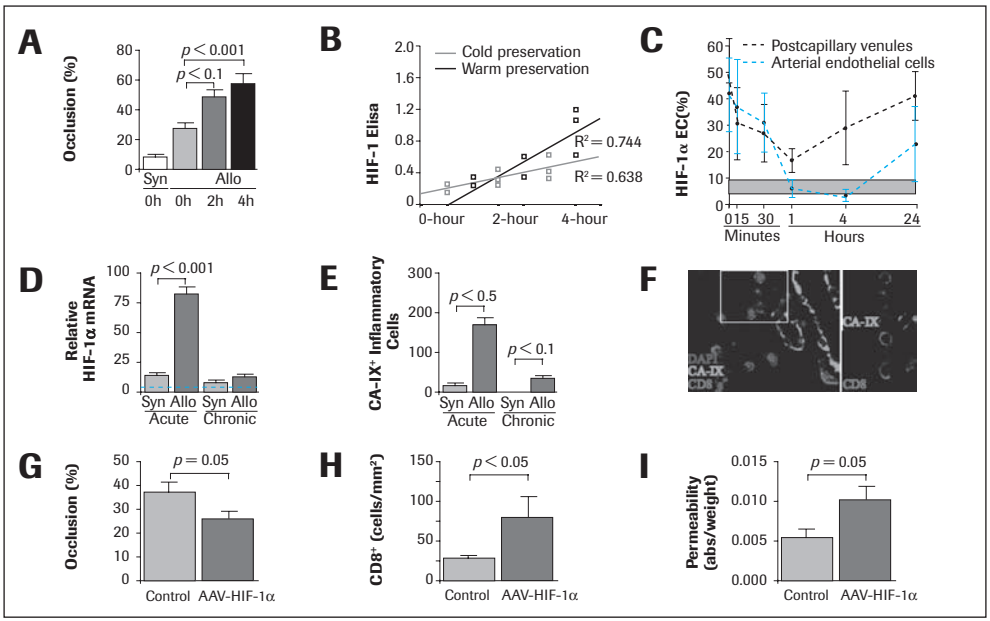

Figure 1. A) *Rat heterotopic heart transplantations were performed between MHC-mismatched strains from DA to WF rats (allo), or from DA to DA rats (syn). In the chronic rejection model, allograft recipients received cyclosporine 2 mg/kg/d during the first 7 days and 1 mg/kg/d thereafter and the grafts were harvested 60 days after transplantation. Allografts were subjected to 0-, 2-, and 4-hour ischemia time before transplantation. Prolonged cold preservation prior to transplantation significantly increased arterial occlusion in chronically rejecting allografts.* ***B)*** *HIF-1 stabilization was determined by ELISA from nuclear protein extracts. In non-transplanted hearts, graft cold ischemia time correlated with prominent HIF-1 protein stabilization (grey line). Additional warm ischemia significantly aggravated HIF-1 stabilization (black line).* ***C)*** *Immunohistochemistry was used to detect nuclear HIF-1 protein in syngrafts that were harvested at different time points after reperfusion. Reperfusion destabilized HIF-1 in endothelial cells shortly after return of circulation. Grey area represents values in non-transplanted hearts.* ***D)*** *Real time RT-PCR was used to determine HIF-1α mRNA levels in allografts that were harvested at 5 days and did not receive immunosuppression. Acute rejection significantly upregulated total HIF-1α mRNA.* ***E)*** *Acute rejection also increased HIF-1 activity in graft infiltrating inflammatory cells determined by immunoreactivity against HIF-1 target gene CAIX.* ***F)*** *CAIX co-localized with CD8⁺ inflammatory cells in acutely rejecting cardiac allografts as determined by double immunofluoresence staining.* ***G-I)*** *Allografts were perfused with adeno-associated virus encoding stable form of HIF-1α using the chronic rejection model.* ***G)*** *HIF-1 gene transfer protected against the development of CAV but increased* ***H)*** *inflammatory cell influx and* ***I)*** *vascular permeability.*

Publications

1. Keränen M, Nykänen A, MD, Krebs R, et al. Ischemia-reperfusion and alloimmune response activate hypoxia-inducible factor-1 in rat cardiac allografts; *(Manuscript submitted)*
2. Keränen M, Nykänen A, Krebs R, et al. AAV-mediated gene transfer of stable HIF-1α ameliorates rat cardiac allograft arterosclerosis, but increases permeability and inflammation; *(Manuscript in preparation)*
3. Keränen M, Nykänen A, Krebs R, et al. Ischemia-reperfusion and alloimmune response activate hypoxia-inducible factor-1 in rat cardiac allografts; *(Abstract Submitted to World Transplant Congress 2006)*

Prof. James McCluskey, Principal Investigator
Dr. Jamie Rossjohn, Co-Investigator
Dr. Andrew Brooks, Co-Investigator
Dr. Anthony Purcell, Research Associate

University of Melbourne, Victoria, Australia

Defining the Molecular Basis of T Cell Allorecognition

Original Aims

The molecular basis of T cell allorecognition remains very poorly understood despite being of fundamental importance to understanding transplantation rejection. This proposal aimed to compare the 3-dimensional structure and binding kinetics of commonly used alloreactive human T cell receptors complexed to their cognate anti-viral ligands and to their allogeneic ligand(s).

Summary of work-to-date and achievements

Epstein Barr Virus (EBV) is a ubiquitous human pathogen that chronically infects up to 90% of the population. Persistent viral infection is characterised by viral latency and periods of viral replication that are kept in check by a strong antiviral cytotoxic T lymphocyte (CTL) response. Despite its large genome size, CTL immunity to EBV focuses on only a few viral determinants that in HLA $B8^{+}$ individuals include the immunodominant antigen (Ag) FLRGRAYGL (FLR) from EBNA-3 protein. Despite a potential repertoire of $>10^{12}$ $\alpha\beta$ T cell receptors (TcR) the HLA B8-restricted cytolytic T cell response to the FLR latent Ag is strikingly limited in the TcR $\alpha\beta$ sequences that are selected. Even in unrelated individuals this response is dominated by a single highly restricted TcR $\alpha\beta$ clonotype that can be present to levels up to 10% of infected individuals. These same CTLs also alloreact upon cells expressing HLAB*4402 and B*4405 but not HLA-B*4403. In order to understand the basis for this phenomenon, and to better understand T cell allorecognition in general, we have begun solving the protein structures involved in these events. We first determined the 1.9Å crystal structure of the HLA B8-FLR peptide complex which crystallises as a dimer in the asymmetric unit. A bulged conformation of the bound peptide was observed that provides a structural basis for the critical role of the P7 tyrosine residue in T cell receptor binding. The peptide also induces backbone and side chain conformational changes in HLA B8 that are transmitted along the peptide-binding groove in a domino effect. The crystallographic HLA B8 FLR dimer is oriented such that both peptide ligands are projected in the same plane and suggests one model of the higher order MHC peptide complex that could be involved in formation of the class-I Ag-loading complex or in T cell receptor signalling.

We next determined the crystal structure of the anti-HLA-B*/FLR–specific "public" TcR to 1.5Å, representing a significant advance on previously determined TcR structures. This crystal structure reveals that five of the six hypervariable loops adopt novel conformations providing a unique combining site that contains a deep pocket predicted to overlay the HLA B8-peptide complex. The findings hinted at a structural basis for the immunodominance of this clonotype in the immune response to EBV. This was further cemented when we solved the structure of this immunodominant or "public" TcR complexed with the HLA-B*/FLR binary ligand. Residues encoded by each of the highly selected genetic elements of an immunodominant clonotype recognising EBV were critical to the Ag specificity of the receptor. Upon recognising Ag, the immunodominant TcR undergoes extensive conformational changes in the complementarity-determining regions (CDRs), including the disruption of the canonical structures of the germline-encoded CDR1 and CDR2 loops to produce an enhanced fit with the HLA-peptide complex. TcR ligation induces conformational changes in the TcR constant domain thought to form part of the docking site for CD3ε. These findings indicate that TcR immunodominance is associated with structural properties conferring receptor specificity and suggest a novel structural link between TcR ligation and intracellular signalling.

Energetics of T cell receptor interactions with MHCp

We also dissected the energetic landscape of MHCp recognition by the LC13 immunodominant αβ TcR commonly used in the immune response to EBV. The impact of natural and systematic substitutions in the CDR loops on ligand binding was quantitated and evaluated in the context of the high-resolution atomic detail of each component of the TcR/MHCp complex in its liganded and unliganded state. Surprisingly, the germline-encoded CDR1 and CDR2 loops contributed only minimal energy through direct recognition of the Ag and instead they played a major role in stabilising the ligated CDR3 loops. Accordingly, the energetic basis for recognition, including the initial recognition events, was dictated by a combination of public, non-germline and germline-encoded residues in the CDR3α and CDR3β loops. Therefore the energetic burden of different CDR loops in TcR/MHCp interaction is not fixed and apparently shifts reflecting inherent adaptability of the TcR in ligating different ligands.

TCR antagonism by altered peptide ligands

The next set of experiments examined the potential of antagonist ligands to inhibit T cell alloreactivity. Alloreactive T lymphocytes are central mediators of graft-versus-host disease and allograft rejection. The public LC13 CTL clonotype has allospecificity for the alloantigens HLAB* 4402 and B*4405 despite being, driven by cross-reactive stimulation with the common, persistent herpesvirus EBV. Since such alloreactive memory CTL expansions have the potential to influence transplantation outcome, altered peptide ligands (APLs) of the target HLAB* 0801-binding EBV peptide, FLRGRAYGL, were screened as specific antagonists for this immunodominant clonotype. One APL, FLRGRFYGL, exerted powerful antagonism of a prototypic T cell clone expressing this immunodominant TcR when co-stimulated with target

cells presenting B*0801FLRGRAYGL. Significantly, this APL also reduced the lysis of allogeneic target cells expressing HLA-B*4402 by up to 99%. The affinities of the agonist and antagonist complexes for the public TcR, measured using solution and solid-phase assays, were 8 μM and 138 μM respectively. Surprisingly the half-life of the agonist and antagonist complexes were similar, yet the association rate for the antagonist complex was significantly slower. These observations were further supported by structural studies that suggested a large conformational hurdle was required to ligate the immunodominant TcR to the HLA-B*0801 antagonist complex. By defining an antagonist APL against an immunodominant alloreactive TcR, these findings raise the prospect of exploiting such peptides to inhibit clinical alloreactivity, particularly against clonal T cell expansions that react with alloantigens.

Thermodynamics of T cell receptor interactions with MHCp

We next examined the thermodynamics of the LC13 TCR interaction with HLA-B8/FLR. Current evidence suggests a general thermodynamic pattern of enthalpically driven, entropically disfavoured αβ-TcR ligation of p-MHC I consistent with inherently flexible hypervariable loops of the TcR being stabilised upon ligation. To evaluate whether this underlying energetic signature determines MHC restriction, we examined the thermodynamic basis of the immunodominant LC13 αβTcR interacting with its cognate pMHC I ligand. Surprisingly, we observe this interaction to have low enthalpic contributions and to be governed by favourable entropic forces, despite large-scale conformational changes in the TcR upon ligation. The findings suggest that the thermodynamic principles underlying MHC restriction may not solely reside at the TcR–pMHC I interface. We conclude that extrinsic molecular factors, such as co-receptor ligation, conformational adjustments involved in TcR signalling or constraints dictated by higher order arrangement of ligated TcRs, might play a greater role in guiding MHC restriction than previously appreciated.

Single residue MHC I polymorphisms induce dramatic T cell alloreactivity

In other experiments we explored the impact of single residue polymorphisms on the T cell alloreactivity between members of the HLA-B44 group of related allotypes. HLA-B*4402 and B*4403 are naturally occurring MHC class-I alleles that are both found at a high frequency in all human populations and yet they only differ by one amino acid on the α2 helix (B*4402 Asp156->B*4403 Leu). $CD8^+$ T lymphocytes discriminate between HLA-B*4402 and B*4403 and these allotypes stimulate strong allogeneic responses reflecting their known barrier to haemopoietic stem cell transplantation. While HLA-B*4402 and B*4403 share>95% of their peptide repertoire, B*4403 presents many more unique peptides than B*4402, consistent with the stronger T cell alloreactivity observed towards B*4403 compared with B*4402. Crystal structures of B*4402 and B*4403 show how the polymorphism at position 156 is completely buried and yet alters both the peptide and the heavy chain conformation, relaxing ligand selection by B*4403 compared with B*4402. Thus, the naturally selected single residue polymorphism between HLA-B*4402 and B*4403 modifies both peptide repertoire and T cell

recognition, and is reflected in the paradoxically strong alloreactivity that occurs across this single residue class-I mismatch. The findings also suggest that the maintenance of this dimorphism in diverse human populations is related to the differential selection of developing and mature T cells by these two allotypes.

MHC I polymorphism also affects the pathway of Ag presentation

It is well known that HLA class-I polymorphism creates diversity in epitope specificity and T cell repertoire. We also showed that HLA polymorphism also controls the choice of Ag presentation pathway. A single amino acid polymorphism that distinguishes HLA-B*4402 (Asp116) from B*4405 (Tyr116) permits B*4405 to constitutively acquire peptides without any detectable incorporation into the TAP-associated peptide loading complex (PLC) even under conditions of extreme peptide starvation. This mode of peptide capture is less susceptible to viral interference than the conventional loading pathway used by HLA-B*4402 that involves assembly of class-I molecules within the PLC. Thus, B*4402 and B*4405 are at opposite extremes of a natural spectrum in HLA class-I dependence upon the PLC for Ag presentation. These findings unveil a new layer of MHC polymorphism that affects the generic pathway of Ag loading revealing an unsuspected evolutionary trade-off in selection for optimal HLA class-I loading versus effective pathogen evasion.

T cell recognition of bulged longer peptides

Although HLA class-I alleles can bind epitopes up to 14 amino acids in length, little is known about the immunogenicity of these determinants, or the nature of any responding T cell repertoire. Here we describe an HLA-B*3508-restricted CTL response with public TcR usage recognising a 13-mer epitope (LPEPLPQGQLTAY) of EBV. The high-resolution structure of this complex reveals a rigid, centrally bulged peptide in which only the N-terminal face is critical for recognition by the dominant clonotype, SB27. The SB27 TcR associates slowly (Kd 11 μM, kon 6600 $M^{-1}s^{-1}$) onto the bulged HLA-B*3508 complex, suggesting significant remodelling at the TcR/pMHC interface upon engagement. Remarkably, HLA-B*3501LPEPLPQGQLTAY, which differs from HLAB* 3508 by a single amino acid polymorphism (Leu 156->Arg), also interacts with the dominant TcR (Kd 44 μM), but without any detectable CTL response in HLA-B*3501^{+} individuals. The Leu 156->Arg substitution results in a broadening of the Ag-binding cleft in HLA-B*3508, thereby representing a critical feature that enables productive engagement of the public TcR. The use of a public TcR in this CTL response apparently reflects a dominant role of the prominent pMHC I surface that is highly dependent on polymorphic HLA class-I residues inaccessible to the TcR. It is not known whether the TCR repertoire has sufficient plasticity to recognise all longer determinants during the anti-viral CTL response. Here we show that unrelated individuals infected with EBV also generate a significant CTL response directed towards an HLA-B*3501- restricted, 11-mer epitope from the BZLF1 Ag. The 11-mer determinant adopts a highly bulged conformation with seven of the peptide side chains being solvent-exposed and available for TCR interaction. Such a complex

potentially creates a structural challenge for TCR co-recognition of both HLA-B*3501 and the peptide Ag. Surprisingly, unrelated B*3501 donors recognising the 11-mer use identical or closely related αβ TcR sequences that share particular CDR3 motifs. Within the small number of dominant CTL clonotypes observed, each have discrete fine specificity for the exposed sidechain residues of the peptide. The data show that bulged viral peptides are indeed immunogenic but suggest that the highly constrained TCR repertoire reflects a limit to TCR diversity when responding to some unusual MHCp ligands. Thus, our data show that unusually long MHC class-I-restricted epitopes play an important role in immunity, but their bulged conformation represents a barrier to αβ T cell receptor (TCR)-MHC I docking. To understand how this is achieved while still preserving MHC restriction, we also determined the structure of a TCR complexed to an HLA-B*3508-13-mer epitope. This complex is atypical of TCR/pMHC I interactions, adopting an orthogonal binding mode and being dominated at the interface by peptide-mediated interactions. The TCR assumes two distinct orientations, swivelling on top of the centrally bulged, rigid peptide such that only limited contacts are made with MHC I. Although the TCR-peptide recognition resembles an antibody-Ag interaction, the TCR-MHC I contacts define a minimal 'generic' footprint of MHC restriction.

Future Goals

We have two current aims. The first is to identify the allopeptide responsible for the T cell alloreactivity of the LC13 clonotype with HLA-B*4402/05. This information would be used to determine the structure of the LC13 TcR in complex with its alloligand. This will provide the first glimpse of a naturally alloreactive T cell receptor interacting with allogeneic MHCp enabling a comparison to its cognate self-+viral ligand (HLA-B8/FLR). The second is to identify the allopeptide involved in the B35-restricted alloresponse mediated by CTL clone SB27.

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Dr. Su Metcalfe, Principal Investigator
Miss Poorni Muthukumarana, Ph.D. Student

University of Cambridge, Addenbrookes Hospital, Cambridge, UK

The Role of Axotrophin in Regulatory Transplantation Tolerance

The focus for this 12-month funding period has been to establish a role for axotrophin in transplantation tolerance.

Report Summary

Axotrophin null mouse colony

The axotrophin null mouse is central to this project and we imported founders from Dr. Gary Lyons, Madison, University of Wisconsin, to set up a colony in our university animal house. This took several months due to local import restrictions and health controls requiring holding of stock off-site and their rederivation, eventually bringing embryos into the university housing. A breeding colony was then founded and experimental work is now initiated. As a back-up to the new Cambridge-based colony we have also cryopreserved ~400 axotrophin heterozygous embryos.

CBA mouse heart allografts in axotrophin-null BALB/c recipients

(i) The original importation of axotrophin null mice included littermates for a pilot transplantation study. Under our routine tolerogenic alternate day regimen of 12 doses of anti-CD4/8 blocking antibodies, two null recipients bore their grafts beyond 100 days post-transplant. The control null recipients (no therapy) rejected grafts between 8-11 days. (ii) In a second study, using the new Cambridge-based colony, the therapeutic mab dose was reduced to 6 doses given on alternate days. This regimen induces long-term graft survival in wild-type controls. By providing less tolerogenic cover, the truncated regimen also provides a more stringent test of the effect of loss of axotrophin and we found that the axotrophin null recipients rejected their graft at around 21 days, in contrast to wild-type littermates which show long-term survival. We conclude that axotrophin contributes to generation of transplantation tolerance *in vivo*.

Ex vivo cellular and molecular analyses of recipient splenocytes

Spleen cells from the allografted axotrophin null mice were harvested at time of rejection or after 100 days, and analysed *ex vivo* according to the following protocols:

A: Mitogenic responsiveness to the following stimuli

(i) control
(ii) concanavalin A
(iii) LPS
(iv) PMA/ionomycin
(v) anti-CD3/antiCD28

This tests for any effect of axotrophin on T and B lymphocytes under different stimuli. The results are to be compared to responsiveness of untransplanted mice that are null or wild-type for axotrophin.

B: FACS profile CD3; CD4; CD8; CD19; CD25; CD62L

Here different cell sub-populations are being assessed to look for levels of expression of their respective markers, for numerical changes per sub-population, and for population-related shifts in activation status (for example, related to the known hyperproliferation response in axotrophin-null T cells *in vitro*). In future, more extensive profiling will include intracellular targets including STAT3, SOCS3, and Foxp3 using our newly installed FACSCanto for six colour analyses per sample.

C: *Ex vivo* response to donor antigen

We have established a routine set of *ex vivo* analyses to "fingerprint" the splenocyte response status at time of isolation (0 hour) and thereafter following challenge with donor antigen (48 hours; 120 hours - time of reboost - and 123 hours) using the graft tolerant, or graft rejected splenocytes.

Samples for profiling are taken as follows:

cytokines (daily: IL2, IL4, IL6, IL7, IL10, INFγ, LIF)

proteins proteins are separated into (i) cytoplasmic proteins, (ii) nuclear proteins, and (iii) DNA-bound proteins and each fraction is analysed by Western blot for STATs 1,3,4,5 & 6, Foxp3, c-kit, LIF-R, gp130, calcineurin, Fyn, CD4, ubiquitin, and actin (load control and fraction control)

mRNA is prepared for RT-PCR of selected targets including STAT3, SOCS3, Foxp3, calcineurin, and axotrophin where relevant.

Aliquots of all samples are stored at -80°C, providing a library of documented materials for direct comparison with new *ex vivo/in vivo* transplant studies (e.g. LIF-null recipients). With time, this approach effectively accrues fingerprints of allo-immune response profiles associated with axotrophin and with LIF, both in tolerance (where this occurs) and in rejection.

Significant features of the axotrophin null recipients so far observed are:

- greatly enlarged spleen (~ twice normal);
- predominance of lymphoblasts in the splenocyte population upon isolation;
- flow-cytometric identification of a large population of $CD3^+$ blasts;
- suppression of INFγ in tolerance, but high expression of INFγ in rejection:

  rejection = 8714 pg/ml

  tolerance = 13 pg/ml

  at 24 hours, 12 dose mab therapy series
- LIF release in tolerance is double that in rejection;
- Foxp3 is low in both tolerance and rejection.

These findings reveal a marked *in vivo* phenotype in axotrophin-null recipients (splenomegally) with loss of axotrophin correlating with (i) loss of homeostatic regulation of graft-activated splenocytes, (ii) accentuated INFγ in rejection, and (iii) reduced Foxp3 expression. This would be in accord with our working hypothesis that axotrophin functions in feedback control of the immune response and plays a role in the induction of transplantation tolerance.

Lack of axotrophin is associated with low thymic Foxp3 and high thymic LIF transcript levels.
Our collaborator, Professor Garry Lyons, University of Wisconsin, has compared thymic transcripts from BALB/c mouse littermates that were null, heterozygous, or wild-type for *axotrophin*. This revealed a reduction of both Foxp3 and neuropilin 1 in the absence of axotrophin whilst other markers of Treg including CTLA-4, lymphocyte activation gene (LAG-3), and GITR showed no consistent changes. Furthermore, lack of axotrophin resulted in increased transcripts for both LIF and the LIF-response gene, SOCS3, whilst there was abundant constitutive expression of the LIF receptor which was independent of axotrophin. This is in accord with our previous finding that loss of axotrophin results in excessive secretion of LIF protein and suggests functional activity of LIF in the thymus that is normally regulated by axotrophin. Probing of the same sample set is shown in figure 1.

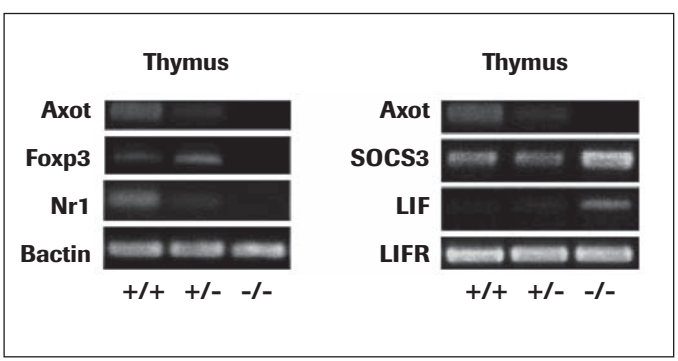

Figure 1. Analysis of thymus transcripts.

Axotrophin protein

The structure of the axotrophin protein is unknown. As part of the project, I employed a student to undertake a bioinformatic survey of the axotrophin protein within the vertebrates (human, dog, rat, mouse, Xenopus, puffer fish, and zebra fish), looking for conserved sequences as a clue to functional sites. A single conserved domain (RINGv) is predicted to have E3 ligase activity: the remaining structure is disordered and thus likely to have multiple interactive protein partners. Two highly conserved short motifs exist: their significance is as yet unknown. This work is now being extended through my collaboration with Dr. Mike Taussig, Chairman of the European Science Foundation Programme on Integrated Approaches to Functional Genomics. I am already interacting with partners in this programme, namely Toby Gibson (EMBL, Heidelburg) who is an expert on disordered proteins, and Professor Mathias Uhlen (Human Proteome Resource, Stockholm) who is preparing high quality antibodies against human proteins. Together we plan to pursue the structure of axotrophin and to raise good antibodies to enable probing of axotrophin (both human and mouse) *in vivo*, *ex vivo* and *in vitro*.

LIF-null mice

A colony of LIF-null mice will be established in Cambridge from a UK-based founder stock. These are to be used for transplantation plus *ex vivo* analyses and for cross breeding onto the axotrophin null background.

Axotrophin expression in clinical transplant recipients (with Dr. Allan Kirk and Dr. Allan Hess)

In two collaborative clinical studies, preliminary analyses on Foxp3 and axotrophin expression in graft biopsies suggest a correlation with graft function[7].

Axotrophin in vascular endothelial cells

Using RNAi of HUVEC, in collaboration with Dr. Cris Print (University of Auckland) and Dr. Steve Charnock-Jones (University of Cambridge), we have identified genes of the vascular endothelium that appear to be regulated by axotrophin including key mediators of vascular function. For illustration, please see
http://www.health.auckland.ac.nz/molmedpath/research/print.htm.

We speculate that axotrophin is active at the vascular-immune interface: this concept will be tested in the future as it has particular relevance to chronic vascular allo-rejection, where chronic inflammation may undermine a protective role of axotrophin/LIF, and where LIF-based therapy may reinforce protection.

Publications

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Dr. Wei-Ping Min, Principal Investigator
Prof. David White, Co-Investigator
Prof. Robert Zhong, Co-Investigator
Prof. Anthony Jevnikar, Collaborator
Prof. Bertha Garcia, Collaborator

University of Western Ontario, London, Canada

Novel Strategy for Inducing Transplant Tolerance by Genetically Modifying Dendritic Cells

Abstract

We have been very successful in completing this project. We have achieved all goals designed in the proposal. Through this ROTRF-supported research, we have published nine full articles and nine abstracts in high-ranking journals. We presented ten papers as oral presentations and in the poster sections of international academic meetings. Seven fellows received their training through this ROTRF-supported project. Among them, three fellows received eight international or national awards.

To date we have accomplished the following achievements:

1. Immune modulation by tolerogenic dendritic cells after gene silencing CD40. This paper is the first report that CD40-siRNA treated DC can be used as a source of Tol-DC for induction of antigen-specific tolerance and Treg formation.

2. Immune modulation and tolerance induction by RelB-silenced dendritic cells. This study is the first demonstration of induction of transplant tolerance using *in vitro* generated tolerogenic DC through RNA interference. Tolerance induced by RelB-silenced DC is associated with immune modulation through Treg formation.

3. Preventing renal ischemia-reperfusion injury using small interfering RNA by targeting complement 3 gene. A mouse warm ischemia model was used to examine effect of C3-siRNA on preventing IRI. Blocking the expression of C3 with C3 siRNA resulted in a reduction in IRI, as evidenced by maintained renal function and reduced histopathological change. C3 siRNA protects against lethal kidney ischemia.

4. *In vivo* DC-specific gene silencing (ongoing project). We have developed siRNA-loaded immunoliposomes bearing CD11c-specific antibodies for DC-targeted siRNA delivery.

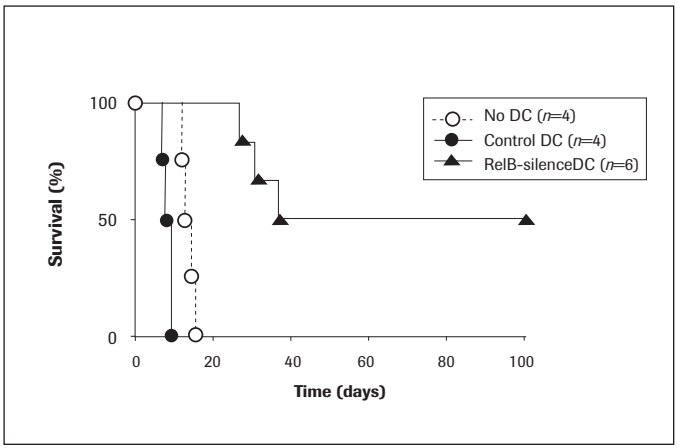

Figure 1. Induction of tolerance in allogeneic heart transplantation by RelB-silenced DC. *7-day cultured donor (C57/BL6)-derived DC were transfected with RelB-siRNA (RelB-silenced DC) or GL2-siRNA (control DC). Subsequently, cells were injected intravenously (5 x 10^5 cells/mouse) into recipient (BALB/c) mice. 7 days after DC treatment, an allogeneic (C57/BL6 to BALB/c) heart transplantation was performed. Data demonstrate survival rate of grafts after transplantation (* $p < 0.001$).*

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17. Li M, Lian D, Ichim TE, Zhong R, Min W-P. *In vivo* immune modulation by *in vitro*-generated tolerogenic dendritic cells in murine heart transplantation. *American J Transplantation* 2004: 4: 600; *(Abstract)*.
18. Min W-P, Lian D, Li M, Ichim TE, et al. Synergistic infectious tolerance by adoptive transfer of tolerogenic dendritic cells and regularity T cells. *Am J Transplant* 2004; 4:412; *(Abstract)*.

Dr. Volker Nickeleit, Principal Investigator

University of North Carolina, Chapel Hill, USA

BK-Virus Load Measurements in Kidneys: New Strategies for Assessing the Risk of BK-Virus Nephropathy

There is increasing evidence that donor-derived risk factors significantly contribute to the development of BK-virus nephropathy (BKN) post-kidney transplantation. Our sponsored pilot analysis evaluated BK-virus load levels and antibody titers in the general population (i.e. the potential pool of organ donors) by quantitative PCR analysis and by the hemagglutination inhibition assay (HIA) at time of autopsy in 40 patients (78 native kidneys). We wanted to better define potential 'donor' risk constellations for the development of BKN post grafting.

Of the patients, 58% (23/40) showed latent BK-virus infections that were not associated with morphologic changes or a specific immunohistochemical staining profile (using antibodies directed against the VP- or SV40T regions). The most common site of latent BK-virus infections was the urothelium of the ureters and the urinary bladders (53%, 21/40) whereas only a minority of patients (18%, 7/40) revealed BK-virus in the kidneys, and none in the blood. BK-virus DNA could be amplified in 11/78 native kidneys (14%) in the cortex and/or medulla. In 8% (6/78) and 5% (4/78), respectively either multiple intrarenal foci of latent BK-virus or high intrarenal viral load levels (up to 17,000 BK copies per 25,000 cell equivalents) were noted. In comparison, latent viral loads in the bladder and ureters were low (highest readings between 1,000 and 40 copies per 25,000 cell equivalents, respectively). Positive anti-BK-virus antibody titers (HIA titers $>$ 1:128) were found in 98% of patients (39/40); the antibody titers did not correlate with latent viral load levels. Although patients with latent BK-virus renal infections tended to be older [70 years versus 58 years (median) in the negative group] significant differences regarding sex, race or age were not found.

These data indicate that a minority of native kidneys harbors latent BK-viruses with high viral loads (i.e. multifocal renal involvement or hot spots) seen in 5% to 8% of organs. Latent (intrarenal) BK-virus infections can only be detected by molecular techniques and are not reflected by plasma antibody titers. Since BKN has a prevalence of 1% to 10% it is tempting to speculate that those 8% of native kidneys with high latent BK virus loads - once transplanted – constitute a significant risk for the development of disease. Future studies will specifically investigate the association of "donor kidneys" and "BKN".

Publications

1. Nickelet V. Latent polyoma-BK-virus loads in the urogenital tract of non-immunocompromised patients. *Polyomavirus-Associated Nephropathy Interdisciplinary Conference* 2005; *(Abstract)*.

Dr. Hamid Rabb, Principal Investigator
Dr. Lorraine Racusen, Consultant
Dr. Peter Heeger, Consultant
Dr. Sam Mohapatra, Consultant

Johns Hopkins University School of Medicine, Baltimore, USA

Mechanisms of T Cell Modulation of Renal Ischemia Reperfusion Injury

Summary

Ischemia reperfusion injury (IRI) to the allograft is a major limiting factor for early transplant success, and by unclear mechanisms, predisposes to an increased incidence of acute rejection and long-term allograft. We had found that T cells are direct mediators of kidney IRI, which provides the theoretical basis for T cell manipulation to prevent and treat IRI[1]. However the role for the T cell in IRI is not explained by classic models of T cell function or adaptive immunity. The aims of the present study were to begin to explore the mechanisms by which T cells mediated IRI. We began to explore (aim 1) what specific T cell would be most important in the pathogenesis of renal IRI. We first hypothesized that the Th1 CD4 cell played a pathophysiologic role. We used STAT4-deficient mice, which are deficient in Th1 CD4 T cell function. We found that these mice had deficient interferon-γ (IFN-γ) production by intracellular T cell staining, and had moderate protection from renal IRI (Fig. 1)[2]. We also studied the STAT6-deficient mice and to our surprise, these mice, deficient in IL-4 production and Th2 T cells, had worse renal IRI (Fig. 2). Thus, CD4 T cells potentially played an injurious or protective role in IRI depending on their precise phenotype. These results were independently found in liver IRI by the UCLA group, making this conclusion of higher impact for transplantation of other organs besides kidney[3].

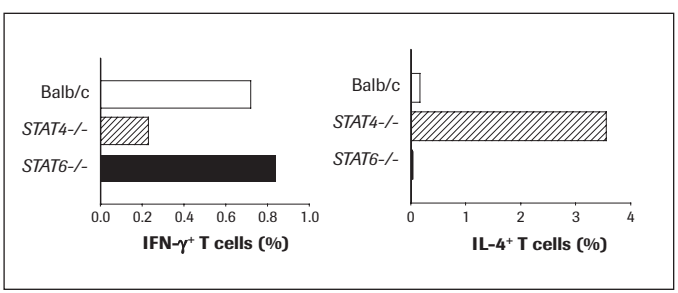

Figure 1. T cells from STAT4-/- mice demonstrated a reduced production of IFN-γ and vigorous production of IL-4 production.
In contrast, T cells from STAT6-/- mice had an opposite production of cytokine pattern with active IFN-γ production and minimum IL-4 production.

The non-heart beating deceased donor represents a major opportunity to expand the organ donor pool. However, warm IRI severely limits the number of organs that can be used. We then hypothesized that the same concepts for isolated renal IRI could be extrapolated to

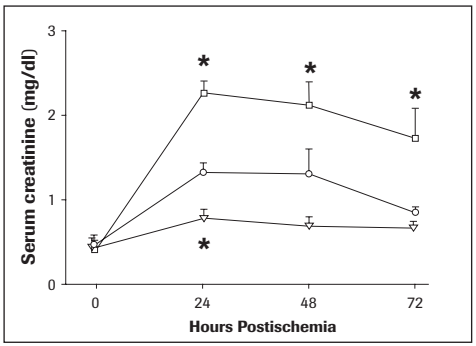

Figure 2. Renal function analysis. *STAT6$^{-/-}$ mice had significantly worse renal function compared with wild-type (WT) Balb/c (and STAT4$^{-/-}$) at 24, 48 and 72 hours post-ischemia.STAT4$^{-/-}$ mice were modestly protected compared with Balb/c, and only at 24 hours post-ischemia. *p < 0.05 versus Balb/c WT mice (n = 12/each strain). (circle: Balb/c WT, triangle: STAT4$^{-/-}$, square: STAT6$^{-/-}$).*

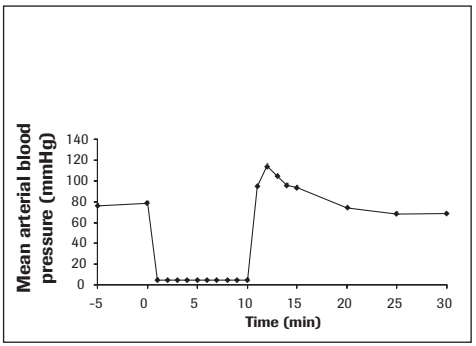

Figure 3. Mean arterial blood pressure pre-, during and post-cardiac arrest.

whole body IRI. We developed a novel model of kidney injury after whole body IRI after cardiac arrest in mice (Fig. 3), and evaluated the role for T cells in improving organ quality. We found that T cell deficiency conferred a marked protection from both functional and structural consequences of ischemic injury to the kidney after the non-heart beating state[3]. This was associated with a partial reduction in pro-inflammatory cytokine production, and particularly, less expression of ICAM-1 (Fig. 4). Based on our data, we predict that T cell modulation of the non-heart beating human organ donor would increase the number of organs available for transplantation[4].

A second major aim was to test the hypothesis that T cell receptor MHC II interactions play an important role in the T cell-mediated renal dysfunction. We found that TCR $\alpha\beta$-deficient mice were protected from IRI compared to WT littermate controls (Fig. 5). This was associated with reduced histologic injury and significantly less postischemic TNF production at the protein level (Fig. 6). However, TCR $\alpha\beta$ deficiency did not affect neutrophil or macrophage trafficking to postischemic kidney. In contrast TCR $\gamma\delta$- deficiency led to minimal protection in renal function, however, was associated with reduced histologic injury after IRI. These data demonstrate, for the first time, that the TCR plays a direct, though modest role in the pathogenesis of kidney IRI, and identifies the engagement of alloantigen-specific pathways in injury responses[5].

We also started to investigate the role of TCR transgenic mice in IRI in an effort to probe for antigen specificity in IRI. A large number of expensive knockout mice needed to be evaluated to establish the optimal conditions for T cell expansion in this model, and were beyond the

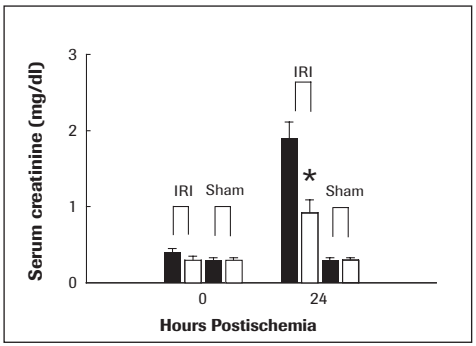

Figure 4. T cell-deficient mice are functionally protected from renal injury following cardiac arrest. *SCr was significantly reduced at 24 hours (*p< 0.05) after WBIRI in T cell-deficient mice (white bars) compared with SCr in WT mice (black bars)*

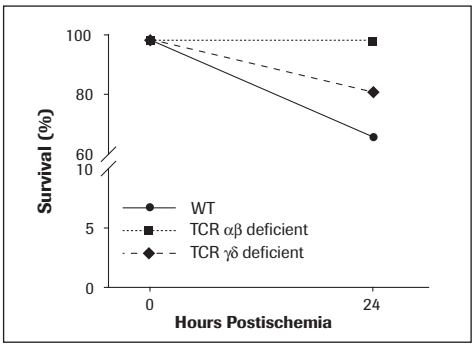

Figure 5. Survival of TCR-deficient mice and WT mice at 24 hours after IRI (n=12 in each group).

scope of the grant to complete. However, preliminary data generated with the support of the ROTRF grant were used to successfully obtain a large NIH R01 in order to examine the above questions in greater depth. In addition, the ROTRF grant served as a successful training vehicle for a number of trainees. Three post-doctoral fellows who were in part supported by this grant have gone on to faculty positions (Naoko Yokota MD, PhD: Japan, Melissa Burne-Taney PhD: Johns Hopkins, Vladimir Savransky MD, PhD: Johns Hopkins) and one is still in training (Dolores Ascon, PhD).

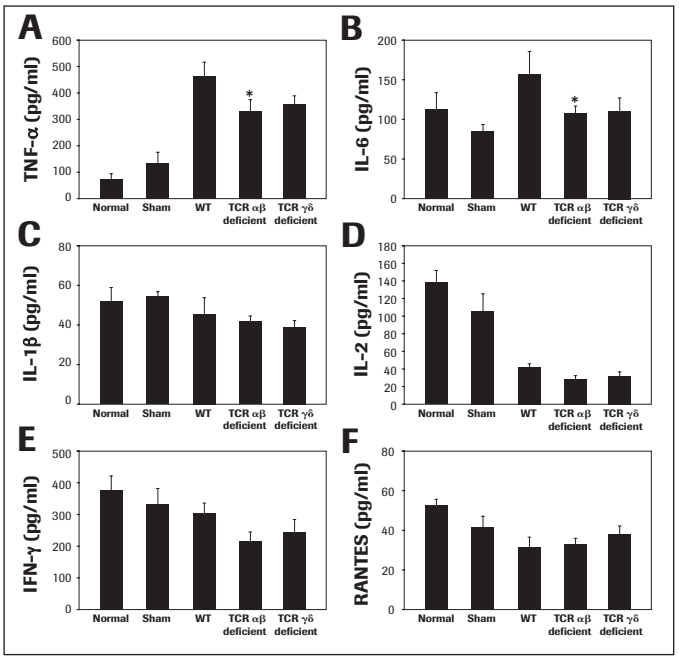

Figure 6. Pro-inflammatory cytokine and chemokine levels (pg/ml) at 24 hours after IRI in kidney tissue homogenate. A) *TNF-α,* ***B)*** *IL-6,* ***C)*** *IL-1β,* ***D)*** *IL-2,* ***E)*** *INF-γ, and* ***F)*** *RANTES. * Indicates significant difference between WT and TCR αβ-deficient mice, $p < 0.05$.*

Publications

** Publications directly deriving from the ROTRF grant.*

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Prof. Robert C. Robbins, Principal Investigator
(Successor to Prof. B. Reitz)
Dr. Dominic C. Borie, Co-Investigator

Stanford University School of Medicine, Stanford, USA

Function of Janus Kinases in Endothelial Cells and Significance in Chronic Allograft Vasculopathy

The laboratory is continuing investigation of the JAK3 pathway vis-à-vis immunotolerance and transplanted graft rejection. Currently, we have employed the use of a novel aortic transplant model in mice to assess the importance of the JAK3 pathway in the development of transplanted graft vasculopathy. We have chosen the mouse model of aortic transplantation as it affords the opportunity to utilize JAK3 knockout mice. By evaluating the host response to transplanted vessels in the JAK3 knockouts, we will definitively establish the regulatory role of JAK3 in the development of rejection vasculopathy, and pave the way for effective pharmacologic manipulation of this pathway.

At present we have established the murine aortic transplant model in our laboratory, successfully performing the operation in both syngeneic (n=9) and allogeneic (n=9) recipients. We have explanted the transplanted grafts and applied computer-assisted micro-imaging techniques to establish the immune-mediated vasculopathy that ensues following transplant into an allogeneic animal (Fig. 1). Over the next month, we will carry-out the transplantations in both JAK3 knockouts and allogeneic recipients receiving cyclosporine for immunosuppression. At the end of this phase, we will have data comparing the rejection response from four separate groups: (1) JAK3 knockouts, (2) allogeneic recipients, (3) allogeneic recipients treated with cyclosporine, and (4) syngeneic recepients.

Based on these results, we will move towards pharmacologically blocking the JAK3 pathway to induce immunosuppression following transplant.

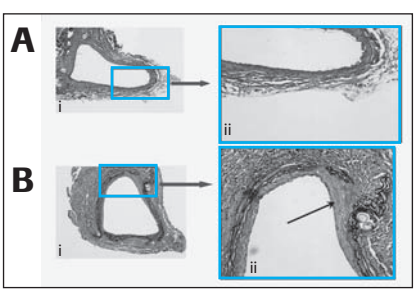

Figure 1. Histological evaluation of perfusion-fixed aortic interposition grafts in syngeneic A) and allogeneic B) hosts. *Upper panel* ***A)*** *depicts normal vessel morphology with no luminal narrowing (i, 100x). Enlarged view (ii, 200x) demonstrates normal intima and media. Lower panel* ***B)*** *shows features typical of immune-mediated vasculopathy, including luminal narrowing by nearly 30%. Enlarged view (ii, 200x) shows intimal hyperplasia (black arrow).*

Prof. Mauro Sandrin, Principal Investigator

Austin Research Institute, Heidelberg, Australia

Reduction of Galα(1,3)Gal for Xenotransplantation: Studies of HAR/DXR

Aim 1. iGb3S GKO**:** A DNA targeting construct to inactivate exon 5 (encoding the catalytic domain) has been constructed and transfected into embryonic stem cells. An ES cell clone containing the targeted disruption has been obtained and once confirmed will be inserted into blastocysts to generate the knockout mouse. We anticipate this in 2006 and ROTRF will be acknowledged in the resulting publication.

Aim 2. Elimination of Gal by glycosyltransferase competition was completed and reported in previous years of the grant. However, we characterised the anti-iGb3antibody response: The aim of these studies was to examine the antibody response of α1,3GT$^{-/-}$ mice to iGb3 and to examine the molecular mechanisms of presentation. Human 293 embryonic kidney cells were transfected with α1,3GT or iGb3S cDNAs and clonal lines were produced that expressed comparable levels of the Gal carbohydrate for both protein and glycolipid. These were used to immunise either α1,3GT$^{-/-}$ and (α1,3GT$^{-/-}$ x CD1$^{-/-}$) mice with and without anti-CD4 antibody treatment. Anti-Galα(1,3)Gal antibody levels were measured by LISA. Strong anti-Gal antibody responses were observed in α1,3GT$^{-/-}$ mice immunised with α1,3GT-293 cells. A much weaker but significant anti-Gal response was observed in mice immunized with iGb3S-293 cells. Non-immunised mice contained very low levels of anti-Gal antibodies, and this level was not increased by immunisation with mock transfected 293 cells. Similar responses to immunisation with the three cell lines were observed in the (α1,3GT o/o x CD1 o/o) mice. All antibody responses were ablated by CD4 treatment of the recipients.
Gal-α(1,3)Gal synthesised by iGb3S is immunogenic in α1,3GT$^{-/-}$ mice. The elicited antibodies are T cell dependent and CD1 independent. This suggests an alternate uncharacterised pathway for glycolipid presentation.

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5. Sandrin MS. Are glycosyltransferases still relevant for xenotransplantation? Oral presentation, *The 35th meeting of the Australasian Society for Immunology* 2005; *(Abstract)*.

Dr. Koichi Shimizu, Principal Investigator
Dr. Richard N. Mitchell, Consultant
Dr. Peter Libby, Consultant

Brigham and Women's Hospital, Harvard Medical School, Boston, USA

Source of Intimal Smooth Muscle-Like Cells in Aortic Allograft Arteriopathy

Graft arterial disease (GAD) limits long-term solid organ allograft survival. The vast majority of intimal smooth muscle-like cells (SMLC) derives from host precursor cells, but the mechanisms of their recruitment and proliferation are not understood[1]. Recent clinical and animal studies have demonstrated the presence of certain chemokines in allografts during the development of GAD lesions[2-5]. This study tested the hypothesis that specific chemokines could recruit SMLC in GAD. To characterize and evaluate specific functions of intimal SMLC, we prepared primary cultures of intimal SMLC from murine aortic allografts. After 8-12 weeks murine aortic allografts no longer contain original donor medial smooth muscle cells (SMCs), permitting selective isolation of intimal SMLC.

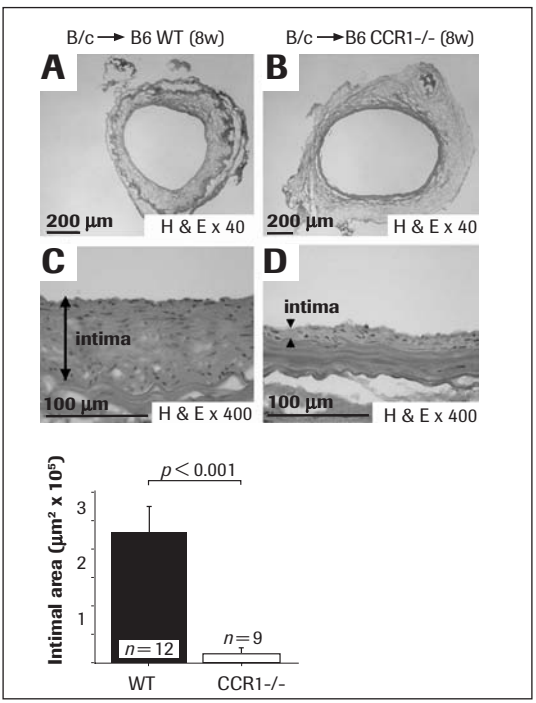

Figure 1. (A-D) *Intimal lesions of aortic allografts:* ***(A, C)*** *wild-type (WT) recipients versus* ***(B, D)*** *CCR1-deficient recipients.* ***E)*** *Allografts in CCR1-deficient hosts show significantly reduced GAD lesions (intimal area, 1.42 ± 3.23 x 10^4 µm², n=9, p<0.001) compared to allografts in WT recipients (2.28 ± 1.60 x 10^5 µm², n=12).*

We compared primary cultures of intimal SMLC from murine aortic allografts with primary medial SMC cultures from naïve aortas. RNase protection and DNA microarray analyses demonstrated that treatment with IFN-γ and TNF-α induced CCR1 mRNA expression only by SMLC, but no chemokine receptor mRNAs in medial SMCs. MIP-1α binding and immunohistochemistry revealed the CCR1 expression on SMLC but not on medial SMCs. CCR1 ligands, C10 and RANTES, induced concentration-dependent migration and proliferation of SMLC, but not medial SMCs. RANTES induced phosphorylation of c-jun N-terminal kinase (JNK) in SMLC and the JNK specific inhibitor (SP600125) abrogated RANTES-induced SMLC proliferation. Finally, aortic allografts in CCR1-deficient hosts showed significantly reduced GAD compared to allografts in WT recipients (Fig. 1). Thus, intimal SMLC uniquely express functional CCR1 chemokine receptor that plays an important role in GAD by regulating their recruitment and proliferation.

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Oral presentations

1. *Experimental Biology, FASEB Annual Meetings 2001.*
2. *Second Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology 2001.*
3. *74th Scientific Session of the American Heart Association 2001.*
4. *Annual meeting of American Society of Transplantation 2002.*
5. *75th Scientific Session of the American Heart Association 2002.*
6. *Annual meeting of American Society of Transplantation 2003.*
7. *76th Scientific Session of the American Heart Association 2003.*
8. *Annual meeting of American Society of Transplantation 2004.*
9. *77th Scientific Session of the American Heart Association 2005.*
10. *Experimental Biology, FASEB Annual Meetings 2005.*

Dr. Rakesh Sindhi, Principal Investigator
Ms Amy Magill, Research Technician
Ms Xiaoting Tang, Research Technician
Dr. Janine Janosky, Consultant
Dr. Jeff Pasley, Consultant
Dr. Suren Sehgal, Consultant

Children's Hospital of Pittsburgh, Pittsburgh, USA

Designing Biomarker-Assisted Clinical Trials for Immunosuppressants

Abstract

Current dose- and concentration-controlled evaluation of immunosuppressants has a 50% efficacy failure rate. Biomarker-assisted clinical trials may limit this failure rate by fostering individualization of immunosuppression and reducing risks of immunosuppression. To this end, 1-year funding from ROTRF was used to look for novel biomarkers of immunosuppression. Two parallel techniques were deployed on residual blood and plasma from pediatric recipients receiving a controlled immunosuppressive protocol. The protocol consisted of induction with rabbit, anti-human thymocyte globulin (rATG) followed by steroid-free monotherapy with tacrolimus or sirolimus. The techniques consisted of

1. screening of residual plasma for protein spectra using SELDI-time-of-flight mass spectrometry, and

2. high throughput cellular/subcellular imaging using a) a multilaser-flow cytometer equipped with 488 nm, 633 and 407 nm excitation lasers, and b) computerized subcellular fluorescent imaging with a light microscopy-based system.

The results will be used to solicit additional funding for i) identification, quantitation, and validation, and ii) to explore effects: concentration relationships between biomarkers and immunosuppressant concentrations in whole blood. This may validate drug-sensitive biomarkers, which have a greater dynamic range than clinical endpoints such as rejection. These may serve as surrogate (mechanistic) endpoints in clinical trials of immunosuppressants.

Results

1. Screening of residual plasma for protein spectra (SELDI-TOF-MS)

Preliminary work using whole blood from normal human subjects, reconstituted with varying

concentrations of calcineurin inhibitors and antiproliferative drugs, and stimulated with the mitogens phorbol-myristic acid and ionomycin, revealed several protein peaks in the range of M/Z ratio <5000 Da (e.g. 4469, 4626 and 4792 Da), which could serve as potential targets of immunosuppressants. This pattern was seen on an anion exchange chromatographic surface. However, the results were not reproduced, and were quite different in plasma derived from whole blood of actual pediatric subjects receiving TAC (Fig. 1).

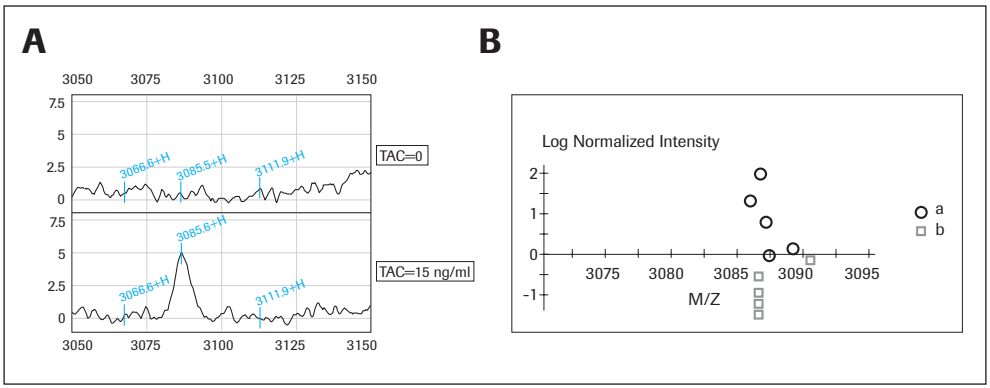

Figure 1. Screening and preliminary identification of a novel protein marker of tacrolimus in patient samples. *A preliminary search for a protein marker of TAC IM was conducted by screening cryopreserved plasma from five children, obtained before and after administration of TAC, by IMAC3 (Cu^{2+}) chip array analysis of mass spectra on SELDI-TOF-MS. All five children were enrolled in protocol RO1AI49156. The results of this preliminary case study are depicted.* ***A)*** *For one of these protein peaks, M/Z 3086 Da, the plasma samples obtained after TAC administration demonstrated increased expression compared with that seen in the absence of TAC. This is shown for one child.* ***B)*** *The signal intensities of this protein peak before (Group b, □), and after TAC (Group a, ○) segregated into 2 groups. The t-test for these 2 groups has a p-value of 0.009. This protein peak may therefore represent a TAC-specific marker. Further study is required to interpret this finding. The signal-to-noise ratio is summarized for each subject in* ***Table 1*** *and grouped as before (specimens 1b through 5b) and after TAC (specimens 1a through 5a). For a signal/noise ratio to be considered as having changed significantly, its value must increase to >2.0. Also summarized in this table are the respective concentrations of TAC in ng/ml of whole blood, present in the post-transplant specimens of these five children (specimens 1a through 5a). Of note, subject 3 with the lowest signal to noise ratio (2.2) after TAC (specimen 3a) also had the lowest concentration of TAC (5 ng/ml).*

2. a) Developing cellular endpoints for immunosuppression

Serial monitoring of pediatric liver recipients had suggested to us that the risk of cellular rejection was related to increased donor-specific immunoreactivity[1]. An immunoreactivity index by classical ^{3}H-thymidine MLR was defined as the multiple by which proliferative response to donor cells exceeded that to third-party lymphocytes. The resulting immunoreactivity index of >1 suggested increased rejection risk[2,3]. It was associated with protracted requirement for increased immunosuppression, and a significantly increased risk of recurrent or delayed

Subject	Signal/Noise	M/Z	TAC (ng/ml)
1b	1.7	3087.036	0
2b	1.6	3087.838	0
3b	2.0	3088.913	0
4b	1.6	3088.169	0
5b	1.1	3086.096	0
1a	3.0	3087.071	15.7
2a	3.0	3087.341	13.6
3a	2.2	3089.055	5
4a	5.4	3085.869	14.7
5a	8.5	3086.636	12.9

Table 1. *For the remaining 4 children, the data further suggest that higher signal-to-noise ratios of 5.4 to 8.5 in subjects 4a and 5a were not necessarily associated with higher TAC whole blood concentrations than subjects 1a and 2a, with signal-to-noise ratios of 3. If this protein marker were to represent a biomarker of toxicity of TAC, these observations would imply that subjects 4 and 5 would be more likely to experience drug toxicity than subjects 1 and 3 despite similarities in TAC concentrations. An alternative view would suggest that if this 3086 Da protein was a marker of efficacy, subjects 4 and 5 would potentially experience more benefit, because this efficacy marker was expressed to a greater degree in these subjects, than in subjects 1 and 2, who had comparable TAC concentrations. Further study is required to interpret these findings.*

rejection compared with those demonstrating decreased rejection-risk suggested by an immunoreactivity index <1. Because the MLR takes 5 days to perform, a more rapidly responsive lymphocyte subpopulation was sought by multi-laser flow cytometry in recipient cells subjected to MLR-type co-cultures for 24 hours instead of 5-days.

Subject population: Sixteen children with liver transplants (0.45-18 years) receiving regimen described in section 1. Peripheral lymphocytes (PBL) from each child were stained with 1 μM CFSE, and incubated for 24 hours with irradiated donor (HLA-DR matched) and third-party (HLA-DR-unmatched) PBL at a 1:1 ratio. Frequencies of naïve ($CD45RA^+$), memory ($CD45RO^+$) and double-positive ($CD45RA^+RO^+$) $CD4^+$, $CD8^+$ and $CD19^+$ cells expressing the activation marker CD25 to varying degrees (CD25hi, CD25lo and CD25neg) were measured by seven-color flow cytometry. For each of 27 subphenotypes (e.g. $CD4^+25hiCD45RO^+$), cells demonstrating ≥ 3-fold dilution of CFSE (log scale) due to activation-induced proliferation, were analyzed. The immunoreactivity index (IR) for each phenotype was calculated as the multiple by which donor-induced proliferation exceeded that due to third-party antigen. This was correlated with a similar index determined by 5-day, ^{3}H-thymidine MLR. IR>1 reflected increased rejection risk (Rej-risk). IR<1 reflected decreased Rej-risk. IR derived from both types of MLR was correlated.

Results: Subjects included 8 non-rejectors (Non-Rej), 7 Rej, and one child off immunosuppression due to PTLD. IR calculated by the proliferative response of $CD4^+25hiCD45RO^+$ cells in CFSE-MLR correlated well with IR calculated by 5-day MLR ($r^2=0.60$). The correlation

between the remaining 26 subpopulations of T and B cells evaluated in CFSE-MLR and 5-day MLR was poor (Table 2, r^2 values for double-positive populations not shown). The 5-day MLR demonstrated IR<1 in 7 of 8 Non-Rej, the child with PTLD, and 2 of 7 Rej. CFSE-MLR based on $CD4^+CD25hiCD45RO^+$ cells indicated IR>1 in 7 of 7 Rej, after the Rej episode, and in 2 of 8 Non-Rej, during the first 60 days after LTx (Table 3, Fig. 2). The subject with PTLD and 6 of 8 Non-Rej demonstrated IR<1 in CFSE-MLR ($CD4^+CD25hiCD45RO^+$; Table 3, Fig. 2).

	$CD45RO^+$ (memory)			**$CD45RA^+$ (naïve)**		
	$CD4^+$	**$CD8^+$**	**$CD19^+$**	**$CD4^+$**	**$CD8^+$**	**$CD19^+$**
CD25hi	**0.60**	0.008115	0.18237	0.158925	0.031801	0.210317
CD25lo	0.000386	0.00577	0.000665	0.01498	0.001914	0.206267
CD25neg	0.010201	0.000878	0.102253	0.007315	0.020139	0.008108

Table 2. Regression (r^2) values relating proliferative response of T and B cell subpopulations in CFSE-MLR to 5-day MLR.

Technique	**Non-Rej IR<1 *n*=8**	**PTLD (off) IR<1, *n*=1**	**Rejectors, IR>1, *n*=7**
^{3}H-thymidine MLR	7/8	1/1	5/7
CFSE-MLR ($CD4^+25hi45RO^{++}$)	6/8	1/1	7/7

Table 3. Results of IR determinations by CFSE and classical MLR in rejecting and non-rejecting subjects (Non-Rej), and in a subject who was not receiving any immunosuppression due to PTLD.

Conclusions: Memory T helper cells, which express high amounts of activation marker CD25 may express donor-specific alloreactivity more rapidly, and as effectively as classical ^{3}H-thymidine MLR. This may facilitate rapid identification of rejection risk and safer strategies to minimize clinical immunosuppression.

2 b) Developing subcellular endpoints for immunosuppression

Specimens that had been stained with fluorochrome-labeled antibodies for flow cytometry were also imaged in a six-channel light microscopy-based, ArrayScanII. This platform proved less sensitive (unable to detect the same proportion of cells stained with a given fluorochrome) than the flow cytometer. Further, light microscopy failed to discriminate between emission spectra of fluorochromes such as FITC and phycoerythrin (PE) despite manufacturer's assertions. The manufacturer was unable to provide additional advice regarding suitable optical filters that would facilitate discrimination between emission spectra of commonly used fluorochromes. Therefore, the platform and the technology were abandoned.

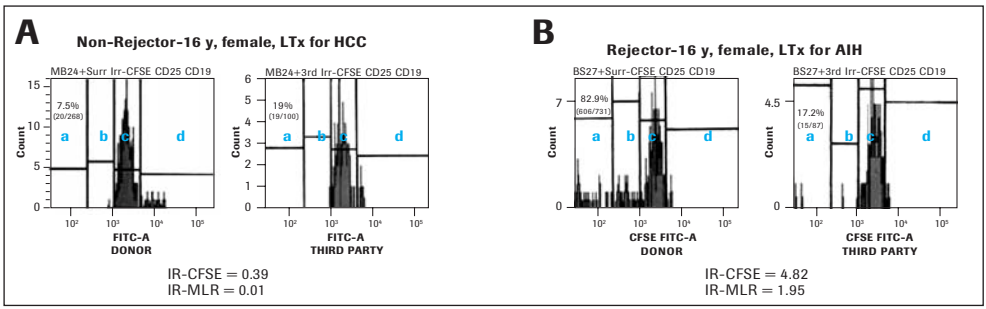

Figure 2. A) *Left and right panels represent histograms from flow cytometric analysis of CFSE-stained T helper cells ($CD4^+25hi45RO^+$) from a non-rejecting 16-year old adolescent. She received LTx for hepatocellular carcinoma. In both panels, the area labeled* ***a*** *contains recipient PBL with the least amount of fluorescence due to CFSE. This results from proliferation-induced dilution of CFSE in recipient T helpers co-cultured with donor PBL (left panel) and third-party PBL (right panel). The ratio of donor-reactive recipient T helper frequencies and third-party-reactive recipient T helper cell frequencies is 0.39. This is the IR derived from CFSE-labeled $CD4^+25hi45RO^+$ cells. The IR derived from the classical ^{3}H-thymidine MLR from the same patient is 0.01. Both values suggest decreased donor reactivity relative to third-party reactivity.* ***B)*** *Left and right panels represent histograms from flow cytometric analysis of CFSE-stained T helper cells ($CD4^+25hi45RO^+$) from a 16-year old adolescent with recurrent rejection. She received LTx for autoimmune hepatitis (AIH). In both panels, the area labeled* ***a*** *contain recipient T helper cells with the least amount of fluorescence due to CFSE. This results from proliferation-induced dilution of CFSE in recipient T helpers co-cultured with donor (left panel) and third-party PBL (right panel). The ratio of donor-reactive recipient T helper frequencies and third-party-reactive recipient T helper cell frequencies is 4.82. This is the IR derived from CFSE-labeled recipient $CD4^+25hi45RO^+$ cells. The IR derived from the classical ^{3}H- thymidine MLR from the same patient is 1.95. Both values suggest increased donor-reactivity.*

Summary

In this technology-testing phase, 1-year funding provided by the ROTRF suggests that it may be possible to screen plasma from transplant recipients for new low-molecular-weight protein markers of immunosuppression using SELDI-TOF-MS. Potential candidate peaks (e.g. m/z 3086 Da), will need to be validated in several more patients, and in multiple specimens from the same patient during an interdose interval, for reproducibility and effect: concentration relationships. Thereafter, identification by tryptic digestion and 2-D Gel/MS-MS/MALDI-TOF-MS procedures is needed.

Of more immediate promise is the finding that high content fluorescent imaging is possible with a multi-laser flow cytometer. This can allow enumeration and functional characterization of finely typed cell subsets in co-culture experiments within a day. Using seven-color/nine-parameter format, we were able to screen nine subpopulations each of T helper, T cytotoxic, and B cells for their proliferative response to donor and third-party antigen in a single co-culture experiment. The most activated (CD25high) subpopulation of memory T helpers appeared most able to replicate the results of the classical 5-day MLR, indicating all rejectors as having

a continued risk of rejection shortly after the rejection event. As described by us recently, such measurements can be adapted to test the response to immunosuppressive regimens in "rare" populations with a statistical level of certainty, using small sample sizes[3]. These concepts have been aimed at increasing the efficiency of clinical drug testing with mechanistic biomarkers. They remain the subject of intense investigation in our laboratory.

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Dr. Peter Terness, Principal Investigator

University of Heidelberg, Heidelberg, Germany

Using the Immunosuppressive IDO Gene for Prevention of Allograft Rejection

The aim of our project was to use indoleamine 2,3 dioxygenase (IDO), a protein which has been shown to suppress the maternal immune response against the foetus during pregnancy, for suppression of graft rejection. To this end, the gene was cloned into a replication-defective adenovirus and expressed in dendritic cells (DCs). Transgenic DCs were able to suppress the allogeneic T cell response[1]. Thus, a stimulatory cell was transformed into a tolerogenic one. Interestingly, when the DCs own IDO gene was upregulated by treatment with IFN-γ they did not acquire immunosuppressive properties[2]. The relevance of these findings for the immunoregulatory role of IDO was discussed in detail[3]. IDO is an enzyme which degrades tryptophan. Our studies showed that the resulting tryptophan metabolites mediate T cell suppression. In order to test the function of these metabolites *in vivo*, rats transplanted with allogeneic skin were treated daily with these compounds. As expected, the treatment induced a significant prolongation of allograft survival (Fig. 1)[4]. In a parallel experiment we tried to generate suppressive DCs by incubating them with mitomycin C (MMC). MMC is an alkylating agent which partially inhibits RNA transcription and protein synthesis. Following treatment with MMC the DCs downregulated their costimulatory (CD80, CD86) and adhesion molecules (ICAM-1), and strongly suppressed the allogeneic T cell response[5]. Taken together, our studies indicate two ways of generating suppressive DCs, which have the potential of inhibiting graft rejection, one by expression of the IDO-transgene and the other by treatment with MMC.

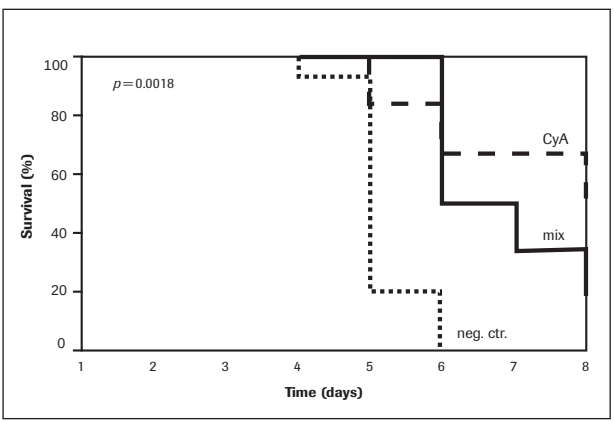

Figure 1. The effect of IDO-induced tryptophan metabolites on rat skin allograft survival. *Skin was transplanted from BN to LEW rats. Recipients received daily s.c. injections with 15 mg/kg metabolite mixture (kynurenine and 3-hydroxyanthranilic acid; n=6) or cyclosporin A (positive control; n=6). The negative control received saline solution (n=5) or no treatment (n=10). Kaplan-Meier survival curves are shown. The metabolite mixture significantly prolonged graft survival (p=0.0018).*

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Dr. Jacques-P. Tremblay, Principal Investigator
Dr. David Rothstein, Co-Investigator

CHUL Research Center, Sainte-Foy, Quebec, Canada

Development of Central Immunological Tolerance towards Myoblast Transplantation

Our long-term aim is to develop a treatment to increase the muscle strength of critical muscles in patients with Duchenne muscular dystrophy (DMD). Our strategy is based on the transplantation of allogeneic myoblasts (or eventually allogeneic stem cells) without requiring sustained immunosuppression. Thus, in this grant application, we aimed to induce immunological tolerance towards allogeneic donor cells, muscle fibers formed by the fusion of these donor cells, and dystrophin (which acts as a neo-antigen for DMD patients who lack this protein). Tolerogenic protocol(s) successful in myoblast transplantation will also be directly applicable for solid organ transplantation.

Our aim was to develop "central" immunological tolerance to myoblast transplantation (MT). We have induced central tolerance to MT using donor bone-marrow transplantation (BMT) after conditioning with anti-CD45RB, anti-CD40L and low dose (non-myeloablative) total body irradiation. We have confirmed and extended our pilot studies by determining the requirement for both anti-CD40L and anti-CD45RB in the regimen as well as demonstrating donor-specific tolerance to skin and/or myoblast allografts[1]. We have subsequently modified the protocol used in aim 1A by minimizing the conditioning regimen by reducing or eliminating irradiation and using initially busulfan and subsequently treosulfan alone in combination with cyclophosphamide[2]. To determine the extent of the tolerance we have been able to perform both skin and MT in the same mice and have examined the durability and GVHD in long-term graft survivors. These results are included in the manuscript.

Thus, the ROTRF grant has been extremely useful. It has been the only grant which has supported this research work and permitted us to obtain very good results which are the basis for a currently pending NIH application to continue this type of research.

Publications

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Dr. Anne M. VanBuskirk, Principal Investigator
Ms. Julie Dierksheide, Research Associate

Ohio State University, Colombus, USA

Cytokine Inhibition of CTL Reactivation and Post-Transplant Lymphoproliferative Disorder

Specific Aims

We were funded for 1 year to investigate 1) if TGF-β acts through the APC or directly on the T cell to inhibit CTL restimulation, 2) determine if pre-exposure of APC to TGF-β prior to antigen exposure will inhibit CTL restimulation, 3) determine if cell contact is required and 4) determine which type of antigen-presenting cell is the relevant population.

Results

We made considerable progress in our single year of funding. This progress is evidenced by presentations at regional and national meetings, a manuscript published in Blood and a second manuscript ready to be submitted. Our specific scientific results and their significance are outlined below.

1. We began our studies by determining whether TGF-β could inhibit CTL restimulation from purified $CD3^+$ cells as well as from unseparated PBL. We found that memory CTL were efficiently restimulated from purified $CD3^+$ cells in the absence or presence of TGF-β. In contrast, CTL restimulation was inhibited by TGF-β when the CTL arose from whole PBL (Fig. 1). These data suggest that TGF-β was acting through a cell other than $CD3^+$ cells to alter CTL restimulation. These data indicate several important points regarding memory CTL restimulation. First, memory CTL can be effectively restimulated when the only APC is the autologous EBV-LCL stimulator. Secondly, this restimulation is not altered by TGF-β. However, if other APC are present, TGF-β can reduce CTL restimulation.

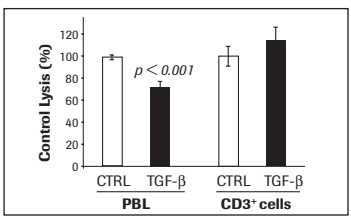

Figure1. TGF-β can inhibit CTL restimulation from whole PBL, but not purified $CD3^+$ cells. *PBL or purified $CD3^+$ were cultured with autologues EBV-LCL stimulators in the presence or absence of TGF-β for 5-7 days. CTL activity against the EBV-LCL was assessed using non-radioactive methodes. Data are from a representative experiment, with similar data obtained with 3 different donors.*

2. We next determined whether exposure to TGF-β prior to antigen exposure would alter APC function and inhibit CTL restimulation. Our previous data indicated that APC exposed to

antigen and TGF-β in PBL could not only inhibit CTL restimulation, but could also inhibit a different memory T cell function, *trans vivo* DTH (data not shown). To assess this, we isolated APC and treated them overnight with TGF-β before adding in the $CD3^+$ cells and the autologous EBV-LCL stimulators. CTL activity was assessed after 5-7 days. We determined that overnight exposure to TGF-β was sufficient to alter APC function so that CTL restimulation was inhibited (data not shown).

3. We attempted to determine whether cell-cell contact was required for APC to mediate the TGF-β mediated inhibition using trans-well technology. Unfortunately, due to technical considerations, we were unable to obtain conclusive data on this aspect of the research proposal.

4. The last of the proposed specific aims was to determine which type of APC was the relevant population. We obtained APC by several methods, and determined that $CD14^+$ monocytes are the relevant population that can inhibit CTL restimulation after treatment with TGF-β. We have not ruled out a contribution of monocyte-derived dendritic cells (mo-DC), but given the relatively rapid culture period, 18 hours with TGF-β, then 5-7 days with $CD3^+$ cells, we believe that mo-DC are unlikely to be the major cell type involved.

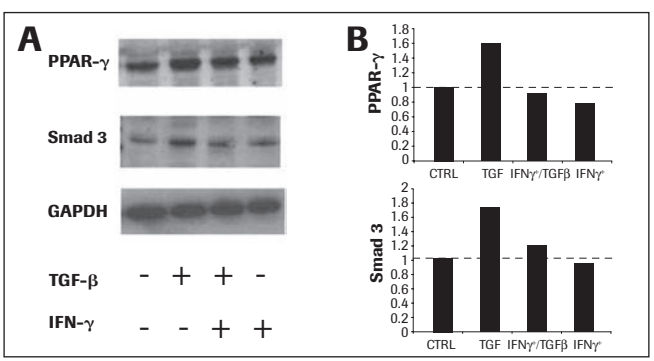

Figure 2. IFN-γ reduces smad 3 and PPAR-γ induces by TGF-β. A) *THP-1 celles were incubated with cyokines and the level of smad 3 and PPAR-γ expression assessed by immunoblotting.* ***B)*** *Histogram of the densitometry data. PPAR-γ/GAPDH ratio was determined and control expression arbitrarily set at 1.*

5. In studies that were not specifically outlined in the proposal, but were a natural outgrowth of our results, we began to investigate the relationship between IFN-γ and TGF-β and how these cytokines alter APC function in CTL restimulation. First, we demonstrated that exogenous IFN-γ could prevent TGF-β-mediated inhibition of CTL restimulation (data not shown).

6. Second, we assessed several parameters of TGF-β and IFN-γ signaling. Using flow cytometry, we showed that IFN-γ increases phosphorylated STAT-1, and that this is unaffected by TGF-β (data not shown). In contrast, TGF-β increases both smad 3 and peroxisome proliferator-activated receptor-gamma (PPAR-γ), and these molecules are down-regulated by the addition of IFN-γ, as shown by Western blot and flow cytometry (Fig. 2,3). It is

noteworthy that detection of PPAR-γ by flow cytometry has, to date, not been reported. The PPAR-γ results were also observed in primary APC (data not shown), where APC from two different donors were tested.

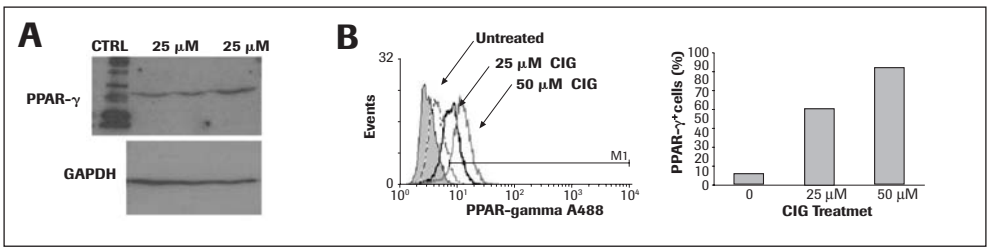

Figure 3. Ciglatazone treatment increases PPAR -γ. *THP-1 cells were treaded overnight with ciglitazone. PPAR-γ was detected by* ***A)*** *immunoblotting or* ***B)*** *flow cytometry. Shaded histogram is isotype control.* ***C)*** *The percent cells staining positive for PPAR-γ in the histogram overlay are shown schematically in the graph.*

7. Finally, to assess effects on functionality, we treated primary APC with the PPAR-γ specific ligand ciglitazone (cig) and assessed CTL restimulation. We show that the cig-treated APC have increased expression of PPAR-γ (Fig. 4A). Importantly, the cig-treated APC significantly inhibited memory CTL restimulation (Fig. 4B). To ensure the most rigorous test for inhibition, the CTL were tested using the LCL growth inhibition assay. This is a very stringent test of CTL activity, as the CTL must kill sufficient LCL to show reduced growth compared to LCL alone after 2 weeks. Specificity is demonstrated using a third party LCL. CTL restimulated in the presence of either TGF-β-treated or cig-treated APC had reduced ability to kill autologous LCL.

Conclusions and significance

Our data show that TGF-β inhibits CTL restimulation by altering $CD14^+$ APC function rather than through direct action on the $CD3^+$ cells. Further, IFN-γ can block this negative activity of TGF-β and prevent inhibition of CTL restimulation. Our data investigating the relationship between IFN-γ and TGF-β indicate that TGF-β does not block IFN-γ-induced phosphorylated STAT-1. This indicates that if TGF-β blocks IFN-γ signaling, it is at a point farther along the signaling pathway. In contrast, IFN-γ can inhibit TGF-β induced smad 3 and PPAR-γ. Smad 3 is early in the TGF-β signaling pathway. How the TGF-β and PPAR-γ pathways are related is essentially unknown. Our data suggest that at least some of the TGF-β effects on CTL restimulation may be mediated by PPAR-γ, in that treatment of APC with a PPAR-γ-specific ligand, cig, results in cells able to inhibit CTL restimulation.

The significance of the findings supported by our ROTRF grant is that they add to our understanding of why some transplant patients develop the devastating complication of PTLD, and begin to provide clues to how we can better treat this condition or avoid it altogether.

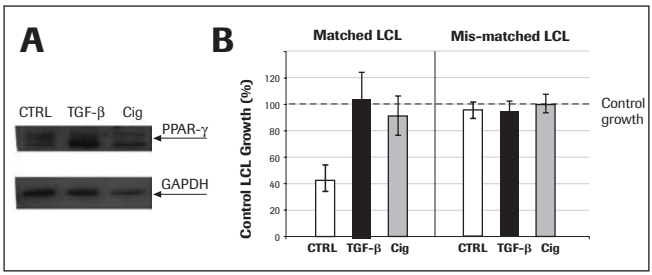

Figure 4. Ciglitazone-treaded APC have increased PPAR-γ and inhibit memory CTL restimulation. *$CD14^+$ APC were isolated from PBL and treated with 25 μM ciglitazone for 18 hours. After washing away supernatant and non-adherent cells, APC were lysed for* ***A)*** *immunoblotting or* ***B)*** *cultured with autologous $CD3^+$ cells and autologous EBV-LCL stimulators. After 5 days, CTL were tested against autologous and third-party LCL in an LCL Growth Inhibition assay. Data are shown as mean percent control growth of 6 replicates plus standard deviation.*

Healthy adults have EBV-reactive immunity that protects them from the life-long EBV infection, although in PTLD patients, that EBV memory immune response does not function properly. It is well established that cellular immunity, particularly CTL activity, is crucial in protecting from or clearing PTLD. Unfortunately, the factors underlying memory CTL restimulation are not well understood. We demonstrate that TGF-β can inhibit memory CTL restimulation, and that this inhibition is through the APC. Our data indicate that at least some of the TGF-β effects may be mediated through PPAR-γ. IFN-γ can overcome this inhibition. Although EBV-LCL are capable of presenting antigen and restimulating memory CTL, that restimulation can be overridden if TGF-β- exposed APC are present. It is important to remember that *in vivo*, $CD3^+$ cells will be exposed to not only the EBV-transformed cells, but also "professional" APC. Further, TGF-β is ubiquitous and calcineurin inhibitors can induce this cytokine. An additional consequence of the immunosuppression medications is reduced levels of IFN-γ. Therefore, APC would certainly have the opportunity to be exposed to TGF-β in the absence of IFN-γ. Our data argue that colocalization of TGF-β and APC with low/absent IFN-γ results in weak CTL restimulation. Therefore, we hypothesize that PTLD develops more easily when TGF-β is present or IFN-γ is decreased. Thus, reducing TGF-β or increasing IFN-γ should reduce PTLD. Our data regarding PPAR-γ suggest that PPAR-γ agonists may reduce APC function and could therefore contribute to PTLD development. This is of clinical interest given the use of thiazolidone drugs (PPAR-γ activators such as rosiglitazone) to treat type II diabetes and insulin resistance. In the future, we plan to continue our studies and test these new hypotheses *in vivo* using the hu-PBL SCID mouse model.

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2. VanBuskirk AM, Nye KJ, Lesinski G, et al. TGF-β inhibits CTL restimulation via APC and induces PPAR-γ; *(In preparation)*.
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4. Nye KJ, Hatton J, Duncan FJ, et al. Update on the immunobiology of post-transplant malignancies. Oral presentation, *Great Lakes Transplant Immunology Forum* 2004; *(Abstract)*.

6. How to apply for an ROTRF grant

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