R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

October 2004

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

Funding Cycle XII of the Roche Organ Transplantation Research Foundation (ROTRF) started with the submission of the Letters of Intent up to 1 April 2004. In this cycle, ten new investigators have been awarded a grant, and the ROTRF wishes them success in pursuing their research objectives.

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. The reports included in this and previous Biannual Reports, the high-quality papers published in peer-reviewed journals, and the presentations of the scientists at international meetings are the proof of the excellent work carried out by the investigators.

With the launch at the end of 2004 of a new initiative to fund more clinical research projects in transplantation, the ROTRF awards now two types of grants: conventional research grants and clinical research grants. This new clinical initiative aims to support research projects using human clinical material and patients, addressing 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. The response of investigators to this initiative has been particularly positive, with about 40% of the total number of Letters of Intent submitted in Cycle XIII being applications for clinical research grants.

For the first time, the ROTRF will be present at the American Transplant Congress in Seattle not only with its traditional booth but also with a Mini-Symposium on 24 May 2005. The Symposium, chaired by ROTRF Trustees Prof. Andrew Bradley and Prof. Allan Kirk, will give the opportunity to four ROTRF grantees to present the results achieved during their ROTRF-funded research.

We would like to thank F. Hoffmann-La Roche Ltd for its uninterrupted support of the Foundation. In addition, the ROTRF activities would never have taken place without the competent and enthusiastic commitment and input of the ROTRF Board of Trustees, the ROTRF Scientific Advisory Committee, and the very talented ROTRF grantees.

On behalf of the Board of Trustees

Philip F. Halloran, MD, PhD

2. Facts and Figures

Funding Cycle XII – Letters of Intent Submission in April 2004

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that ten applications have been awarded grants in the latest cycle of funding. The grants allocated in this cycle total 2.1 million Swiss francs (CHF). The Board of Trustees and the Scientific Advisory Committee (SAC) of the ROTRF were once again very pleased with the high quality and innovation demonstrated in the applications received.

The ROTRF received 96 Letters of Intent in funding cycle XII up to the deadline submission (April 2004) from scientists around the world. Of the applications, 46.9% were received from Europe, the major countries being the UK (10.4%), France (10.4%) and Germany (7.3%), while 45.8% came from the United States (39.6%) and Canada (6.2%). Asia (4.2%), Australia/New Zealand (2.1%) and South America (1%) accounted for the remaining 7.3% of the applications.

Based on the evaluation and reviews of the Scientific Advisory Committee, the Board of Trustees invited the 22 top-ranked applicants to submit a Full Paper Application. After thorough review of the Full Paper Applications, the Board of Trustees awarded ten full or partial grants of up to 300'000 CHF to five applicants from the USA, two from the UK and one each from Finland, Canada and France.

The abstracts of these newly awarded grants are published in the first pages of this Biannual Report and on the ROTRF homepage. The funded projects focus on the induction of tolerance, mechanisms of long-term graft survival, organ rejection, and identification of donor organ characteristics required for the optimal organ function and survival after transplantation.

Statistics on Applications to the ROTRF

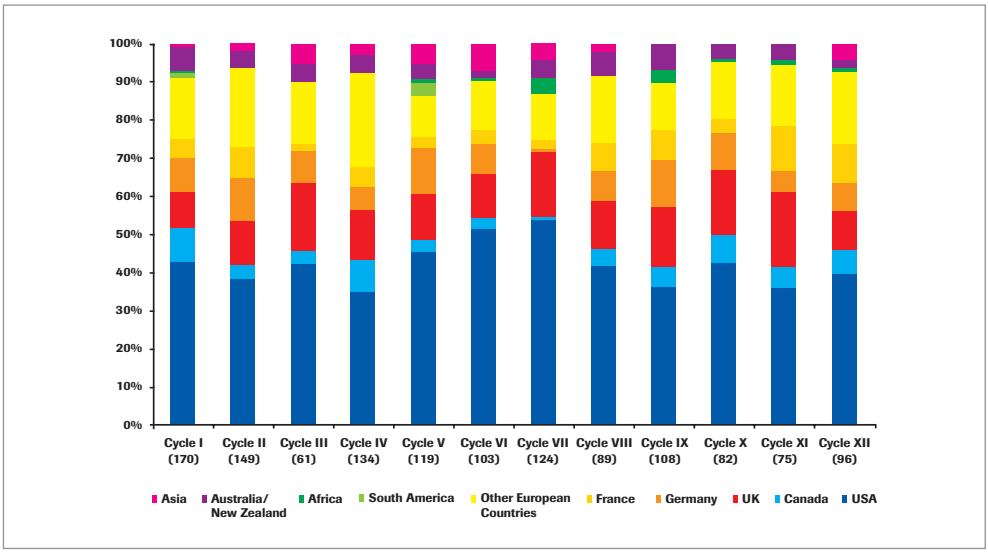


Figure 1. *Geographical distribution of the applicants who submitted Letters of Intent (LOI) during the first twelve ROTRF funding cycles. The total number of LOIs submitted per cycle is shown in brackets.*

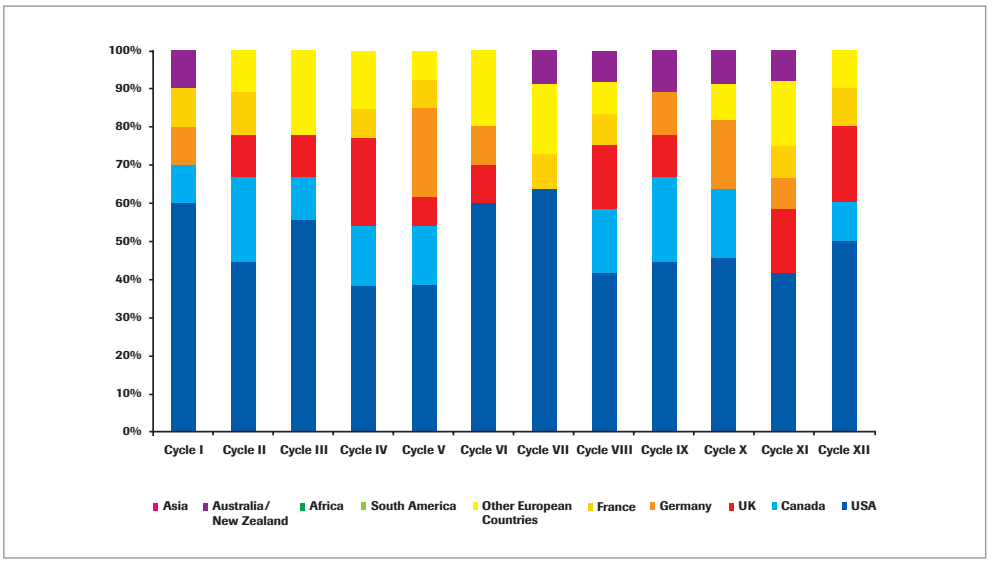
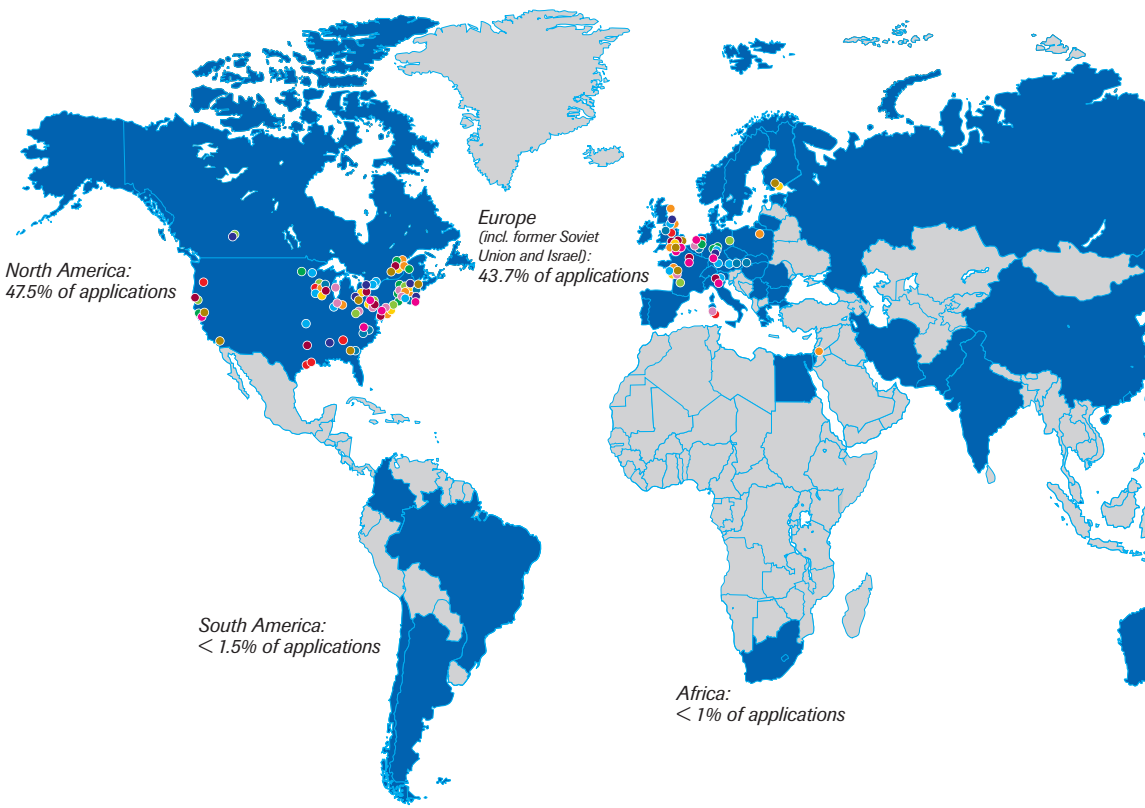
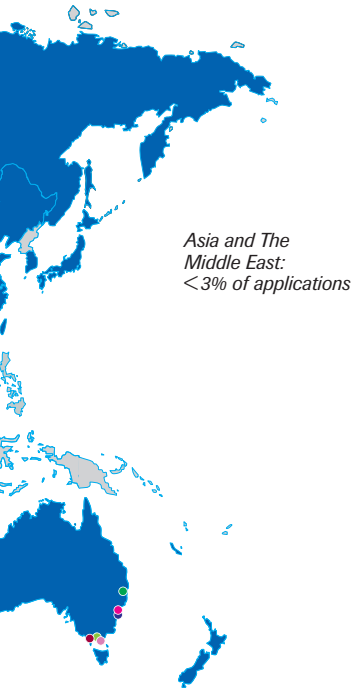


Figure 2. *Geographical distribution of the applicants who were awarded ROTRF grants during the first twelve ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF Applications Worldwide





Asia and The Middle East: < 3% of applications

Australia and New Zealand: < 5% of applications

at least one application ever received

no application received

Cycle I Grantees

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

Cycle II Grantees

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

Cycle III Grantees

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

Cycle IV Grantees

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 Grantees

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 Grantees

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

Cycle VII Grantees

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 Grantees

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 Grantees

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

Cycle X Grantees

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 Grantees

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 Grantees

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

3. Grant Awards in Cycle XII

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

INSERM, Nantes, France

Characterization of Tolerance-Related and Induced Transcript, a New Member of the CD20 Family

The Gene Search system tool was used to identify new molecules in a model of allograft tolerance and develop new means of promoting long-term acceptance of allografts and improve the outcome of organ transplantation. We cloned the cDNA for a rat gene specifically expressed in graft-infiltrating cells in tolerated allografts. We named it TORID, for tolerance-related and induced transcript. Analysis of the predicted amino acid sequence of TORID and its human counterpart LR8, showed despite a distinct chromosomal location, a yet undescribed homology with the four-transmembrane CD20/FcεRIβ family proteins.

TORID mRNA is found in a number of rat tissues and lymphoid organs especially. Among the different subpopulations of freshly isolated leukocytes, macrophages and dendritic cells (DCs), a strong expression of TORID was found. TORID protein localizes to the nuclear envelope, which suggests an intracellular function. Interestingly, expression of TORID is dramatically decreased following macrophage or DC maturation/activation, and over-expression of TORID in DCs through viral transduction induces the development of immature cells refractory to further stimulation. Moreover, infection of allografts with an adenovirus encoding TORID significantly prolongs allograft survival. This suggests that TORID may be involved in functions of immature myeloid cells, and processes of induction or maintenance of allograft tolerance.

This project addresses the expression and functional role of TORID in immune responses particularly in transplantation using different approaches of over-expression and inhibition of gene expression. Characterization of TORID may lead to the development of a new means of promoting long-term acceptance of allografts.

Dr. Claude Daniel, Principal Investigator

INRS-Institut Armand-Frappier, Laval, Canada

The Role of Histocompatibility Antigens in the Regulation of Allograft Rejection

After organ transplantation, the recipient immune system is activated by differences between self and donor histocompatibility antigens, which is defined as alloreactivity. This alloreactivity is still a major hindrance to the success of organ transplantation. Host effector T cells can recognize the donor histocompatibility antigens either directly as intact molecules, or indirectly after presentation by self antigen-presenting cells. These two pathways imply different routes of activation of the recipient immune system and different mediators of graft rejection. Furthermore, numerous studies have demonstrated the crucial role played by cytotoxic T cells and antibodies in acute rejection. Their contribution to chronic rejection remain however to be confirmed.

The goal of our research program is to elucidate the mechanisms responsible for graft rejection following T cell activation by direct and indirect alloreactivity pathways. A better understanding of these mechanisms will facilitate the design and the discovery of drugs and therapies that can more specifically interfere with graft rejection.

Dr. Robert L. Fairchild, Principal Investigator

Cleveland Clinic Foundation, Cleveland, USA

Short-Term Therapies Directed at Inflammation and T Cell Activation

Rejection of organs transplanted to treat end-stage heart and kidney disease remains a serious problem in medicine leading to loss of the transplanted organ and the potential death of the patient if a new organ is not available. To inhibit rejection, patients are faced with taking immunosuppressive drugs every day that create many new problems including increased susceptibility to infection and malignancy. These problems have necessitated the design of other strategies to inhibit transplant rejection. The facet of allograft rejection not attenuated by current immunosuppression strategies is the overt inflammation imposed on the graft at the time of the transplant operation. Surgical tissue trauma and other events inherent in the operation quickly induce recipient leukocytes to infiltrate the allograft tissue. Studies from this laboratory have used a heart transplant model in mice to show that this early infiltration causes considerable tissue damage and facilitates the recruitment of cells that eventually mediate rejection of the heart graft.

We have recently observed that attenuation of this early leukocyte infiltration with a 3-day course of therapy directed at the cells that mediate rejection inhibits the rejection of the allografts. The experiments in this proposed study are designed to understand the underlying mechanisms that prevent rejection of the heart grafts in this model. Complete understanding of the components and mechanisms underlying the abrogation of cardiac allograft rejection using this strategy will allow us to extend these studies into other models where the clinical potential of this novel strategy can be tested and may lead to the ability to avoid the immuno-suppressive drugs currently used to inhibit rejection.

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

Yale University, New Haven, USA

Role of Innate Immunity in Transplantation Tolerance

Toll-like receptors are critical innate immune receptors that detect the presence of microbial invasion. Recent *in vitro* studies have demonstrated that Toll-like receptors can affect the function of T regulatory cells. However, the role of Toll-like receptors in immunological tolerance, specifically transplantation tolerance remains obscure. Our preliminary data provides evidence that signaling via the Toll-like receptor signal adaptor, MyD88, inhibits tolerance in the adult. Furthermore, we provide evidence that Toll-like receptor expression is reduced in the neonate.

Since neonatal tolerance models are critical for our understanding of the basic mechanisms of immune tolerance, we propose to examine whether Toll-like receptor immune function is reduced in the neonate and whether this effect is critical for neonatal transplantation tolerance by promoting the generation of functional regulatory T cells. Therefore, this proposal will determine whether reduced innate immune signaling via Toll-like receptors promotes immunological tolerance.

The information generated will provide a new paradigm in the field that is directly translatable to human allotransplantation and autoimmunity (i.e., the development of inhibitors of Toll-like receptors at the time of tolerance induction). Additionally, this proposal will provide critical information as to whether Toll-like receptor-dependent immune function is impaired in the neonate, a previously unexplored area. Acquisition of this knowledge has the potential to translate into improved therapies for neonatal and childhood infections and the development of more efficacious childhood vaccinations.

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

Imperial College, London, UK

Metabolic Assessment of Human Donor Livers in Transplantation

The success of liver transplantation has led to increased use of 'marginal' grafts and an urgent need to find reliable ways of assessing donor livers both at retrieval and immediately after implantation. Nuclear Magnetic Resonance (NMR) based analysis has been shown to be a good technique for obtaining metabolic profiles of disease and toxicity and for identifying biomarkers of tissue dysfunction. In particular, the technique of Magic Angle Spinning (MAS) NMR spectroscopy combined with computer-based pattern recognition techniques (PR) offers a unique holistic and objective approach to the assessment of donor liver function pre- and post-transplantation.

Our overall aim is to provide a holistic approach for the assessment of the 'quality' of donor livers pre- and post-transplantation with a view to maximizing the success of post-operative graft function and survival. We want to not only enlarge the pool of available donors but also establish criteria for the early detection of post-operative hepatic dysfunction. We will achieve our purpose by using a combination of conventional high resolution NMR spectroscopy and MAS proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis together with ultra high-performance liquid chromatography-MS to obtain a metabolic 'fingerprint' of the donor liver at its retrieval, after cold storage and reperfusion and following its implantation in the recipient. Metabolic 'fingerprints' of biopsy samples will be obtained from donor livers and correlated with graft survival. In addition to the biopsy sample, a series of blood, urine and bile samples will be obtained during the course of the transplant procedure in order to explore any indicators of subsequent graft function. Having devised a successful strategy for identifying suitable donor livers, we would propose to extend the technology to other organs.

Prof. Leszek Ignatowicz, Principal Investigator

Medical College of Georgia, Augusta, USA

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

Long-term acceptance of transplanted solid organs is difficult to achieve and the mechanisms that warrant graft tolerance are still poorly understood. It is believed that if tolerance prevails, many graft-specific lymphocytes must die, whereas the rest of these effector cells have to be overpowered by a subpopulation of regulatory CD4$^+$ T cells. Currently it is also unknown what mechanisms, at the level of a single lymphocyte, direct the CD4$^+$ cell to become a graft invader or graft defender. The most individual feature of lymphocytes is their receptors for antigen. Each person has millions of lymphocytes equipped with unique antigen receptors. Therefore it is expected that the properties of these antigen receptors on individual lymphocytes determine cell fate during an alloresponse.

In our project, we propose to use exclusive genetically manipulated mice that although having a normal number of lymphocytes, these cells express only two to three hundred different receptors for antigens. Using these mice, we propose to determine if lymphocytes functionally committed to attack or to defend a graft express the same or different antigen receptors. Next we will determine if these receptors sense the same or different peptides that remain bound to MHC molecules, and can activate CD4$^+$ lymphocytes. This research should reveal the mechanisms behind the functional commitment of lymphocytes during an alloresponse and examine how affinity of the antigen receptor for alloantigens and self-antigens influence lymphocytes fate.

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 Vasculopathy

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

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

University of Cambridge, Addenbrookes Hospital, Cambridge, UK

Does Axotrophin Regulate Tolerance to Organ Grafts?

Cellular activity is regulated by *on* and *off* switches, ensuring that appropriate responses only occur when they are required. This control is vital in the context of the immune response, and a special type of *regulatory* T lymphocyte normally prevents auto-immune reactivity whilst permitting immune attack against pathogenic microbes. There is great scientific and medical interest in exactly how the regulatory lymphocyte succeeds in preserving this balance. The answer to the question *"What regulates the regulator?"* will advance exploitation of natural immunity for preventing rejection of organ transplants and for successful use of stem cell implants in regenerative medicine.

Axotrophin is a novel gene of unknown function that is found in mammalian stem cells. Using micro-array analyses we have discovered that axotrophin is associated with tolerance to heart grafts in mice. We also found that axotrophin has profound effects in T lymphocytes, effects that might be exploited in treatment of disease. Our aim is to identify how axotrophin functions in organ graft tolerance. To test our hypothesis that axotrophin plays a critical role in regulatory lymphocyte function we will use mice that lack the axotrophin gene. Here we anticipate that attempts to induce transplantation tolerance will fail. Other experiments will use cloned regulatory lymphocytes to ask if "knock down" of axotrophin destroys the regulatory phenotype. The knock down experiments use RNA interference, a highly specific technique that will also allow us to look for a relationship between axotrophin and Foxp3. Foxp3 is a molecular "master switch" in the regulatory T lymphocyte and we hypothesise that axotrophin and Foxp3 are somehow linked in function.

By understanding the regulatory role of axotrophin in lymphocytes, we may eventually develop means to produce donor-specific regulatory T lymphocytes in transplant recipients, so harnessing the power of immune regulation for graft tolerance whilst simultaneously preserving full immune competence to protect the patient from infection.

Prof. Bruce Reitz , Principal Investigator
Dr. Dominic C. Borie, Co-Investigator

Stanford University School of Medicine, Stanford, USA

Can Blockade of Signals in Vascular Cells Prevent Chronic Rejection of Transplants?

Inhibition of the Janus Kinase (JAK) 3 with tyrosine kinase inhibitors is emerging as a new modality of immunosuppression to effectively prevent organ allograft rejection. Although participation of the JAK/signal transduction and activator of transcription (STAT) pathway has been reported in endothelial cells (ECs) activated by a variety of stimuli, its role, and more precisely that of JAK3, in the context of allotransplantation and immunosuppression has not been studied. ECs are key players in the onset of chronic allograft vasculopathy (CAV), a condition characterized by neointimal myoproliferation in response to EC activation, and that plagues organ transplantation. As ECs of organ transplant recipients treated with JAK3 inhibitors will be exposed to the effects of those immunosuppressive molecules, we are curious to characterize the presence and activation profile of JAK3 in ECs in response to allo-immune stimuli represented by allo-antibodies and cytokines that participate in provoking allograft rejection and also by hyperimmune serum. We will subsequently study how JAK3 blockade via pharmacological inhibition, monoclonal antibodies, and RNA interference in cultured ECs affects those cells activation and/or proliferation upon subsequent stimulation. Finally, we will test our hypothesis that grafts and vessels devoid from JAK3 expression ($JAK3^{-/-}$) are less prone to CAV by performing aortic transplants using vessels harvested from donors (allo-immune injury) and by creating catheter-induced endothelial injury (mechanical injury) in $JAK3^{-/-}$ animals. Altogether, this research proposal will provide original insights on the participation of JAK3 in allo-stimulated ECs and will provide critical knowledge of the effects of JAK3 inhibiting strategies on EC function and on the development of CAV.

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 Innate Immune Toll-Like Receptor 4 Activation in Liver Ischemia/ Reperfusion Injury

Liver transplantation has been established as the definitive therapy for patients with end-stage liver disease. Ischemia/reperfusion injury (IRI) is an antigen (Ag)-independent inflammatory event, surrounding organ removal, storage and engraftment. It is not only critical to primary nonfunction or early dysfunction of liver transplants, but also plays an important role in the development of late liver dysfunction leading to chronic rejection. The mechanisms of liver injury following ischemia/reperfusion (I/R) are thought to involve a complex interaction of events initiated by host innate immunities that include Kupffer cell activation, cytokine release, neutrophil activation, increased expression of adhesion molecules, sinusoidal endothelial cell death, and hepatocyte apoptosis. Although the etiology of this post-transplant liver failure is multifactorial, a fundamental question remains to be addressed is what is the molecular nature of the initial innate immune activation leading to the full cascade of IRI.

This study aims at analyzing the Toll-like receptor (TLR) system and its role in initiating liver IRI, by focusing on both endogenous ligands generated during liver I/R, and downstream signaling pathways leading to the activation of effector mechanisms causing hepatocellular damages. TLR system is originally identified as the innate immune receptors in vertebrates to defend infectious agents. Increasing evidence indicates that vertebrates also utilize this sentinel system to distinguish well being from the disease in the absence of infections. Indeed, some intracellular proteins, and degraded cellular products have been shown to stimulate TLRs as endogenous ligands. Thus, results of this study will provide much needed *in vivo* evidence for the establishment of this new paradigm of innate immune functions in non-infectious diseases. This study should help to identify novel therapeutic targets for future clinical application to ameliorate IRI, which will not only improve the outcome of liver transplantation but also expand the organ donor pool (marginal donor more susceptible to IRI) available for transplantation.

4. Progress 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

Type-1 diabetes is caused by autoimmune destruction of insulin-producing pancreatic ß-cells. Two therapies are currently available for treatment of type-1 diabetes: (1) commonly used periodic insulin injections, and (2) transplantation of the pancreas (or islets). Both of these therapies have serious limitations. The former does not completely prevent all the long-term damages, which compromise quality of life and longevity, and the latter is of limited use because of the shortage of donor organs. Additionally, side effects of the necessary lifelong treatment with immunosuppressive drugs make organ transplantation less attractive. Consequently, insulin production from non-ß-cells has been of great interest as an alternative to transplantation. Our continued study is aimed towards providing replacement ß-cells in the form of the recipient's own liver cells, one of the few innately glucose-responsive cells, engineered for glucose-regulated, human insulin secretion as a much needed alternative therapy for insulin-dependent diabetes mellitus (IDDM).

During the course of this study, we generated a number of insulin gene constructs containing varying numbers of "glucose-inducible regulatory elements" (GIREs) derived from the promoter of S14, a quick acting glucose-responsive transcription regulator found in liver; the liver-specific promoter, albumin; and the human insulin cDNA modified so that in the absence of ß-cell specific peptidases in liver, the product may be properly processed by the endogenous protease furin. These gene constructs readily caused insulin expression in hepatocytes; the gene construct 3SAM, containing three GIREs, yielded the most amount of insulin. The insulin produced by 3SAM was biologically active and the amount of insulin produced was time and glucose-concentration dependent. The results of our studies performed *ex vivo* using hepatocytes in cell culture and *in vivo* using streptozotocin-(STZ)-treated diabetic rats were consistent with our strategy of cell engineering, as shown in our published work[1]. The *in vivo* functional efficacy studies demonstrated glucose-regulated insulin secretion from transduced liver cells normalized fasting hyperglycemia and improved glucose tolerance, supporting the feasibility of gene therapy-based treatment for IDDM. The total quantity of insulin produced in our early studies, however, was insufficient to completely correct hyperglycemia in diabetic rats with an unlimited access to food.

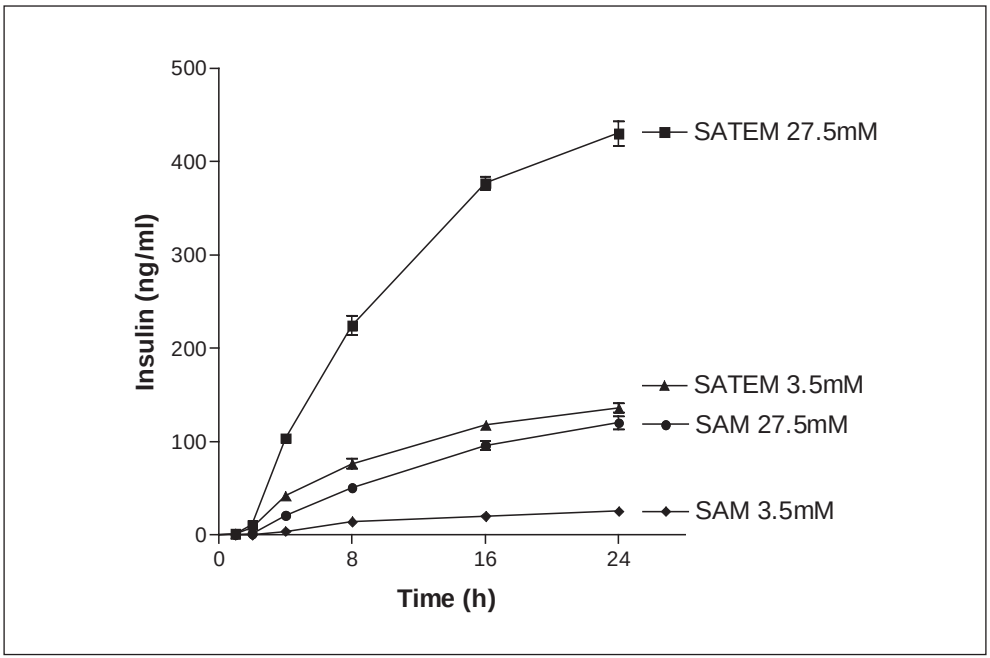


Figure 1. Glucose-dependent insulin production in transfected normal rat hepatocytes.

Our current proposal included modifications in the insulin gene construct and optimizations in the procedures of gene delivery to improve the overall insulin production. We added a translation-enhancer element from vascular endothelial growth factor (VEGF) to our previously tested construct 3SAM, to create a new construct SATEM, which improved the efficiency of insulin mRNA translation, thereby increasing overall amount of insulin production. A side-by-side comparison of normal rat hepatocytes transfected under identical conditions with equal number of replication-defective adenovirus containing either 3SAM or SATEM, revealed that cells expressing SATEM exhibited >3-fold boost in glucose-dependent insulin production when compared with 3SAM (Fig. 1). The two insulin constructs Ad.SAM and Ad.SATEM were then tested for their *in vivo* efficacy in STZ-treated diabetic rats. Blood glucose levels of diabetic rats treated with SATEM were always lower than those treated with 3SAM under identical conditions. Thus, a 9-hour fast was sufficient to correct diabetic hyperglycemia using SATEM, whereas to achieve a comparable correction of hyperglycemia with SAM, approximately 16 hours of fasting was needed. The difference between blood glucose levels after a 9-hour fast between the SATEM and SAM treated group was statistically significant.

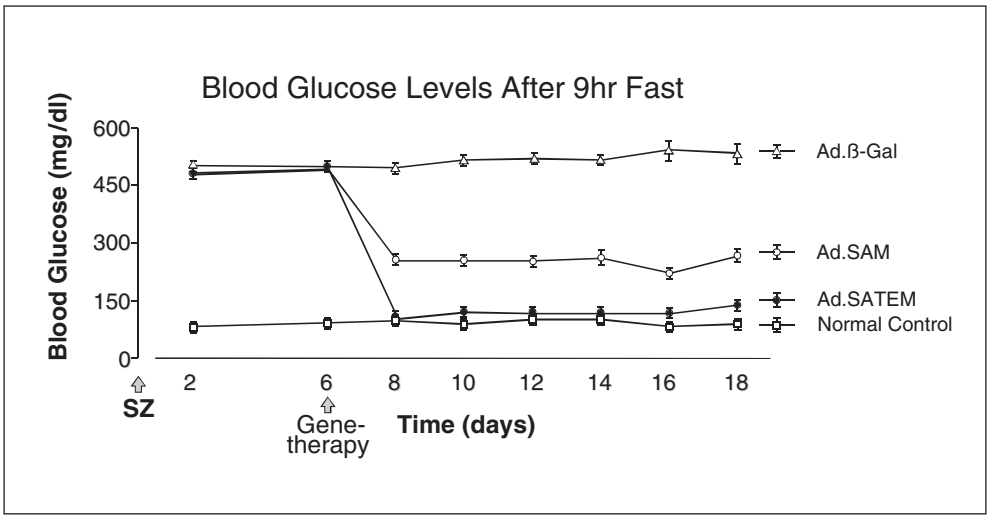


Figure 2. Comparison of glycemic correction caused by insulin gene expression constructs Ad.SAM (Ad.3SAM2) and Ad.SATEM among diabetic rats in vivo. *Each group consisted of 6 STZ diabetic rats and treated with identical amount of adenovirus vector containing the indicated gene construct. Blood glucose levels were recorded before and after 3-, 6-, and 9-hour fasts; the data for 9-hour fast are shown above.*

Human insulin level, as determined by radioimmunoassay, confirmed that SATEM caused glucose-dependent increase in serum insulin levels of diabetic rats, an *in vivo* (14.9 ±1.9 µU/ml versus 5.9 ±1.2 µU/ml in fed *ad lib* and fasted rats, respectively; $p < 0.001$). Similar to our previously described experience[1], the human insulin mRNA could only be detected in the livers of rats subjected to gene therapy and no significant amount of rat insulin mRNA was detectable in the STZ diabetic controls or treated groups, confirming the specificity of insulin expression in liver and near ablation of native ß-cell (Fig. 2).

Due to safety-related concerns associated with use of replication-defective adenovirus, its use in our work is designed to only test efficacy of various insulin constructs in short-term experiments (~4 weeks) as they are generated. However, for long-term expression and reasons of safety, alternatives to the use of adenovirus are needed. At the time when the original proposal was made, retrovirus and adeno-associated virus were included in the list of potential candidate vectors. However, the effectiveness of these vectors was limited due to low titer. Furthermore, it is now clear that these vectors may also not be completely safe. We have recently started focusing our efforts to test methods that avoid viral vectors completely, such as naked injections of plasmid DNA with or without chemicals to form complex. Work is currently under progress with preliminary successful results indicating the feasibility of use of non-viral methods for *in vivo* insulin gene delivery.

In summary, we have made progress on all aspects of the original proposal, including a necessary re-evaluation of method of gene delivery. During this year we will evaluate and optimize the method of delivery of our insulin constructs *in vivo* in treating diabetic animals. Based on our recent and preliminary results using naked DNA injections in diabetic mice, we are optimistic that a significant lowering of diabetic hyperglycemia may be possible by insulin gene expression without using viral vectors.

Publications

1. Alam T, Sollinger HW. Glucose-regulated insulin production in hepatocytes. *Transplantation* 2002; 74:1781-7.
2. Nett PC, Sollinger HW, Alam T. Hepatic insulin gene therapy in insulin-dependent diabetes mellitus. *Am J Transplant* 2003; 3:1197-203. *(Review)*

Dr. Thomas J. Dengler, Principal Investigator
Prof. Stefan Meuer, Research Associate
Prof. Jordan Pober, Research Associate

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Immunomodulation and Protection against Transplant Vasculopathy by Endothelial Precursors

Alloimmune endothelial injury appears to be the initial lesion of transplant vasculopathy. Circulating endothelial progenitor cells (EPC) capable of neovascularization and vascular protection through regeneration have recently been identified. Recent studies have shown cellular chimerism in allograft vessels suggesting a physiologic repair mechanism involving such EPC. We hypothesize that the prevention of vasculopathy development depends on efficient repair of endothelial cell loss in graft vessels. Rapid coverage of endothelial defects will inhibit the inflammatory cascade initiating neointima formation. Graft endothelium replaced by autologous EPC will be resistant to alloimmune attack and may facilitate adaptation through immune-modulating effects; induced expression of angiogenic or antiapoptotic factor may enhance this therapeutic effect.

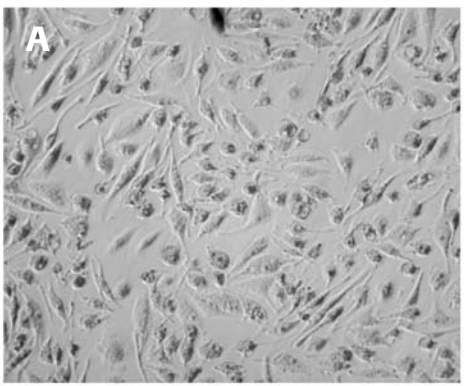
The current project investigates the antigen-presenting and immunomodulatory capacity of autologous EPC and tests if exogenous application of (genetically modified) EPC can achieve cellular chimerism in the endothelium and confer protection against transplant-related vascular injury – the project is structured into 3 main research aims:

1) Isolation and characterization of human EPC
2) Activation of allogeneic and autologous T cells by EPC
3) Endothelial chimerism and vascular protection in hybrid (human endothelium in SCID mouse) vascular animal models including therapeutic genetic modification of endothelial precursors

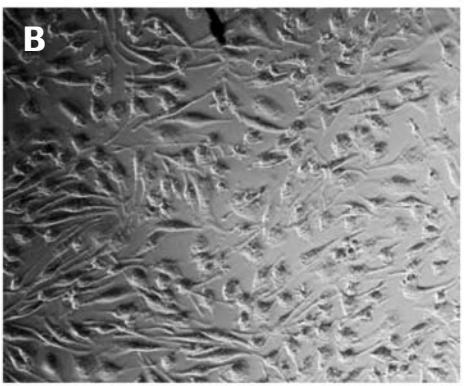
Aim 1: Isolation and characterization of different types of EPC

Two different kinds of EPCs, namely early and late EPCs have been isolated from peripheral blood mononuclear cells (PBMCs) by differential adhesion on fibronectin-coated plates. The existence of heterogeneity in EPCs arising out of peripheral blood has been reported earlier[1]. The early EPCs occurring at day 4 post-plating were spindle-shaped (Figs 1A, B) and were similar to those reported by Asahara *et al*[2]. These cells did not proliferate but survived for a maximum of eight weeks upon bi-weekly change of endothelial medium. In contrast, late EPCs appeared at four weeks post-plating and showed typical cobblestone appearance

similar to that of HUVECs (Figs 2A, B). The late EPCs proliferate for more than 30 doublings and were recently described also as blood outgrowth endothelial cells (BOECs) or late outgrowth endothelial cells (OECs)[1,3,4].

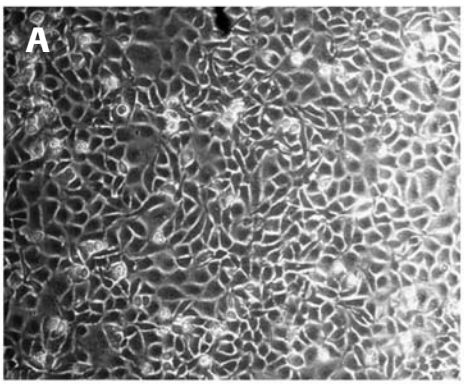


Early EPC 100x magnification

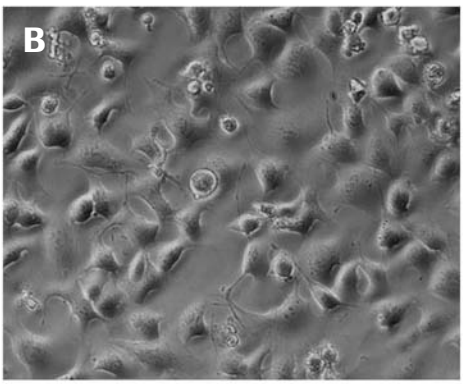


Early EPC 100x phase contrast

Figure 1. Early EPC.



Late EPC 100x magnification



Late EPC 200x phase contrast

Figure 2. Late EPC.

The early EPCs were tested for the uptake of DII–acLDL dye using light microscopy. The dye uptake was stronger when compared to HUVECs (data not shown). The uptake was quantitated by using flow cytometry (data not shown). The early EPCs were tested for binding to Ulex lectin by flow cytometry. The early EPCs displayed a variable expression on day 5/6 and day 20/21 (Table 1). The early EPCs were also analysed for change in endothelial-specific surface-marker expression on day 5 and day 20 by using flow cytometry.

Early EPCs displayed a constant surface expression of CD31. KDR which is specific for haematopoietic stem cell, EPCs and endothelial cells, was increased 2-fold on day 20 when compared to day 5 (Table 1). Also the expression of stem cell markers CD34 and CD133 were 1-11% and 0-3% respectively (Table 1). Among the lymphocyte marker, 70-90% of all cells expressed constant levels of CD45, HLA-1 and HLA-2 (Table 1). CD14 expression was reduced to one fifth during the experimental period. Interestingly, the CD80 and CD86 expression, which were expressed on 1% and 25% of the early EPCs on day 5/6 were completely absent when EPCs were analysed on day 20/21. The results of flow cytometry measurements is summarised in Table 1. EPCs were also tested for their capacity to form tube-like structure in a matrigel. Early EPCs were much less capable of tube formation than either HUVEC or late EPC (Fig. 3); their angiogenic potential was, however, markedly enhanced in conjunction with either HUVEC or late EPC, forming heterolous monolayers and endothelial tubes.

	CD31	**CD34**	**KDR**	**Ulex**	**CD14**	**CD45**	**CD133**	**CD117**
Early EPC day 5/6	77%	1%	16%	32%	50%	78%	0%	0%
Early EPC day 20/21	79%	11%	30%	77%	9%	89%	3%	23%
Late EPC	21%	0%	20%	84%	4%	0%	2%	27%

	HLA1	**HLA2**	**CD58**	**CD80**	**CD83**	**CD86**
Early EPC day 5/6	94%	94%	68%	19%	1%	25%
Early EPC day 20/21	94%	92%	80%	2%	n.d.	n.d.
Late EPC	95%	25%	94%	20%	n.d.	32%

	vWF extracellular	**vWF intracellular**	**CD68 intracellular**
Early EPC day 5/6	n.d.	88%	75%
Late EPC	10%	94%	94%

Table 1. Flow cytometric analysis of surface-antigen expression in early EPC (after isolation and after long-term culture) and in late EPC. *Percentages (%) of positive cells are shown.*

Late EPCs were characterized for the DII–acLDL uptake, tube formation in matrigel and, HIF-α upregulation when infected with Ad HIF. Late EPCs showed a weaker uptake of DIIacLDL when compared with early EPCs using light microscopy. The DII-acLDL uptake was quantitated using flow cytometry. The DII–acLDL uptake by early EPCs, late EPCs and HUVECs were performed simultaneously. Late EPCs were tested for the expression of endothelial-specific markers, stem cell markers and PBMC markers using flow cytometry (Table 1). KDR, CD31 and Ulex lectin expression was positive in 20%, 21% and 84% of late EPCs respectively. Also 94% of the late EPCs expressed vWF upon intracellular staining. Late EPCs were also analysed for the expression of PBMC markers CD14 and CD45 which were relatively negative/expressed by 4% and 0% of late EPCs respectively. Similarly the expression of stem cell markers such CD133 and CD34 were 2% and 0% respectively. In contrast, the expression of CD117 was observed in 27% of the late EPCs. The expression of HLA I and HLA II was observed in 95% and 25% of the late EPCs respectively. The expression of CD86 was observed intracellularly in 94% of the late EPCs. A summary of surface-marker expression in late EPCs is shown (Table 1). Late EPCs were also tested for formation of tubes using a matrigel assay. Late EPCs showed tube formation similar to that of HUVECs (Fig. 3). Interestingly the late EPCs survived for more than 10 days in contrast to HUVECs which were dead within a maximum of 3 days.

In summary, early and late EPCs express rather similar patterns of vascular-type surface molecules, but differ significantly in growth characteristics – for potential therapeutic purposes the cell-line like growth and higher apoptosis-resistance of late EPC with a similar angiogenic potential let this cell type appear particularly suited for animal implantation studies and genetic modification. In cell biological and immunological terms, both cell types express low levels of stem cell markers immediately after isolation, the expression level of which increase somewhat over the further culture period (it is currently unclear whether this represents a maturation process or selection / outgrowth of certain cell subtypes). Interestingly, early as opposed to late EPC display CD14 (especially soon after isolation) and several costimulatory surface antigens including MHC II, placing them more in a monocyte-type lineage suggestive of retained antigen-presenting capabilities. As this expression of CD14 declines with long-term culture (although with persisting MHC II and CD68 expression), this finding may alternatively be explained by an initial contamination by monocyte-like cells that fail to persist in vascular cell medium.

Ongoing studies:

Evaluation of various sources for cell isolation: bone marrow, cord blood and G-CSF-stimulated peripheral blood (in collaboration with local Dept. of Hematology). Further characterization of immunologically-relevant surface antigens (chemokine receptors, immunoregulators).

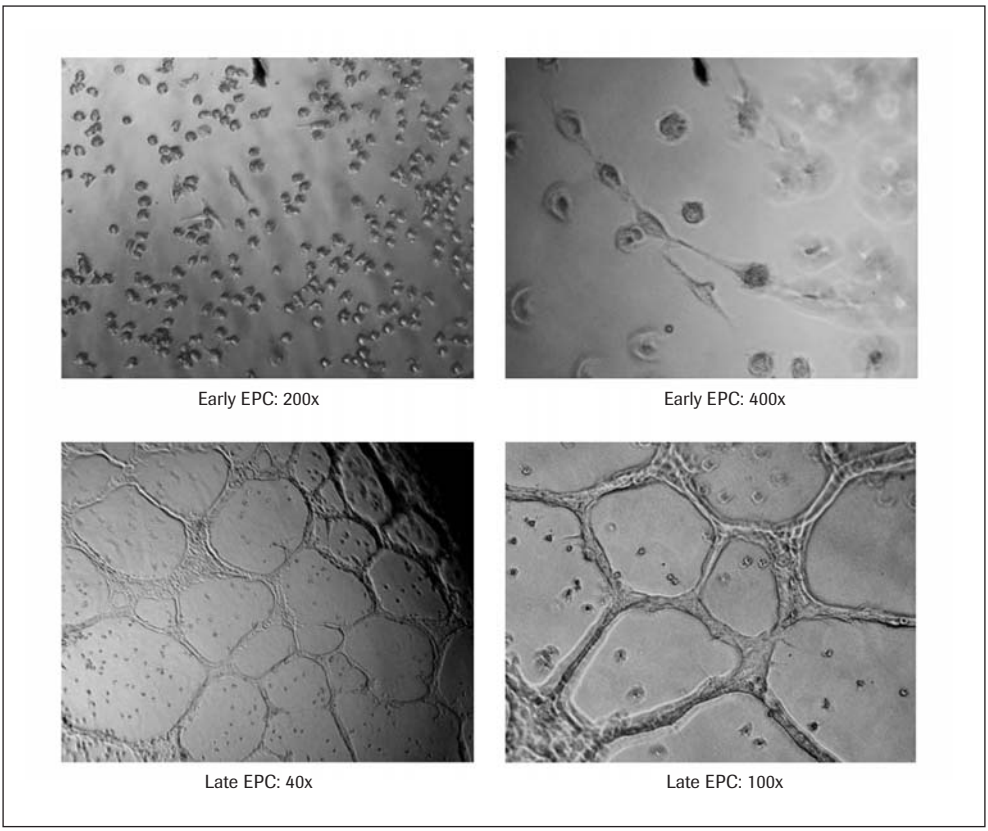


Figure 3. Matrigel tube forming assays (angiogenesis assays).

Genetic modification of EPCs

For studies employing genetically modified endothelial precursors, several adenoviral (or retroviral, for CD32) constructs have been established or are currently under production. In alteration of the original grant proposal, construction of an HGF-encoding adenovirus has been abandoned as several reports have recently been published suggesting only weak or absent therapeutic effects of HGF in tissue repair and especially vascular regeneration setting. Based on our own data and results from a collaborating laboratory (Dr. Jeffrey Schechner, Yale University, USA) indicating marked angiogenetic and anti-apoptotic effects by bcl-2 transduction in endothelial cells a bcl-2-encoding adenovirus has been established instead. Adenoviruses encoding specific transgenes were established using the system developed and published by Vogelstein et al.

cDNA clones for specific transgenes were kind gifts by collaborating researchers: cDNA for a caspase-resistant form of bcl-2 was made available by Prof. A. Bothwell, Yale University, USA, the DNA construct encoding a protease-resistant form of HIF-1α was contributed by Dr. Ratcliffe, Oxford University, Great Britain. Recombinant adenoviruses were produced expressing these respective genes, further referred to as: Ad bcl-2 (caspase-resistant bcl-2) and Ad HIF (protease-resistant HIF-1α). A complete adenovirus encoding EGFP was provided by Dr. U. Merle, Heidelberg University, Germany. A CD32-encoding adenovirus is currently under production. Correct coding sequences in adenoviruses were confirmed by sequencing, specificity of the protein products was ascertained by Western blotting both for bcl-2 and HIF-1α. Successful target cell infection has been achieved for HIF-1α and bcl-2 in fibroblast control cells, HUVEC and both forms of EPC (early and late EPC – data not shown).

For HIF-1α, extensive assessment of induced gene regulation in transgenic endothelial cells and precursors has been performed. The early EPCs were tested for upregulation of HIF-1α upon infection of adenoviral vector Ad HIF using qRT-PCR. Ad HIF expresses HIF-1α and GFP under the control of CMV promotor. The Ad vector Ad GFP served as a control. Early EPCs were infected with either Ad HIF or Ad GFP at a multiplicity of infection (moi) of 1875 and 2500. Infection of early EPCs by Ad HIF resulted in a 25-fold increase versus untransduced EPC, but only a 1.8-fold increase in HIF-1α level in comparison to Ad GFP-infected early EPCs (data not shown). The cause of the unexpected upregulation of HIF-1α in control-transduced early EPC is currently being investigated. The specificity of expression of HIF-1α upon Ad HIF infection in early EPCs was confirmed by Western blotting (data not shown). Initial experiments failed to demonstrate significant effects of HIF-1α overexpression on apoptosis induction by serum starvation or TNF/Actinomycin D (data not shown). The effects on expression of angiogenic molecules on early EPCs, upon HIF-1α is currently being studied.

Late EPCs were tested for the upregulation of HIF-1α upon infection of Ad HIF using qRTPCR. Late EPCs were infected with Ad HIF at a moi of 1, 5 and 25. A dose-dependent increase in the expression of HIF-1α was observed (Fig. 4). The expression of HIF-1α upon Ad HIF infection in late EPCs was confirmed by Western blotting (data not shown). Since HIF-1α expression results in the activation of host of angiogenic molecules, the Ad HIF-infected EPCs were tested for the expression levels of vascular endothelial growth factor (VEGF), placental growth factor (PIGF) using qRT-PCR showing respective upregulation (Fig. 5).

Ongoing studies:

Angiogenesis studies and T cell proliferation assays with HIF-1α transgenic-HUVEC and EPC, implantation of EC gels carrying HIF-1α EPC in SCID mice, target-cell transduction of bcl-2 adenovirus, gene regulation, apoptosis and angiogenesis studies with bcl-2 transgenic EPC.

Aim 2: Endothelial – T cell coculture activation assays

For the evaluation of the immunogenicity and costimulatory potential of endothelial cells or endothelial precursors several coculture cell activation assay systems for T cells (primarily $CD4^+$) and endothelial type-cells as antigen-presenting cells have been developed. In a standard integrational assay, endothelial cells are cocultured with allogeneic CD4 T cells; subsequent T cell activation and proliferation is then quantified by several read-out systems, including ^{3}H-thymidine incorporation, CFSE labeling[5] and cytokine production by way of quantitative RT-PCR from T cells and specific subsets. A novel T cell activation assay was developed, allowing selective evaluation of endothelial cell-dependent costimulation – independent of MHC class II mismatch and the various components of antigen presentation. This novel T cell proliferation assay utilizes HUVEC transgenic for the extracellular portion of the Fcγ receptor type II (CD32). All assays were performed in six replicates. Extensive validation of this CD32-HUVEC-based assay system showed dose-dependent (anti-CD3 mAb) activation of CD4 T cells and inhibition of proliferation by costimulation blockade (e.g. anti-CD58 mAb) similar to assays using native endothelial cells (not shown).

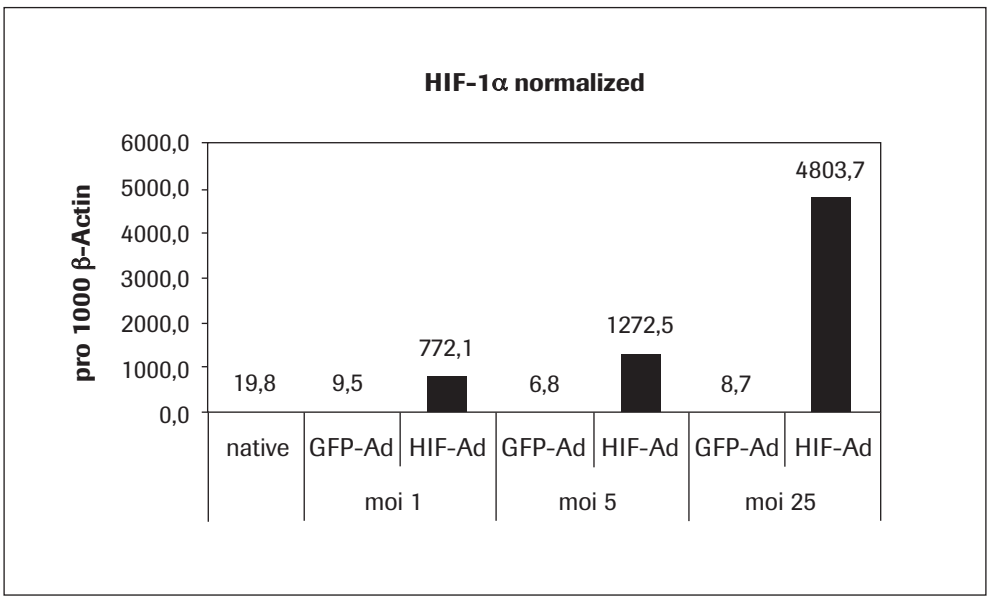


Figure 4. HIF-1α expression in early EPC after adenovirus transduction (normalized to 1000 ß-actin units, qRT-PCR), native, moi 1, 5 and 25.

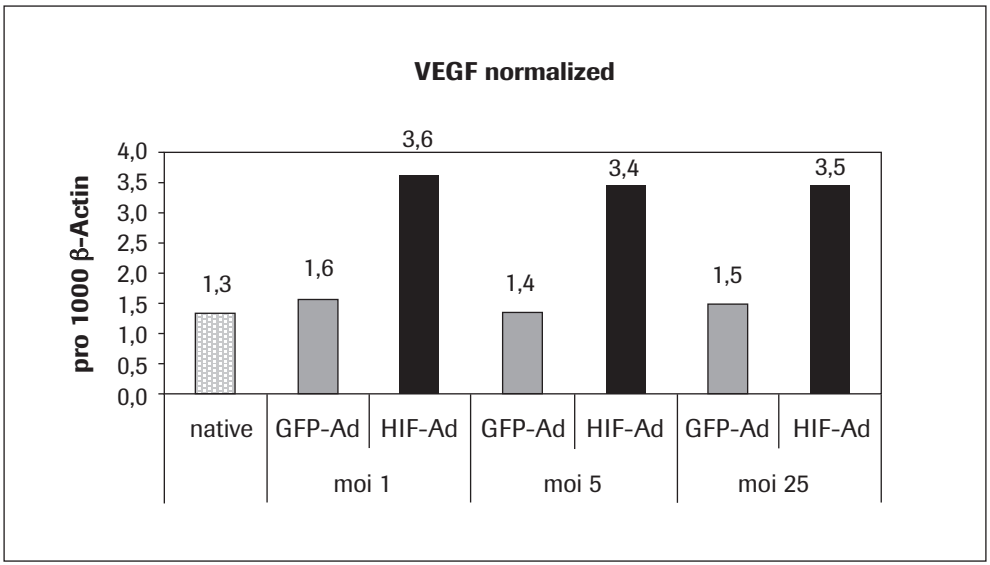


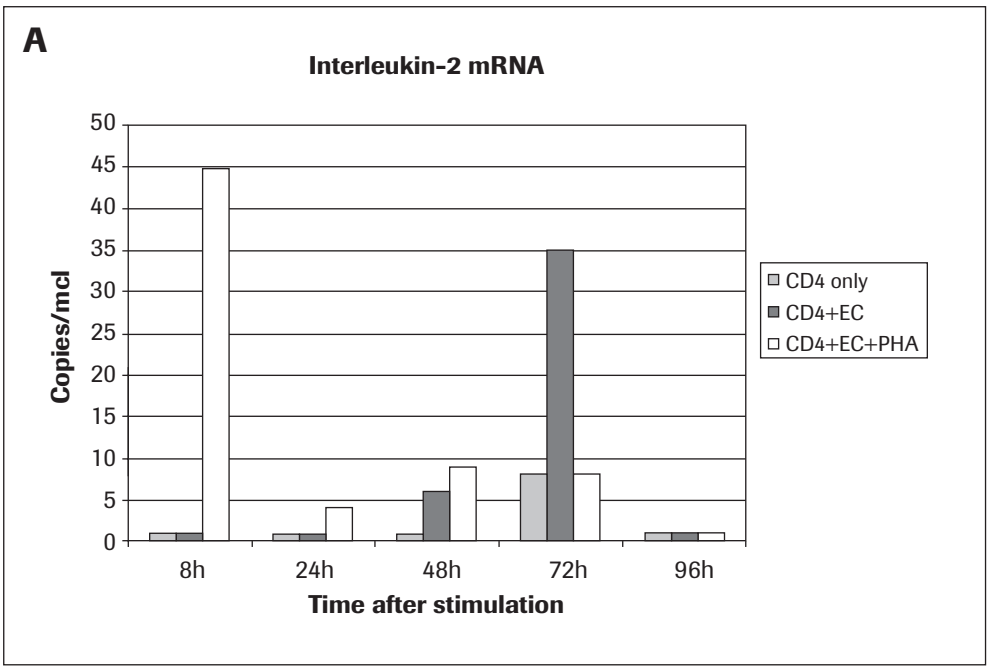
Figure 5. Expression of angiogenic factors VEGF and PlGF in HIF-1α-transduced late EPC (normalized to 1000 β-actin units, qRT-PCR).

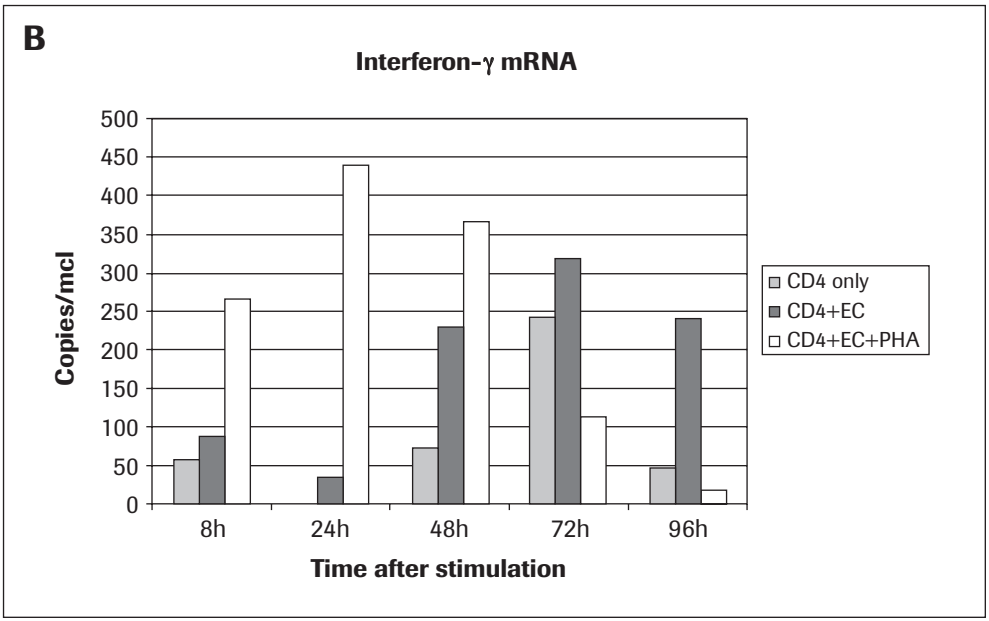
Using these assay systems, endothelial-(HUVEC)-dependent CD4 T cell activation/proliferation was extensively evaluated as it will serve as the reference standard against which T cell proliferation induced by endothelial precursors or endothelial cells derived thereof will be compared.

In the standard assay using native allogeneic HUVEC, maximum CD4 T cell proliferation was observed after 5-6 days of coculture, reaching a stimulation index (thymidine incorporation cpm of stimulated sample/cpm of unstimulated CD4 cell control) of approx. 8-15 – compared to 3-5-fold higher stimulation indices induced by allogeneic professional antigen-presenting cells (e.g. B cells). With the CD32-HUVEC-based assay using 5 µg/ml of anti-CD3 antibody, maximum proliferation measured by thymidine incorporation occurred after 48-72 hours, reaching considerably higher stimulation indices of 20-45, underscoring the better sensitivity of the CD3 mAb-based system. As this assay system is independent of the degree of MHC class II mismatch between coculture cells, the variation of proliferative responses between separate experiments was also markedly smaller than with the standard assay using native allogeneic HUVEC.

As no data on cytokine production on the mRNA level (measured by quantitative RT-PCR) have been described so far for endothelial cell-dependent CD4 T cell activation, an extensive time-course of cytokine mRNA production had to be established. In order to evaluate in detail both activation, Th1/2 polarisation and effector cell differentiation, a broad panel of signature cytokines was analysed, including: IL-2, IFN-γ, T-bet (activation, Th1 polarisation), IL-4, IL-10, GATA-3 (Th2 polarisation), CD40L (CD4 effector differentiation) and foxp3 (T regulatory cell differentiation).

Using native HUVEC without CD3 mAb, maximum cytokine mRNA production was seen as late as 72 hours after stimulation – which is considerably later than following costimulation with B cells or when adding a direct T cell receptor-activating substance such as PHA or CD3 mAb – for most cytokines analysed (Fig. 6A, B). In addition, the predominance of IL-2, IFN-γ and T-bet production (with parallel reduction in the expression of GATA-3) indicates Th1 polarisation induced by endothelial cell stimulation (Fig. 6C). Similar polarisation effects were seen when using the CD32-HUVEC system, however, cytokine mRNA was detectable markedly earlier (8-18 hours after stimulation – data not shown), underscoring the superior sensitivity and activating potential of the CD3 mAb-based coculture system (data not shown).





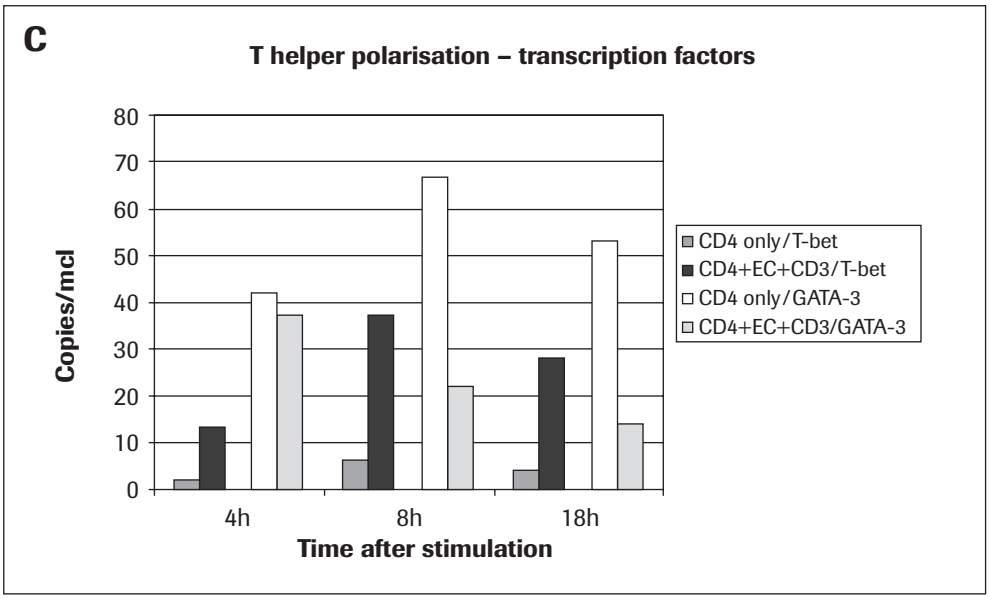


Figure 6. Cytokine mRNA production by CD4 T cells in coculture with non-transgenic, IFN-γ-stimulated HUVEC (qRT-PCR). A. *Time-course of IL-2 mRNA expression.* ***B.*** *Time-course of IFN-γ mRNA expression.* ***C.*** *Time-course of expression of mRNA for T-bet (transcription factor for Th1 polarisation) and GATA-3 (transcription factor for Th 2 polarisation).*

In some pilot experiments, stimulation of CD4 T cells by HUVEC (as described), fibroblasts (representing a non-activating cell type, incapable of T cell costimulation) and endothelial progenitors (late EPC) was compared side-by-side. While fibroblasts – as expected – did not induce significant T cell proliferation (measured by thymidine incorporation), CD4 T cell proliferation induced by late EPC reached approx. 30% of HUVEC-induced proliferation (data not shown) – suggesting a weaker T cell activating potential of this (more stem-cell like, less differentiated) cell type despite its expression of monocyte-like surface receptors that might suggest an improved T cell stimulatory potential, however, these pilot experiments still require rigorous confirmation in repeat experiments.

Ongoing studies:
Comparative activation assays of CD4 T cells stimulated by HUVEC and various types of endothelial precursors (early and late EPC, CD133-derived EPC) with selective evaluation of the costimulatory potential (CD32-HUVEC assay), detailed analysis of cytokine/chemokine production by RT-PCR or ELISA.

Aim 3: Animal models, endothelial chimerism
A hybrid vascular model of 3D cultures of endothelial cells in collagen gels placed in SCID mice was established. The use of this model has been trained and adapted from the laboratory of collaborating researchers (J. Schechner, Yale University, USA) and is now fully functional in our laboratory. For implantation into animals, gels were harvested and trisected approximately 20 hours after formation. Each resulting gel segment was implanted into a s.c. pouch in the anterior abdominal wall of a 5- to 8-week-old SCID mouse (Charles River). The wound was closed with skin staples. After 21-40 days of implantation, gels were harvested from the animals and analysed by immunohistology. Endothelial-cell-derived microvessels linked to the mouse microcirculation and were perfused with mouse blood (Fig. 7).

Angiogenesis (matrigel sprouting assay) and 3D culture experiments in collagen gels were performed comparing HUVEC and early or late EPC, showing considerably greater angiogenetic potential of EPC cells, evidenced by greater microvessel density and less cell loss when using this cell type. In ongoing studies, early/late EPC are compared to HUVEC for their properties in the *in vivo* gels after implantation into SCID mice.

Other studies currently in progress:
Preliminary experiments with injury models after 3D EC gel implantation (reconstitution of allogeneic peripheral blood lymphocytes, MHC antibody challenge). Establishment of aortic interposition grafting in collaboration with Dr. S. Ensminger, Dept. of Surgery, University of Erlangen.

Please refer to Dr. T. Dengler for detailed methods and protocols.

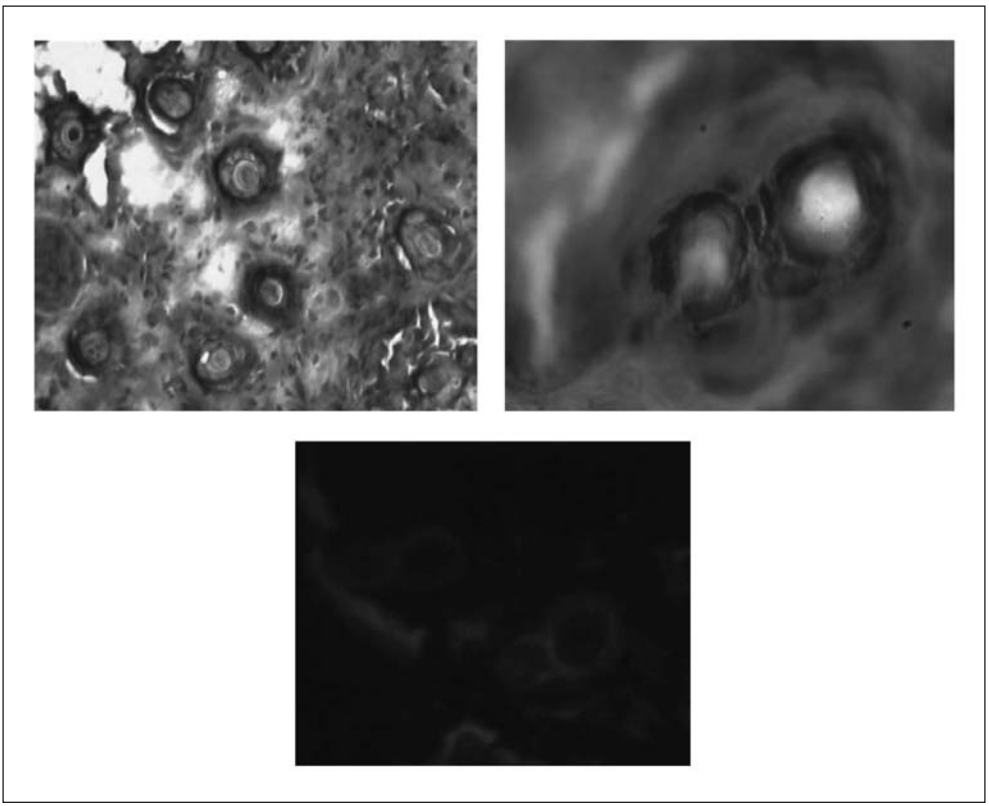


Figure 7. Three-dimensional endothelial cell "tubes" in collagen gels, 3 weeks after implantation in SCID mouse. *Ring-like endothelial cell-lined structures filled with residual cellular material from perfusing blood (upper panels). Immunohistological staining for endothelial marker protein CD31 (lower panel).*

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

Brief background to the study

Chronic graft dysfunction is now the leading clinical problem after renal transplantation. The principal histopathologic lesion seen in this disease is tubular loss with concurrent interstitial fibrosis. Although the severity of acute rejection often correlates with that of subsequent chronic dysfunction, a direct link between these processes has remained elusive.

This study was designed to test the hypothesis that intraepithelial T cells recruited to the renal tubules during acute rejection can directly induce fibrosis by causing tubular epithelial cells to undergo transition to produce a motile population of activated fibroblasts.

A preliminary study of renal allograft tissue sections showed the presence of the S100A4 marker of epithelial to mesenchymal transition in some tubular epithelial cells; the expression of this antigen was heterogeneous both within and between individual tubular cross-sections. Significantly, S100A4-expressing epithelial cells were frequently associated with infiltrating $CD8^+$ T cells, and many coexpressed the Ki67 marker of proliferation. A parallel study of human renal cortical epithelial cells in primary culture demonstrated that S100A4 was induced by stimulation for 72 hours with TGF-β1 and by direct contact with membrane-associated TGF-β on MOLT-16 cells, a model intraepithelial T cell line. Further pilot experiments demonstrated that induction of transition coincided with a significantly increased potential for human renal epithelial cells to invade the tubular basement membrane.

These data are consistent with a model in which intratubular T cells can present TGF-β and directly induce adjacent tubular epithelial cells to transform to proliferating fibroblasts that migrate across the tubular basement membrane, producing fibrotic lesions within the renal interstitium.

The purpose of the present ongoing study is to further investigate the role of allogeneic intratubular T cells in inducing epithelial to mesenchymal transition (EMT) in human tubular epithelial cells: EMT is a recently defined phenomenon that contributes significantly to interstitial fibrosis in animal models of renal fibrosis. The study will also investigate the potential for similar processes to occur during post-transplant fibrosis within lung and liver allografts. Our ultimate aim is that by defining the relationship between T cells involved in chronic graft inflammation and the processes which lead to irretrievable graft damage, we will be able to develop novel strategies to eliminate these T cells and restore organ function.

Progress in the investigation in the first 12 months

For the first phase of the study 68 biopsies were identified in which to establish semi-quantitative assessment of interstitial fibrosis and immunohistochemical procedures. This series represented a cross-section of all renal transplants performed at the Freeman Hospital between 1998 and 2000 and included a proportion of non-heart beating donor cases. Sufficient tissue was remaining for study in 42 of the biopsies and 2 nephrectomies that resulted from histopathologically defined, chronic, irretrievable changes in the tissue. Some time was spent in optimising picro-sirius red staining of interstitial collagens in both normal and diseased tissue – our co-investigating pathologist, who has used picro-sirius red extensively to 'measure' fibrosis in diseases of the liver, was involved in this optimisation. All biopsies, nephrectomies and normal controls have now been stained by this method and await analysis using Leica QWin software which has recently become available. Intensive training in the use of this software has been received by Dr. Helen Robertson and our specialist microscopist collaborator, Dr. Trevor Booth (funded by the Bio-Imaging Unit in the University of Newcastle). The QWin image analysis software is particularly suited to semi-quantitative analysis of histological staining such as that produced by picro-sirius red and immunohistochemistry. Semi-quantitative analysis of interstitial collagens will be performed within the next few months.

Immunohistochemistry has also been carried out on most of the first phase biopsies to detect S100A4, phosphoSmads (see below), CD3 and various combinations of these – the nephrectomy tissue has proved to be invaluable as positive control material and archival normal kidney tissue blocks, from kidneys not used for transplant, have been used to establish expression of various proteins in non-transplanted, normal renal tissue.

We have completed development and have validated a number of *in vitro* models to examine the role played by allospecific T cells during the induction of EMT. These include:

1. Creation of primary $CD103^+$ T cell lines which express bio-active TGF-β (and can also coexpress the regulatory cell marker FOXP3).
2. Development of electrophysiological models of epithelium function to assess the biological significance and reversibility of T cell-mediated EMT.
3. Demonstration that TGF-β elicits signalling in activated human T cells and renal epithelial cells through phosphorylation and nuclear translocation of the Smad 2 and/or 3 proteins.
4. Demonstration that BMP-7 induces phosphorylation of the antagonistic Smad 1/5/8 protein system in human renal epithelial cells (this is associated with reduction in markers of EMT).

In December 2003, a notable review[1], which has influenced the emphasis of our ongoing work, was published in JCI by leading world experts in epithelial-mesenchymal transition in kidney, Kalluri and Neilson. We have responded to the work discussed in this review to include analysis of phosphoSmad expression in both cultured cortical renal epithelial cells and tissue sections, as definitive evidence of the activity of the TGF-β family of molecules, which includes BMP-7. Preliminary results of this work are exciting – in particular the relationship between phosphoSmad and S100A4 expression in acute and chronic rejection. At present we are preparing a manuscript for publication later this year. We have also taken the opportunity to share ideas with Dr Kalluri. An important outcome of this discussion was ratification of the concept of reversibility of EMT and the idea of repopulation of tubules in the presence of the TGF-β antagonist, BMP-7.

Immunohistochemical study of the expression of S100A4, as evidence of the potential for epithelial to mesenchymal transition, has now been undertaken in explants of liver from patients undergoing liver transplants. Thus it has been possible to establish a pattern of expression of S100A4 in primary liver diseases including those with an immune component – PBC and PSC – as well as alcoholic liver disease and metastatic liver. S100A4 was absent from small bile ducts in a biopsy taken immediately prior to transplant. It is clear that S100A4 is expressed in epithelial cells of small bile ducts and in the ductular reaction and, as in the transplanted kidney, is associated with CD3 infiltrates and pSmad 2/3 expression. This study is continuing and will proceed to protocol and diagnostic biopsies from post-transplant liver.

Primary human biliary epithelial cells are now available (commercially and by local culture using methodology developed by our group). Work to establish a coculture model with TGF-β presenting T cells will begin shortly. Furthermore, we have established that S100A4 is expressed in bronchiolar epithelium during acute rejection of the transplanted lung, and studies of the regulation of EMT in cultured human airway epithelial cells have commenced.

Dissemination of results

Within the first few months of the study a manuscript stemming from preliminary work on which our application to ROTRF for funding was based was accepted for publication[2]. In addition, Professor Kirby was invited to present this work to the Annual Congress of the British Transplantation Society in April 2004. More recent data (including phosphoSmad expression) was presented at the American Transplant Congress in Boston in May 2004[3,4]. To attend the ATC, Dr. Robertson received funding from both the Royal Society (UK) and the British Society for Immunology in addition to a contribution from the University of Newcastle. In August 2004, Prof. Kirby talked at the XX International Congress of the Transplantation Society in Vienna on epithelial to mesenchymal transition, also by invitation. In all of these publications and presentations we have gratefully acknowledged ROTRF funding. Also at the Congress in Vienna, another member of our group (Dr. Chris Ward) presented early observations of EMT processes within airway epithelium following lung transplantation[5].

In addition to our published studies of EMT following renal transplantation, we are currently preparing manuscripts showing results of a detailed analysis of EMT during vanishing bileduct syndrome in the liver, and bronchiolitis obliterans in the transplanted lung – both of these observations are novel.

For the first time, the description of EMT in human renal allograft recipients was published in JASN earlier this year[6].

Publications

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NK Cells in Transplantation

The original specific aims of this grant were: (1) to analyze the role of NK cells post-transplant, (2) to determine the expression of NK cell activation receptors after transplant and (3) to determine if signaling through NK cell receptors induces cytokine production and cytotoxicity. We made important progress and have significant findings in each of these aims.

NK cells infiltrate liver allografts early after transplantation[1]

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[2]. LIMC were labeled with monoclonal antibodies against NKR-P1 and TCR-$\alpha\beta$ for analysis by two-color flow cytometry. One day after transplantation NKR-P1 NK cells constitute 56.4 $\pm$ 6.2% (n=3) of the LIMC isolated from the allograft while 18.6 $\pm$ 3.0% (n=3) of the LIMC are α/β TCR T cells. To determine if the NK cells are of donor or recipient origin NK cells were labeled with anti-$RT1^{a,b}$ antibodies. At 24 hour post-transplant 22.9 $\pm$ 6.1% (n=3) of the LIMC are NK cells of recipient origin ($RT1^{a,b}$ negative) demonstrating that a substantial population of recipient-derived NK cells infiltrate allogeneic liver grafts during rejection.

To further evaluate the kinetics of NK cell infiltration after transplantation, LIMC isolated from allografts were analyzed for the proportion of NK cells at multiple time points post-transplant (6 and 12 hours, 1, 2, 3, 5, and 7 days post-transplant). NK cells of recipient origin infiltrate the grafts as early as 6 hours post-transplant. The proportion of NK cells peaked within the first 24 hours post-transplant and decreased by day 2 as the proportion of T cells increased in the allograft. Interestingly, we noted that the proportion of recipient-derived NK cells began to increase again 5 days after transplant in allografts and comprised 26.6 $\pm$ 8.3% of the total number of cells at day 7 post-transplant. In syngeneic liver grafts the proportion of NK cells reaches its peak at 12 hours post-transplant and then decreased to pre-transplant levels suggesting an innate response to transplant injury is important in the early infiltration of NK cells. These data suggest that there is a bimodal infiltration of NK cells into liver allografts, early as a result of non-specific surgical stress and later at the time that effector cells are infiltrating the allograft.

Since IFN-γ is a key immunoregulatory cytokine that promotes acute rejection through upregulation of MHC expression, direct cytotoxicity and induction of chemokine expression we analyzed the levels of serum IFN-γ in the first 7 days post-transplant. Peak levels of IFN-γ in the serum of allograft recipients were detected on day 3 post-transplant. NK cells, in addition to T cells, are capable of IFN-γ production. To determine more directly which cells in the graft produce IFN-γ we isolated LIMC from allografts and performed cell-surface and intracellular staining using monoclonal antibodies against NKR-P1, TCR-α/β, RT1A^{a,b}, and IFN-γ. Flow cytometric analyses revealed that 18% of NK cells and 9% of T cells in the graft produced IFN-γ at day 2 post-transplant (Fig. 1).

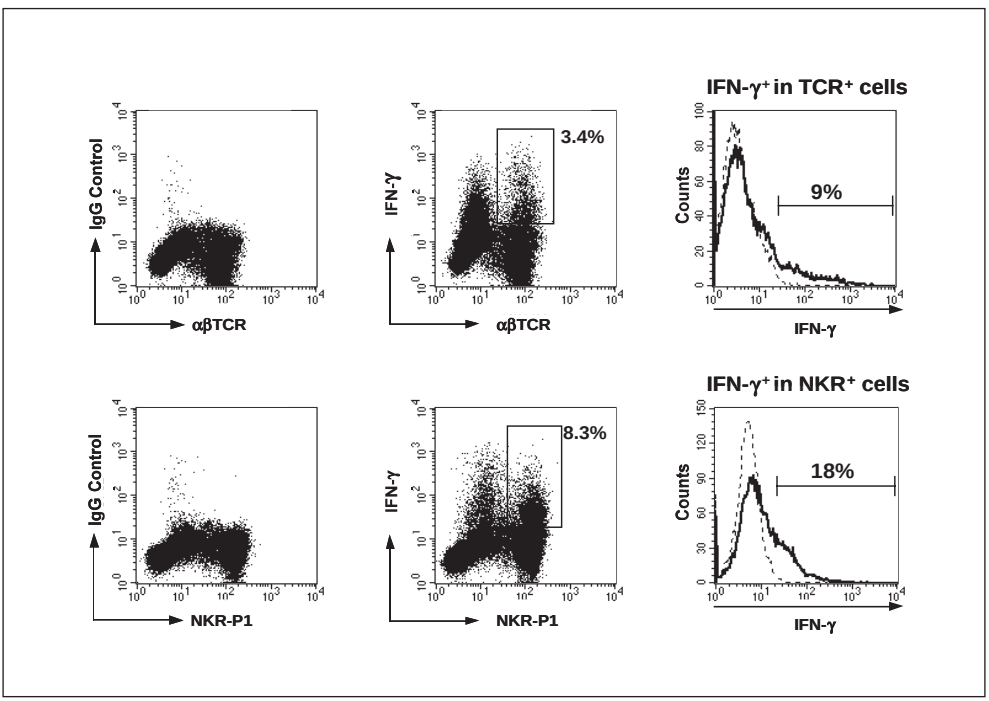

Figure 1. Expression of IFN-γ by T cells (top) and NK cells (bottom) isolated from the graft. *T cells and NK cells were isolated directly from an allograft 2 days post-transplant and analyzed for IFN-γ expression by intracellular cytokine staining. The isotype control is shown on the left and by the light dashed line in the histogram. Note that on day 2 post-transplant NK cells are a relatively small component of the infiltrate as compared to T cells. Similar results were obtained in three separate experiments.*

It should be noted that these values are for cells taken directly from the allograft without additional *in vitro* activation. These data, along with our previous studies, suggest that recipient-derived NK cells recruited to the allograft early after transplantation produce IFN-γ, which can induce expression of the chemokines IP-10, fractalkine and MCP-1α further promoting the infiltration of NK cells and other potential effector cells. Innate immunity and, NK cells in particular, may be important in the early events leading to allograft rejection.

Graft survival is prolonged in the absence of NK cells

To define the role of NK cells after liver transplantation we studied the effect of AGM1 on allograft survival. Allograft recipients treated with a single dose of AGM1 on day 1 had significantly prolonged survival compared to control rats. We demonstrate that treatment with AGM1 results in prolonged survival of all liver allograft recipients and that 30% of these rats demonstrate indefinite survival and donor specific non-responsiveness. In AGM1-treated rats who survived for >100 days, we performed skin grafts from both DA (donor) and PVG (third party) rats to investigate whether donor-specific tolerance was induced. As expected, the PVG (third party) graft was rejected at 14 days. In contrast, the DA (donor) graft showed no evidence of rejection.

Rapamycin inhibits NK cell function

The direct effects of immunosuppressive drugs on NK cell function remains unclear. NK cells were purified from normal Lewis rat spleens and primary NK cell lines were established by standard protocols and analyzed for proliferation in the presence of increasing doses of CsA (0-100 ng/ml), FK506 (FK, 0.1-100 ng/ml) or rapamycin (RAPA) (0-10 ng/ml). NK cells demonstrated robust proliferation both in the absence and presence of CsA (data not shown). In experiments conducted in parallel we confirmed that CsA inhibited proliferation in an MLR (data not shown). In contrast, FK inhibited proliferation only at high doses and RAPA significantly ($p < 0.05$) inhibited the proliferation of NK cells in a dose-dependent manner. Since IFN-γ is such a pivotal cytokine in the response to alloantigen and since we have shown NK cells produce abundant IFN-γ in allografts, we analyzed the effects of CSA, FK and RAPA on IFN-γ production by NK cells. Primary NK cells secrete robust levels of IFN-γ. Neither CSA, FK nor RAPA inhibits IFN-γ production by NK cells (data not shown).

To determine if immunosuppressive drugs alters the ability of NK cells to kill, cytotoxicity against tumor targets was analyzed both in the presence and absence of immunosuppressive drugs (CSA, 100 ng/ml, FK, 100 ng/ml and RAPA, 10 ng/ml). CSA and FK did not effect NK cell–mediated killing while cytotoxicity was significantly ($p < 0.05$) inhibited in the presence of RAPA (Fig. 2).

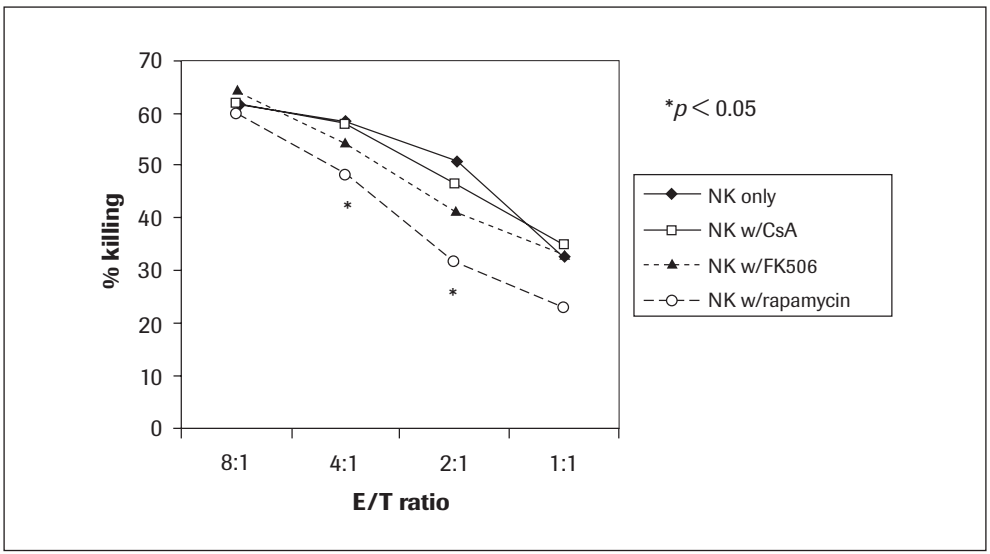

Figure 2. Effects of immunosuppressive drugs on cytotoxicity. *Primary NK cells were isolated and purified prior to incubation with CsA (100 ng/ml), FK506 (1.0 ng/ml), or RAPA (1.0 ng/ml) for 24 hours. Cells were counted and replated with labeled Yac-1 targets at the E:T ratios indicated (X-axis). The % killing (experimental-spontaneous) is plotted on the Y-axis. The variability between triplicates was <10%. These data are representative of three separate experiments.*

These data indicate that activated NK cells may contribute to the rejection response in CSA- and FK-treated allograft recipients. Furthermore, NK cells retain effector function in the presence of CSA and FK and thus could actively contribute to the elimination of virally infected or transformed cells in transplant patients.

In contrast, NK cell proliferation and killing are significantly diminished by RAPA treatment. Understanding the differential effects of these immunosuppressive drugs on NK cell effector function is important in clinical transplantation.

Generation of anti-rNKp30 monoclonal antibodies

Since rNKp30 is a novel molecule and antibodies were not available, we initially raised both anti-peptide and anti-recombinant rNKp30 rabbit antisera that have been quite useful[3]. Additionally, we have generated a panel of nine anti-rNKp30 mAbs using a fusion protein of the extracellular domain of NKp30 and human IgG1-Fc as the immunogen. All of these antibodies are IgG1,κ and bind specifically to rNKp30-transfected 293 cells, rNKp30-transfected RNK-16 cells (rat NK cell line), IL-2-activated rat NK cells and NK cells isolated from rat PBL and spleen (Fig. 3 and data not shown).

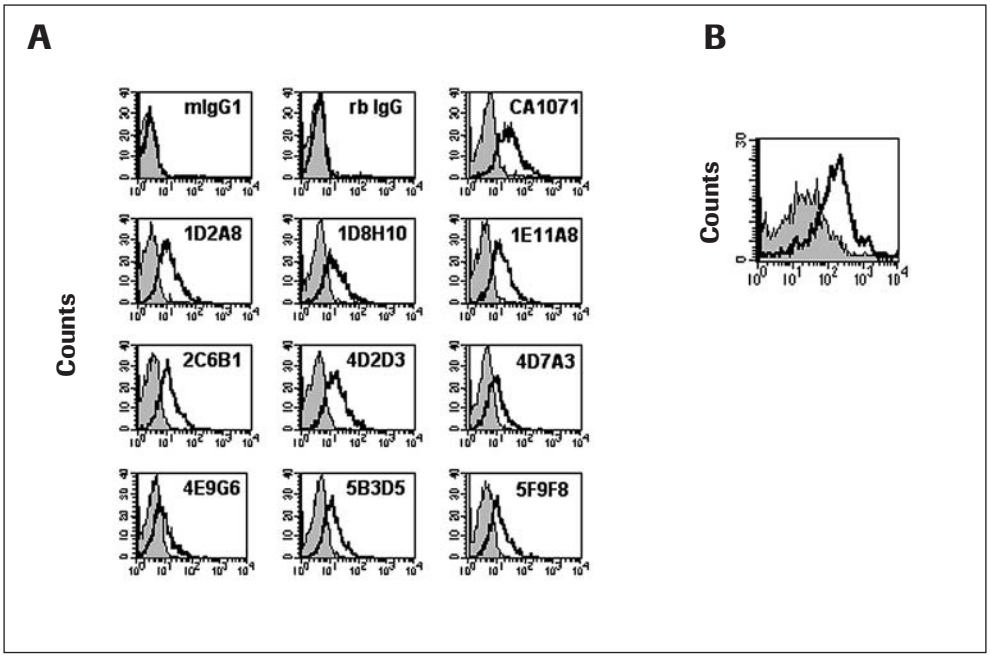

Figure 3. Mouse anti-rat NKp30 monoclonal antibodies recognize rNKp30 on the surface of transfected RNK-16 NK cells (A) and rat splenic NK cells (B). A. *NKp30 mAbs (last three rows) were used to stain wild-type NKp30⁻ RNK-16 cells (filled histogram) or a stably transfected clone of NKp30⁺ RNK-16 cells (empty histogram). All NKp30 mAbs were biotinylated and a streptavidin-PE secondary reagent was used to detect the antibody. The top row shows staining with the isotype control (mIgG1), rabbit antisera (rbIgG), and our polyclonal anti-rNKp30 antisera (CA1071).* ***B.*** *Mouse anti-rat NKp30 mAb clone 5F9F8 recognizes NKp30 on the surface of rat splenic NK cells (NKR-P1⁺, CD8⁺). Staining with the isotype control (filled histogram) is shown. Similar results were obtained for the other antibodies shown in* ***A****.*

RNK-16 NK cells expressing rNKp30 are cytotoxic

The rat leukemic cell line, RNK-16 is an NK cell line that demonstrates many of the effector functions of primary NK cells. For this reason this cell line has been a valuable resource to specifically analyze the functional properties of transfected human and murine NK cell receptors[4]. Since we determined that RNK-16 does not express an endogenous rat NKp30 receptor we transfected RNK-16 with a flag-tagged rNKp30. RNK-16 cells growing in log-phase were electroporated with FLAG-rNKp30 pCDNA3.1. Stable clones were selected in G418-containing media and analyzed for rNKp30 expression by flow cytometry and Western blot. Importantly RNK-16 cells stably transfected with rNKp30 are functionally cytolytic against YAC-1 target cells (data not shown) and demonstrate specific cytotoxicity in a redirected lysis assay. The functional activity of NKp30 was determined by a FcγR-dependent redirected lysis assay using rNKp30⁺ RNK-16 cells (clone 2B3) as effector cells and labeled FcR⁺P815 target

cells. Lysis of the target cells was augmented by treatment of the effector cells with anti-rNKp30 antibody suggesting that rNKp30 activated cytotoxicity. RNKp30-mediated cytotoxicity is specific as antibodies directed to other molecules expressed on RNK-16 (for example CD8) did not induce killing (Fig. 4A). Moreover, rNKp30-mediated cytotoxicity is dose-dependent since increased killing is observed with increased amounts of NKp30-specific antibody (Fig. 4B). These data are extremely important since they show for the first time that NKp30 stimulation of rodent NK cells activates cytotoxicity.

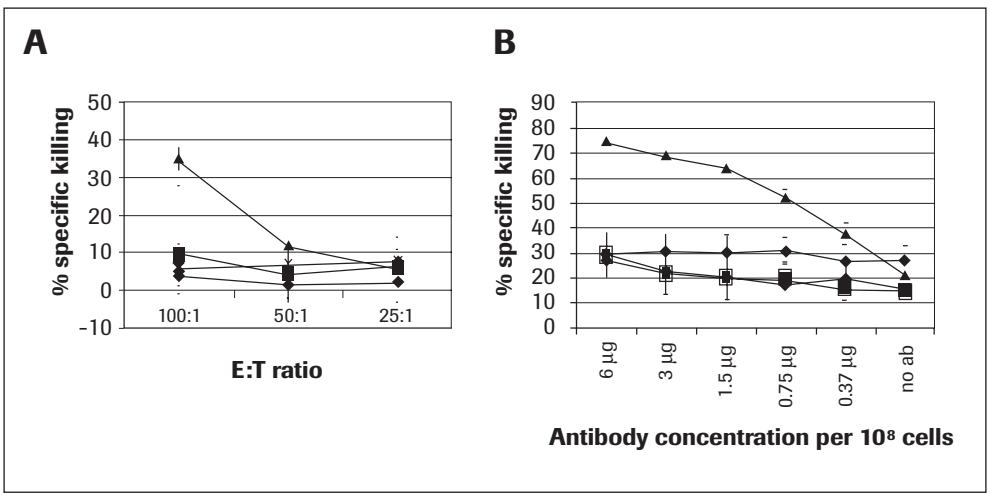

Figure 4. NKp30 activates cytotoxicity. *Four hour killing assays were performed using rNKp30⁺ RNK-16 cells as effector cells and labeled FcR⁺P815 target cells.* ***A.*** *The effector cells were incubated with 2 µg/10⁶ cells of anti-rNKp30 antibody (triangles), an isotype control (diamonds), an irrelevant antibody (aCD30, squares), or an antibody that recognizes another receptor on rat NK cells (αCD8, stars) and plated at various E:T ratios.* ***B.*** *The effector cells were incubated with increasing concentrations of anti-rNKp30 antibody (triangles) or isotype control (diamonds). As an additional control, untransfected RNK-16 cells were incubated with anti-rNKp30 antibody (squares) or isotype control (circles) and plated at the same 100:1 E:T ratio.*

NKp30 activation leads to IFN-γ production

We demonstrate that rNKp30 activation induces the secretion of cytokines from rNKp30-transfected RNK-16 cells, rat PBL and rat splenocytes (Fig. 5 and data not shown). Antibodies directed to rNKp30 induced production of IFNγ. Interestingly, antibodies specific for other rat NK activation receptors NKR-P1A and Ly49s (which we have confirmed are expressed on the surface of the NK cells) did not induce IFN-γ production.

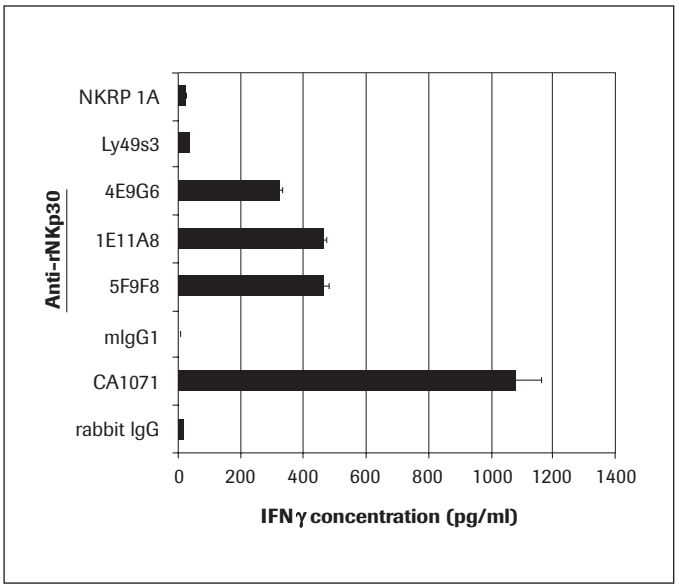

Figure 5. NKp30 activation induces IFN-γ production from rat splenocytes.
Freshly isolated rat splenocytes were cultured with plate-bound antibodies for 24 hours and supernatants were assayed by ELISA for rat IFN-γ production. Three anti-rNKp30 mAbs (4E9G6, 1E11A8, and 5F9F8) and the anti-rNKp30 antisera (CA1071) induced production of IFN-γ. Antibodies specific for the rat activation receptors, NKR-P1A and Ly49s3 did not induce IFN-γ production. Incubation of splenocytes with control anti-bodies (mIgG1 and rabbit IgG) did not result in IFN-γ production.

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3. Hsieh CL, Ogura Y, Obara H, Ali UA, Rodriguez GM, Nepomuceno RR, Martinez OM, Krams SM. Identification, cloning, and characterization of a novel rat natural killer receptor, RNKP30: a molecule expressed in liver allografts. *Transplantation* 2004; 77:121-8.
4. Ryan JC, Niemi EC, Nakamura MC. Functional analysis of natural killer cell receptors in the RNK-16 rat leukemic cell line. *Methods Mol Biol* 2000; 121:283-95.

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2. Hsieh CL, Ogura O, Obara H, Ali U, Su WW, Rodriguez G, Nepomuceno R, Martinez OM, Krams SK. NK receptor, rat NKp30, is a polymorphic glycoprotein involved in the immune response during allogeneic liver transplantation. *Am J Transplant* 2004; 4(8):510.
3. Obara H, Martinez OM, Ogura Y, Hsieh CL, Esquivel CO, Krams SM. NK cells recruited to liver allografts are a source of IFN-γ. *Am J Transplant* 2004; 4(8):249.
4. Hsieh C, Obara H, Martinez OM, Krams SM. Rat NK receptor, rNKp30, is expressed in liver allografts and can mediate cytotoxicity. *Transplantation* 2004; 78(2):153.

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Dr. Nancy Ruddle, Research Associate
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Immunologic Ignorance of Transplanted Organs

Specific Aim 1: To test whether tertiary lymphoid organs generated by ectopic production of lymphotoxins and/or chemokines at the site of transplantation break immunologic ignorance

Significant progress has been made. Ectopic production of lymphotoxin-α in a skin allograft (using the RIP-LTa transgenic mouse as a skin donor) caused lymphoid neogenesis in the graft and precipitated rejection in a host that lacks secondary lymphoid tissues. Evidence for the generation of effector and memory cell function is provided. Therefore, these results indicate that tertiary lymphoid organs break ignorance by initiating alloimmune responses within the graft. We are currently performing experiments where congenic Thy1.1 naïve T cells are transferred to test whether they differentiate into effector and memory T cells after encountering alloantigen within tertiary lymphoid organs.

Specific Aim 2: To test whether intragraft inflammation, caused by either innate or adaptive immune responses leads to the genesis of tertiary lymphoid organs

Significant progress has been made. The manuscript abstract is shown below[1].

Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection

Lymphoid neogenesis is the process by which ectopic lymphoid accumulations that resemble lymph nodes arise in non-lymphoid tissues. Such lymphoid accumulations, known as tertiary lymphoid organs, are observed in chronic autoimmunity and they propagate immune pathology by setting up local antigen presenting sites. Whether lymphoid neogenesis occurs in transplanted organs and contributes to rejection is not well understood. To begin to address this question, we retrospectively analyzed 319 murine cardiac allografts for microscopic evidence of lymph node-like structures. We found 78 allografts that had either classical tertiary lymphoid organs, characterized by discrete T and B cell zones and high endothelial venules (HEV) expressing peripheral node addressin (PNAd) (n=34), or PNAd$^+$ HEV without organized lymphoid accumulations (n=44). These changes were present in both short- and long-lived allografts and were invariably associated with rejection. Importantly, they occurred in 78% of allografts undergoing chronic rejection (n=85) but in only 7% of allografts

undergoing primarily acute rejection (n=184). These findings indicate that, like autoimmunity, alloimmunity is associated with lymphoid neogenesis in the target organ and suggest a role for local T cell activation in chronic allograft rejection.

Publication

1. Baddoura FK, Nasr IW, Wrobel B, Li Q, Ruddle NH, Lakkis FG. Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection. *Am J Transplant, in press.*

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

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The Role of Chemokines in Rejection and Bronchiolitis Obliterans Following 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 IP-10 and Mig in allogeneic tracheal transplants. The importance of these chemokines for the development of acute rejection (AR) and bronchiolitis obliterans (BO) was determined by demonstrating decreased tracheal obliteration, fibroproliferation, and inflammation when tracheas were transplanted into CXCR3$^{-/-}$ (the receptor for IP-10 and Mig) mice. We have started to perform experiments in IP-10$^{-/-}$ and Mig$^{-/-}$ mice using similar analysis. These studies have demonstrated a small reduction in the number of lymphocytes recruited into tracheal transplants into Mig$^{-/-}$ recipients but normal lymphocyte recruitment when transplants are performed into IP-10$^{-/-}$ mice. Tracheal transplants into IP-10$^{-/-}$ and Mig$^{-/-}$ mice however have similar fibrosis and luminal obliteration compared to transplants into wild-type recipients.

In separate experiments we have begun to investigate the mechanisms that lead to early upregulation of IP-10 in tracheal allografts. In the original proposal we presented data demonstrating that the early IP-10 expression was derived from donor tissue and partly dependent on NF-κB. Subsequent experiments have shown that hypoxia is an important factor driving the expression of IP-10 in the airways.

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 lungs 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 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 no deaths in the CC10-OVA recipients compared to 100% mortality in the CC10-OVA mice that received wild-type CD8 cells (Fig. 1).

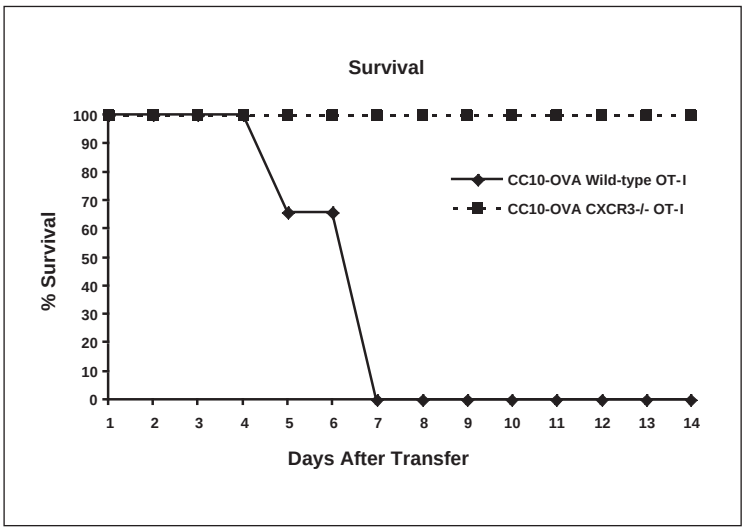

Figure 1. Survival of CC10-OVA mice after injection of either activated wild-type OT-I cells or activated CXCR3$^{-/-}$ OT-I cells.

We have also bred the CC10-OVA mice with Mig$^{-/-}$ mice to form 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. 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, AR, BO, 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 (Table 1).

	NL CD4+	NL CD8+	BO CD4+	BO CD8+
CCR3	9.6±1.5	4.0±0.9	30.9±6.3*	15.9±5.1*
CXCR3	57.1±5.3	69.1±6.1	59.3±7.1	58.0±7.3
CCR4	26.9±3.6	3.4±0.8	38.9±4.8*	11.9±4.3*
CXCR4	47.2±7.5	34.3±6.5	82.2±5.4*	64.3±8.2*
CCR5	48.9±4.6	64.0±4.5	61.0±6.4	61.0±6.7

*$p < 0.05$ BO vs. NL.

Table 1. Chemokine expression profile on BAL lymphocytes (percentage of lymphocytes).

Significance

During the first year of this proposal we have continued to define the molecular signals that control leukocyte 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 preliminary data suggests that expression of Mig and IP-10 are redundant factors in the generation of the acute rejection response.

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 hypoxia may mediate the expression of IP-10 either directly and/or through an NF-κB 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: More quantitative studies on the importance of IP-10 and Mig in the tracheal transplant models are planned. These include detailed flow cytometry, histology, and other measures of fibrosis. We will continue to define the mechanisms of IP-10 upregulation in the model with an emphasis on potential mechanisms by which hypoxia leads to IP-10 expression.

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 knockout 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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Role of Minor Histocompatibility Antigens in Solid Organ Transplant Rejection

Summary

The complex host immune system does not and will not discriminate between an invading pathogen and that of a life-saving allograft. Advances in the past decades have helped to greatly appreciate multiple facets of the immune system and have provided ample tools to manipulate the same. Matching the alleles of the major histocompatibility antigens (MHC) vastly improved the clinical outcomes of transplantations. The role of host-derived T cells in recognizing unique antigenic peptides/MHC complexes on the cells from the grafts has been understood as the major host immune mechanism involved in graft rejections. Although the immune responses mediated by the donor-derived T cells results in destruction of host tissue, graft-versus-host-disease (GvHD), the therapeutic benefits of these T cells in clearing malignant tumors, graft-versus-leukemia (GvL) in patients is extremely attractive as a potential treatment tool. Since T cell responses in MHC-mismatched combination are too deleterious to the host body, a more specific, targeted immune response is sought to harness the capabilities of T cells against malignancies. One such method is to utilize T cells that recognize antigenic peptides generated due to polymorphic differences in *non-MHC loci*. Historically, the antigenic peptides that are generated due to these *'minor'* polymorphic differences in the host and recognized by MHC-matched, donor-derived T cells are defined as 'minor histocompatibility antigens, (mH-Ag)'. Recent findings demonstrate that apart from providing antigenic peptides, full-length proteins of few mH-Ags can also elicit specialized immunological functions. This review discusses a paradigm-shift currently undergoing in the immunobiology of mH-Ags.

Our research supported by ROTRF focuses on two major aspects of minor histocompatibility antigens:

A) The role of minor antigen-derived peptides in eliciting allo-T cell responses and
B) The role of full-length minor histocompatibility antigens in eliciting effector cell functions such as NK cell activation.

Our findings are presented here as a series of manuscripts either published or in preparation.

A) Role of minor antigen-derived peptides in eliciting allo-T cell responses

We have identified and characterized two novel minor histocompatibility antigens, H4 and H7 during the project period of the ROTRF. Two manuscripts were prepared and one has been published[1] and the other is under review[2]. We are in the process of preparing a third manuscript on the mechanism by which a differential proteasomal cleavage of antigenic epitope determines the allo-T cell responses[3].

B) Role of full-length minor histocompatibility antigen, H60 in eliciting effector cell functions such as NK cell activation

Natural killer (NK) cells are the most important effector cells of the innate immune system. NK cells mediate cytotoxicity against infected, transformed cells or normal bone marrow and other tissue grafts by scanning for the normal expression levels of MHC class I molecules on the target cells. NK cells use an array of inhibitory Ly49 receptors to discriminate *'self'* from *'missing-self'* on the basis of the levels of MHC class I molecules. Loss of MHC class I molecules on target cells relieves the murine NK cell of Ly49-mediated inhibition, thus allowing the NK cells to mediate cytotoxicity. Murine NK cells also express NKG2D receptor as their major activating receptor that interact with non-classical MHC class I molecules such as minor histocompatibility antigen, H60 on the target cells. Thus, NK cell function is defined as the outcome of the interplay between the activating and inhibitory receptors.

We have analyzed the ability of NK cells to recognize and eliminate target cells under different MHC mismatched conditions in murine models. The levels and types of MHC class I greatly influenced the ability of NK cells to get activated[4-6].

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Additional Publications

1. Malarkannan S and Pooler LM. Minor H Antigens: Molecular Barriers for successful Organ Transplantations. Ed: Drs D. Wilkes and W. Burlingham, *Immunobiology of Organ Transplantation.* 2004; 7:71-105, Kluwer/Plenum Press.
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Prof. James McCluskey, Principal Investigator
Dr. Jamie Rossjohn, Co-Investigator
Dr. Andrew Brooks, Co-Investigator
Dr. Anthony Purcell, 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 (TcR) 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 characterized by viral latency and periods of viral replication that are kept in check by a strong antiviral cytoxic 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 FLRGRAYGL (FLR) from EBNA-3 protein. Despite a potential repertoire of $>10_{12}$ $\alpha\beta$ TcR, the HLA B8-restricted cytolytic T cell response to the FLR latent antigen 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 $a\beta$ clonotype that can be present to levels up to 10% of infected individuals. These same CTL 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 allorecogntion 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 crystallizes 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 sidechain 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-antigen loading complex or in T cell receptor signalling.

We next determined the crystal structure of the anti-HLA-B*/FLR–specific "public" TcR to 1.5 Å, 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 antigen specificity of the receptor. Upon recognising antigen, the immunodominant TcR undergoes extensive conformational changes in the complementarity determining regions (CDRs), including the disruption of the canonical structures of the germline-encoded CDR1α and CDR2α loops to produce an enhanced fit with the HLA-peptide complex. TcR ligation induces conformational changes in the TcRα 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.

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. 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.

Future goals

Our current aim 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).

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1. Kjer-Nielsen L, Clements CS, Purcell AW, Brooks AG, Whisstock JC, Burrows SR, McCluskey J, Rossjohn J. A structural basis for the selection of dominant αβ T cell receptors in antiviral immunity. *Immunity* 2003; 18(1):53-64.
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3. Webb AI, Dunstone MA, Chen W, Aguilar MI, Chen Q, Jackson H, Chang L, Kjer-Nielsen L, Beddoe T, McCluskey J, Rossjohn J, Purcell AW. Functional and structural characteristics of NY-ESO-1 related HLA-A2 restricted epitopes and the design of a novel immunogenic analogue. *J Biol Chem* 2004; 279(22):23438-46.
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5. Webb AI, Borg NA, Dunstone MA, Kjer-Nielsen L, Beddoe T, McCluskey J, Carbone FR, Bottomley SP, Aguilar MI, Purcell AW, Rossjohn J. The structure of H-2K(b) and K(bm8) complexed to a herpes simplex virus determinant: evidence for a conformational switch that governs T cell repertoire selection and viral resistance. *J Immunol* 2004; 173(1):402-9.

Dr. Anette Melk, Principal Investigator

University of Heidelberg, Heidelberg, Germany

The Importance of Senescence Marker p16^{INK4a} for the Survival of Renal Allografts

Aim of the study

To evaluate whether p16^{INK4a} (p16) limits the ability of renal tissue to withstand peri- and post-transplant stresses and thereby predisposes to the development of allograft nephropathy.

Results

The focus of our work during the first ten months of support has been on starting the transplantation and ischemia-reperfusion experiments in the two different knockout (KO) mouse strains (single p16 KO and double p16/p19^{ARF} KO mice) and appropriate control mice. For this, both strains had to be brought through embryo transfer and are currently bred and aged at the University of Heidelberg's animal facility. In the previous experiments, we had transplanted CBA kidneys into C57BL/6 recipients: We were able to show that p16 mRNA and protein expression significantly increased already 7 days after transplantation in kidneys from old donors when compared to young donor kidneys. Twenty-one days after transplantation an increase in p16 was also seen in young donor kidneys. Since both KO strains are on a C57BL/6 background, we have to confirm these results in a different donor-recipient combination. We have started our transplantation experiments by transplanting C57BL/6 donor kidneys into C3H/He recipients. In this combination that is across full MHC barriers, we receive a full-blown acute rejection comparable to what we had seen in the CBA-C57BL/6 combination. In addition, we are currently transplanting p16/p19 KO donor kidneys into C3H/He recipients. However, we prefer not to discuss this data yet due to the small number of KO mice transplanted in each subgroup. We have also begun the ischemia-reperfusion experiments and are currently performing time courses on C57BL/6 to establish the optimal time point for harvesting the kidneys after injury.

In addition to these experiments, we evaluated the importance of hypertension for the development of senescence. These studies seem particularly important as hypertension is not only a risk factor for allograft nephropathy but also an age-related disease impairing renal function. We studied kidneys from male uninephrectomized Sprague-Dawley rats that either received 1% NaCl and DOCA (100 mg over 6 weeks, n=21) or 1% NaCl alone (controls, n=7). After four weeks, 14 DOCA-treated rats received either spironolactone (SPL, n=7) or

triple therapy (TRP, hydrochlorothiazide, reserpine, hydralazine, $n=7$) for the final two weeks. We studied histopathology and p16 expression (by RT-PCR + immunohistochemistry). DOCA-treated rats developed marked tubular atrophy, interstitial fibrosis and glomerulosclerosis that was alleviated but not reversed by antihypertensive treatment. p16 mRNA expression was 63-fold higher in DOCA-treated rats when compared to controls. Neither treatment significantly reduced p16 expression. p16 mRNA and protein expression showed similar results. Thus renal cells develop irreversible cell-cycle arrest due to mineralocorticoid hypertension that cannot be reversed by antihypertensive treatment. We propose: (1) p16 expression induced by hypertension limits replication and repair and triggers tubular atrophy, interstitial fibrosis and glomerulosclerosis and (2) antihypertensive treatment started after 4 weeks of uncontrolled hypertension is unable to reverse already present p16 expression, but prevents a further increase in p16[1].

In summary, we have begun all aspects of the project. We have interesting new data regarding the relationship between hypertension and senescence. These results may explain the poorer performance of kidneys from hypertensive donors despite good blood pressure control after transplantation.

Publication

1. Abstract submitted to the American Society of Transplantation Meeting 2005.

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 with siRNA

Background and research proposed

T cell-mediated immune rejection is the major barrier in organ transplantation. The immune rejection is mediated by professional antigen-presenting cells called dendritic cells (DC) that initial direct rejection pathway through donor DC and indirect rejection pathway through recipient DC. The presently funded proposal aims to develop new methods for preventing graft rejection through specifically modifying immune reactive genes in DC using a novel gene silencing method, called siRNA.

Work accomplished to date

We have been very successful at initiating this project and accomplishing the initial aims of these experiments. Our achievements to date:

1. We have identified specific silencing sequences of immune genes, including MHC II, CD40, CD80, CD86, IL-12 and RelB, as we proposed in our proposal.
2. We have optimized gene silencing methods. We have currently established 4 new siRNA strategies to silence DC. i) commercially pre-synthesized siRNA (Dharmacon Inc) consisting of 21 base-pair oligonucleotides (Fig. 1), which are ready for use but are costly; ii) siRNA expression vectors (pSilencer™, Ambion Inc) with a *pol III* promoter that drives hairpin RNA expression to form a double-stranded RNA that serves as an endogenously expressed siRNA. Large amounts of pSilencer-siRNA can be prepared through standard molecular cloning techniques for *in vitro* and *in vivo* gene silencing. iii) siRNA-expression cassettes (SEC), which are generated as PCR products consisting of a hairpin siRNA template flanked by promoter and terminator sequences. Once the SEC is transfected into cells, the hairpin siRNA is expressed from the PCR product and leads to gene silencing (data not shown). The advantage of SEC resides in the fact that it is extremely time efficient, which will enable us to rapidly screen for the most potent siRNA amongst many candidate sequences. iv) SEC-vectors. Since SEC yields tend to be small, the effective SEC can be subsequently cloned into a viral or non-viral vector.

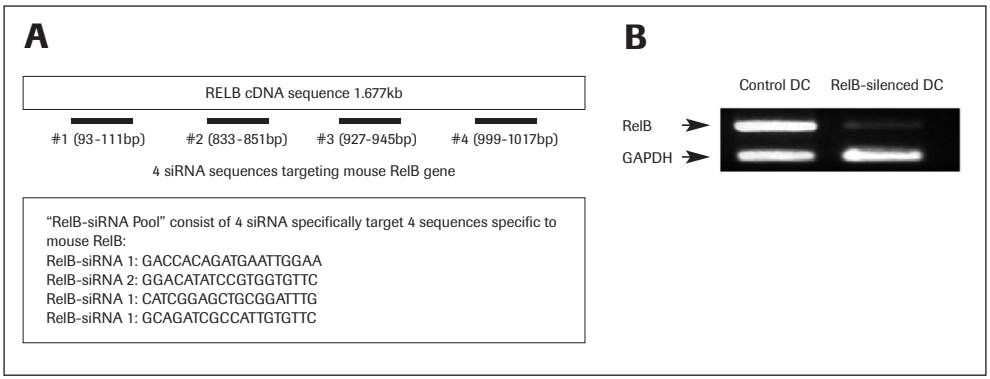

Figure 1. Silencing DC by synthesized siRNA. A. *4 siRNA sequences specific for the murine RelB gene were synthesized and pooled together for DC silencing.* ***B.*** *DC were cultured from bone marrow and transfected with Rel-B-siRNA pool (60 picomoles) on day 7, using GenePorter. 48h after gene-silencing, total RNA was extracted from transfected, non-transfected and mock transfected DC. RT-PCR was performed using the following primers: Sense: CCGAGCTAGGGGCCTTGGGTTCC, Antisense: AGCTCGATGGCGGGCAGGGTCTTG.*

3. We have validated gene silencing by siRNA in DC *in vitro* and *in vivo*. We have compared several siRNA delivery methods, tested persistence of gene silencing in DC (Fig. 2), and succeeded multiple gene silencing in DC (Fig. 3).

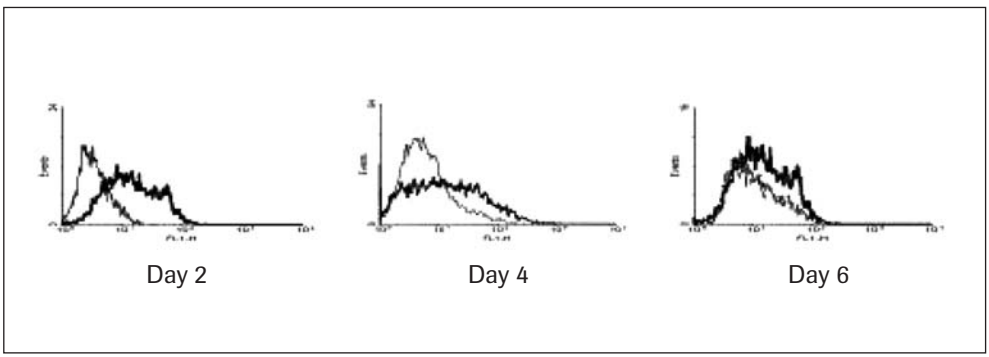

Figure 2. Perstinence of gene-silencing in DC. *DC were cultured for 4 days in 6-well plates. pSilencer-MHC II was added to the culture without transfection reagents. DC were collected at indicated times and stained with anti-IA^b-FITC. The expression of MHC was analyzed by flow cytometry comparing control DC (bolded lines) and silenced DC (fine lines).*

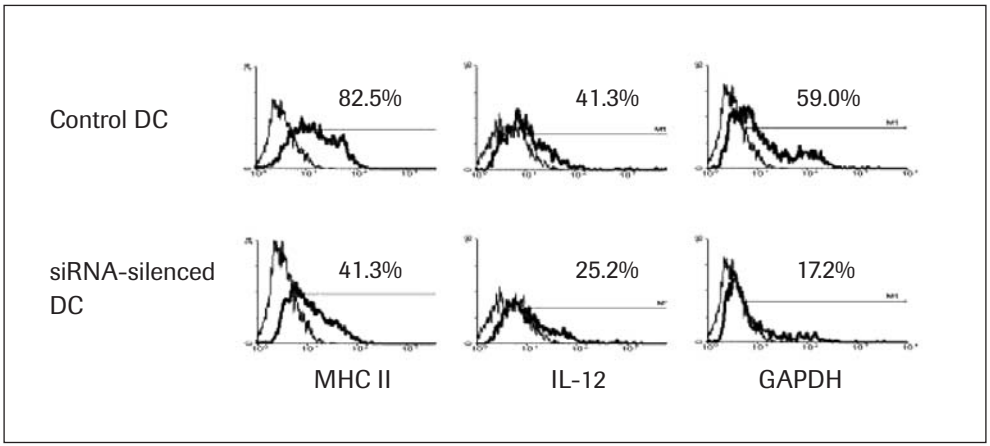

Figure 3. Multi-gene silencing by siRNA. *7-day cultured DC were co-transfected with pSilencer-MHC II, pSilencer-IL2 and pSilencer-GAPDH by GenePorter. 48 hours after gene silencing, the control-siRNA-transfected DC (control-DC, upper panel) and siRNA transfected DC (lower panel) were stained with anti-mouse I-A^b IL-12 and GAPDH (bolded lines) respectively. The isotype controls are shown as broken lines.*

4. We have successfully used IL-12 and CD40 gene-silenced DC for immune modulation (Figs 4-6).

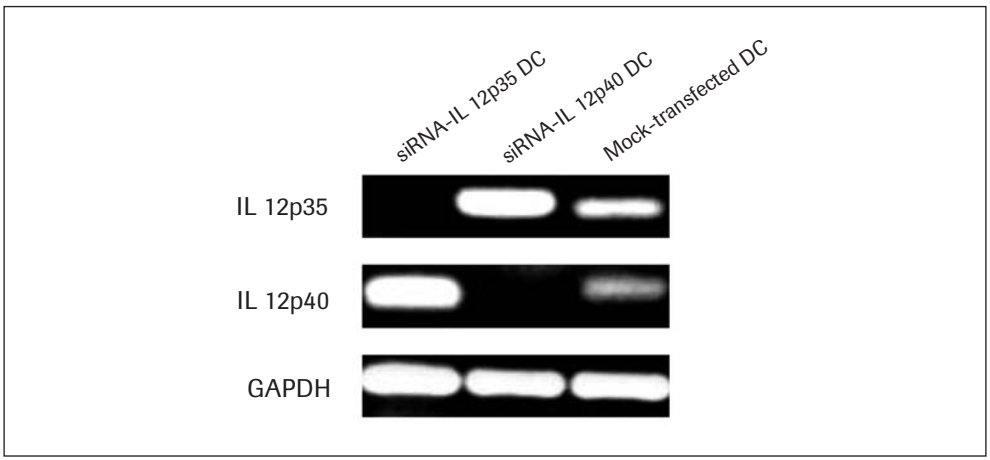

Figure 4. Gene-specific inhibition by siRNA. *DC ($1x10^6$) were transfected with 60 pMol siRNA-IL12p35, siRNA-IL12p40 or GenePorter alone (mock transfected). The transfected DC were activated with 10 ng/ml LPS and 10 ng/ml TNF-α for 24 hours. RNA from the treated DC was extracted by the Trizol method. RT-PCR was performed to assess expression of IL-12p35, IL-12p40 and GAPDH. Data are representative of three independent experiments.*

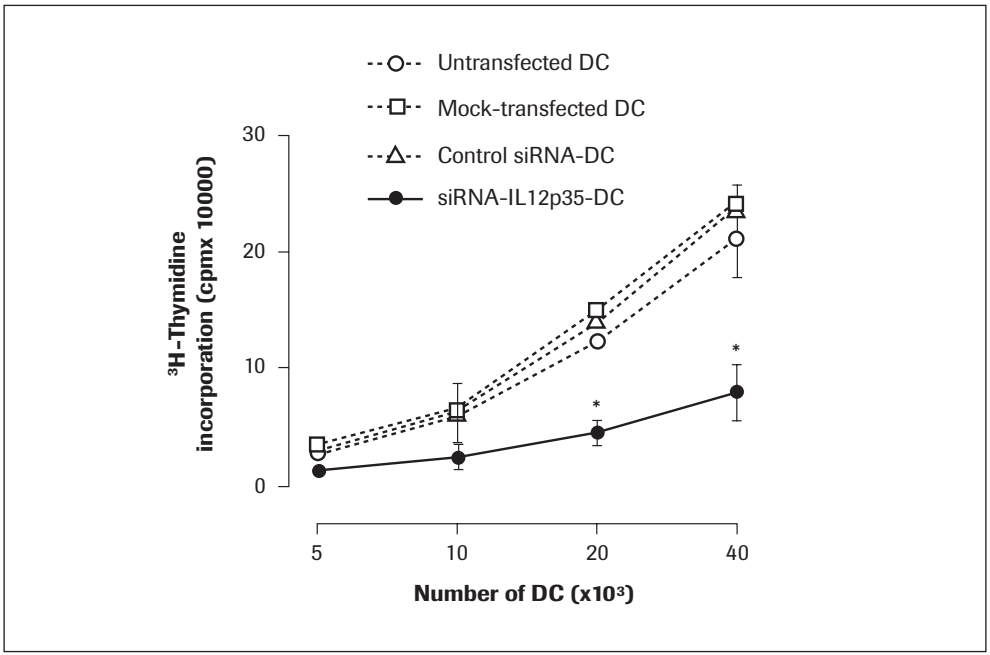

Figure 5. siRNA-IL12p35 silencing inhibits DC allostimulatory ability. *C57BL/6-derived DC (1x10⁶) were untreated (untransfected 0), transfected with GenePorter alone (mock transfected □), transfected with 60 pMol siRNA-INFγ (control siRNA △) or transfected with 60 pMol siRNA-IL12p35 (●) for 24 hours. Allogeneic (BALB/c) T cells (2x10⁵/well) were incubated with siRNA-treated DC at the indicated numbers for 72 hours. Proliferation was determined using [³H]-thymidine incorporation. Data are representative of three independent experiments. (*p < 0.01; by one-way ANOVA and Newman-Keuls test).*

5. We have established a kidney reperfusion ischemia model for testing silencing DC *in vivo* and for kidney transplantation using gene-silenced donor organ.

Ongoing and 2nd year studies

In the upcoming year we will:

1) Study *in vivo* tissue-specific siRNA delivery. We will use immunoliposomes to carry siRNA and specifically silence CD11c⁺ DC
2) Protect organ damage in reperfusion/ischemia injury using siRNA
3) Protect organ rejection in an allogeneic heart transplantation model

Based on our very promising research progress and our excellent research team, we are completely confident that we will complete all the AIMS of the presently-funded research proposal.

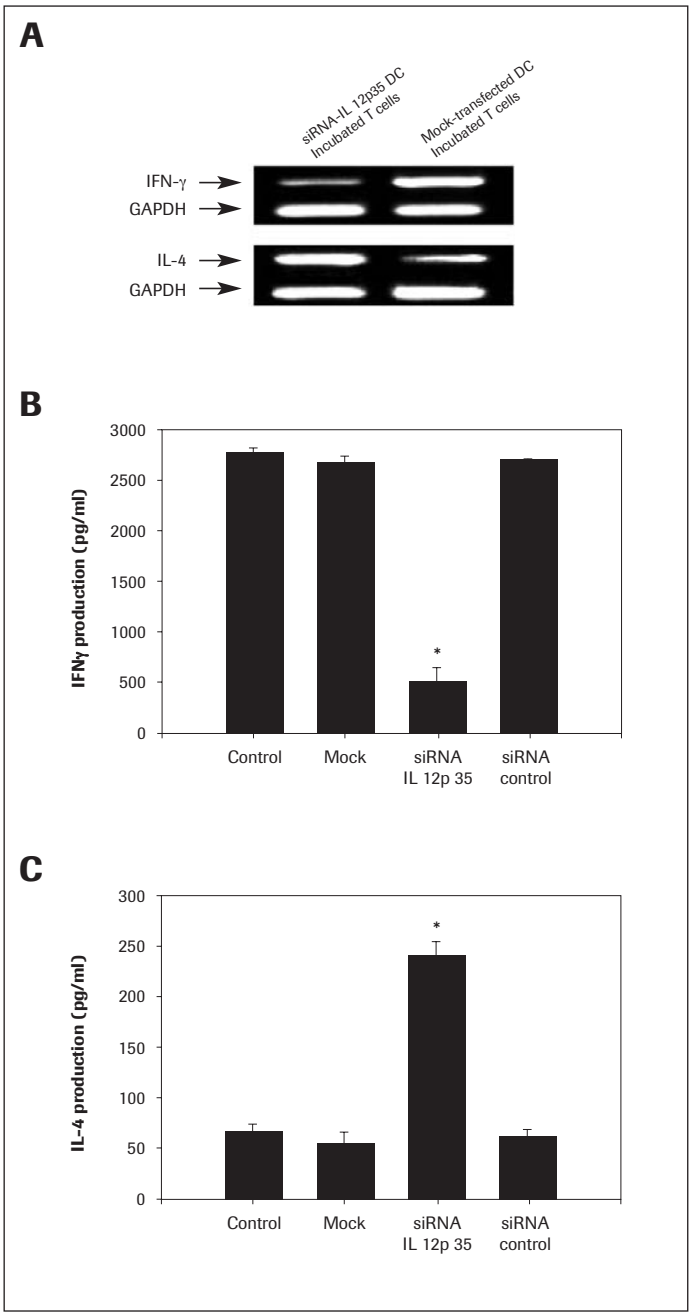

Figure 6. siRNA-IL12p35-transfected DC promote Th2 polarization. ***A.*** *C57/BL6 marrow derived DC were pretreated with GenePorter alone (mock transfected) or transfected with 60 pMol siRNA-IL12p35 for 24 hours. siRNA-treated DC (10^6) were subsequently cultured with allogeneic (BALB/c) T cells ($10x10^6$) for 48 hours. T cells were purified from coculture using a T cell column and RT-PCR was performed for IL-4, IFNγ, and GAPDH.* ***B, C.*** *C57/BL6 bone marrow derived DC were unmainpulated (control), pretreated with GenePorter alone (mock transfected), transfected with 60 pMol siRNA-IL12p35, or 60 pMol siRNA-IFNγ (siRNA control) for 24 hours. siRNA-treated DC (10^6) were subsequently cultured with allogenieic (BALB/c) T cells ($10x10^6$) for 48 hours. Supernatants were collected from the cultures and IFN-γ (Th1 cytokine)* ***(B)*** *and IL-4 (Th2 cytokine)* ***(C)*** *production was assessed by ELISA. (*, $p < 0.05$, **, $p < 0.01$).*

Recruitment of personnel

Dr. Francis Feng, highly experienced with >15 years experience in immunology, molecular biology and various animal models, joined our team.

Publications/abstracts attributed to the award

We have effectively organized and conducted the experiments proposed in the ROTRF application. The research work, based on the first year study, has resulted in six publications and eight presentations. Eight papers have been presented orally at the American Society for Transplantation, American Association for Immunologists, International Congress of Immunology, and International Congress of Transplantation, respectively, in 2004.

Publications

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2. Qian H, Li M, Popov IA, Ichim TE, Zhong R, Rycerz K, Zheng X, Zhang X, Min W-P. Silencing CD40 in dendritic cells by siRNA. *Immunology* 2004; 3:339-48.
3. Ichim TE, Li M, Qian H, Popov IA, Rycerz K, White D, Zhong R, Min W-P. RNA interference: a potent tool for gene-specific therapy. *Am J Transplant* 2004; 4:1227-36. (Mini-review)
4. Qian H, Li M, Ichim TE, Ge W, Popov IA, Rycerz K, White D, Zhong R, Min W-P. Generation of tolerogenic dendritic cells by silencing CD40 using siRNA. *Clin Invest Med* 2004; 27(4):45C. (Abstract)
5. Min W-P, Lian D, Li M, Ichim TE, Popov IA, Rycerz K, Gasia B, Zhong R. Adoptively transferring tolerance by tolerogenic dendritic cells. *Clin Invest Med* 2004; 27(4):152D. (Abstract)
6. Li M, Qian H, Ichim TE, Ge W, Elliott JF, Popov IA, Rycerz K, White D, Zhong R, Min W-P. Silencing dendritic cells by siRNA. *Clin Invest Med* 2004; 27(4):176A. (Abstract)

Prof. Mauro Sandrin, Principal Investigator

Austin Research Institute, Heidelberg, Australia

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

Aim 1: iGb3, a second glycosyltransferase producing Galα(1,3)Gal

We cloned iGb3S cDNA from GGTA1$^{-/-}$ mouse thymus and confirmed mRNA expression in both mouse and pig tissues. The mouse iGb3S gene exhibits alternative splicing of exons that results in a markedly different cytoplasmic tail compared with the rat gene. Transfection of iGb3S cDNA resulted in high levels of cell surface Galα(1,3)Gal synthesized via the isoglobo series pathway, thus demonstrating that mouse iGb3S is an additional enzyme capable of synthesizing the xenoreactive Galα(1,3)Gal epitope. Galα(1,3)Gal synthesized by iGb3S, in contrast to α1,3GT, was resistant to downregulation by competition with α1,2 fucosyltransferase. Moreover, Galα(1,3)Gal synthesized by iGb3S was immunogenic and elicited antibodies in GGTA1$^{-/-}$ mice.

We have designed and developed the targeting strategy to knockout the iGb3S gene, by the flanking of exons 2 to 4 with loxP sites. Targeting constructs are being prepared, and it is planned to transfect C57BL/6 ES cells shortly by Ozgene.

Aim 2: (B) Decreasing Galα(1,3)Gal expression using modified fucosyltranferases

We have previously shown that the cytoplasmic tail of α1,2 fucosyltransferase (FT1) confers its Golgi localisation. We examined the localisation conferred by the N-terminal region of another fucosyltransferase, FT2, which is secreted and acts on different catalytic substrates. The cytoplasmic tail and transmembrane domain of FT2 conferred an intracellular staining pattern that is essentially cytoplasmic and vesicular in nature. Full-length FT2 has a more widespread cytoplasmic staining pattern that includes vesicles. The localisation of the enzyme in Golgi and ER cannot be excluded. We moved the FT2 catalytic domain to the Golgi by replacing its own cytoplasmic tail with that of FT1. After the relocalisation of the catalytic domain of FT2, the enzyme was better than FT1 at competing for substrate with both GT and iGb3S. FT1 had little ability to compete for iGb3.

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

Brigham and Women's Hospital, Boston, USA

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

The arteries of allografted organs typically develop severe, diffuse intimal hyperplastic lesions, leading eventually to lumenal stenoses and to ischemic graft failure; it represents the major long-term limitation to solid organ transplantation. The intimal lesion, graft arterial disease (GAD) also known as transplantation-associated arteriosclerosis, is largely ascribed to an immune-mediated allogeneic response. It can occur in isografts, does not correlate strictly with episodes or severity of acute allograft rejection, and is observed even in the setting of immunosuppression adequate to block acute parenchymal rejection. The entire set of effector mechanisms remain to be elucidated. GAD is an intimal fibroproliferative lesion composed predominantly of smooth muscle-like cells (SMLC) and associated matrix proteins, admixed with mononuclear inflammatory cells. Thus, *we hypothesize that endothelial injury or dysfunction caused by a variety of vascular insults induces a chronic healing response characterized by the recruitment and activation of SMLC.* Proliferation of these cells with ongoing matrix synthesis forms the basis of the GAD lesions. Whether these SMLC derive from donor medial smooth muscle cells (SMC) or from host cells has important implications for targeting therapeutic intervention in GAD, as well as in more conventional atherosclerosis. The lesions consist primarily of SMC and associated extracellular matrix, admixed with infiltrating T cells, macrophages, and other mononuclear leukocytes.

Recent clinical and animal studies have demonstrated the presence of specific chemokines in allografts during acute rejection and during the development of GAD lesions. Moreover, in animal models, blockade of chemokine and chemokine receptor interactions have attenuated leukocyte recruitment into allografts and diminished the development of GAD, pointing towards novel and important therapeutic strategies for this disorder. Inflammatory cells including T cells, neutrophils, and macrophages reside in AAA, although it remains unclear whether these cells simply respond to the pathologic process or incite it. T cells and macrophages can affect atherogenesis by producing various cytokines. In particular, different T cell subsets secrete IFN-γ or IL-4 that characteristically drives opposing effects on a variety of biological processes. $CD4^+$ T helper type-1 (Th1) cells and $CD8^+$ T cytotoxic type-1 (Tc1) cells produce IFN-γ, IL-2, and TNF whereas Th2 and Tc2 cells secrete IL-4, IL-5, and IL-10.

T cell responses polarize toward a Th1/Tc1 phenotype in the presence of IFN-γ, while the critical Th2-inducing cytokine is IL-4 that predisposes them toward Th2/Tc2 T cell responses. IFN-γ assists Th1 development and attenuates Th2 responses by inducing IL-4 transcriptional repressors, while IL-4 promotes Th2 responses.

Distinct patterns of cytokines produced by T cells can modulate chemokine/chemokine receptor expression and theoretically affect the clinical course of disease.

We test the hypothesis that blockade of inflammatory cytokine signaling affects chemokine/chemokine receptor expression, modulating recruitment or accumulation of inflammatory cells and SMLC in the graft and affecting GAD development.

Results

1. To explore the mechanisms by which HMG-CoA reductase inhibitor (statins) affect outcomes in organ transplantation without confounding effects attributable to lipid lowering

To explore the mechanisms by which HMG-CoA reductase inhibitor (statins) affect outcomes in organ transplantation without confounding effects attributable to lipid lowering, we studied heart transplants in normocholesterolemic mice. We chose to conduct the *in vivo* studies with cerivastatin (before its withdrawal from the market) because of its potency and high degree of permeability into non-hepatocytes. Specifically, we tested the hypothesis that a cell-permeant statin can suppress host inflammatory cell recruitment and activation, and thereby modulate the downstream effector mechanisms leading to GAD progression.

We performed heterotopic murine cardiac transplants in total allogeneic or major histocompatibility complex (MHC) class II-mismatched combinations. Transplanted animals received either control chow, chow containing 25 ppm cerivastatin (low dose), or chow containing 125 ppm cerivastatin (high dose). Mean plasma cerivastatin concentrations were 0.0 nM (control), 10.1 nM (low dose), and 21.9 nM (high dose), respectively. Plasma cholesterol levels were the same in all groups. GAD scores decreased in low dose ($p < 0.05$) and high dose ($p < 0.0001$) cerivastatin groups compared to controls, with concomitant reduction in graft infiltrating cells and significantly decreased intragraft RANTES and MCP-1 mRNA expression. Cerivastatin, as well as other statins also reduced RANTES and MCP-1 production in mouse endothelial cells stimulated with IFN-γ and TNF-α *in vitro.*

Clinically achievable levels of an HMG-CoA reductase inhibitor attenuate GAD in murine heart transplants, diminish host inflammatory cell recruitment, and do not alter cholesterol levels. These results indicate that statins can affect arterial biology and inflammation independent of effects on cholesterol metabolism. Experiments are ongoing to identify

whether the effects of statins in chemokine expression can be attributed to diminished recruitment of inflammatory cells and SMLC[1].

2. Heterotopic hearts transplanted into Smad3$^{-/-}$ recipient mice develop an accelerated inflammatory arteriopathy

Vascularized heterotopic abdominal cardiac transplantations were performed using MHC class II-mismatched donor hearts from mice for bm12 (H-2^{bm12}) into recipient C57BL/6 (B6, H-2^{b}) Smad3$^{-/-}$ or B6 Smad3$^{+/+}$ mice. Hearts were harvested for immunohistochemistry analyses after 6 weeks of transplantation, a time point in which we have demonstrated minimal neointimal thickening within the coronary arteries. While coronary arteries of allografts from Smad3$^{+/+}$ recipient mice exhibit minimal neointima formation, there is accelerated intimal hyperplasia within allografts from the Smad3$^{-/-}$ mice. Furthermore, there is marked induction of MCP-1 expression within the neointima of allografts from Smad3$^{-/-}$ mice in comparison to that from Smad3$^{+/+}$ mice suggesting the presence of an inflammatory process. Indeed, nearly all of the neointimal MCP-1 staining colocalized to that of CD11b-positive macrophages, which constituted the majority of cells within the neointima of allografts from Smad3$^{-/-}$ mice. In addition to inflammation within the neointima, perivascular CD11b-positive macrophages were found more abundantly within allografts from Smad3$^{-/-}$ mice than Smad3$^{+/+}$ mice. Perivascular inflammation can contribute to adventitial scarring and arterial lumen narrowing and is an important hallmark in the development of transplant arteriopathy. Collectively, these findings are consistent with lesions found early in the development of transplant arteriopathy, which is typified by an initial preponderance of inflammatory cells to SMLC. Thus, expression of chemokines such as MCP-1 by neointimal macrophages may allow for potentiation of macrophage infiltration resulting in an accelerated inflammatory arteriopathy as found in the allografts from Smad3$^{-/-}$ mice. These data indicate a novel mechanism by which TGF-β1 via Smad3 can suppress inflammatory responses in the vascular system[2].

We presented this work at the American Heart Association conference at Orlando in November 2003, and received honor of finalist for Katz Young Investigator Award[3].

3. Developing a new animal model of aortic aneurysm

To evaluate putative pathways in GAD development, we induced Th1 or Th2 predominant cytokine environments in an inflammatory aortic lesion using murine aortic transplantation into wild-type (WT) hosts or those lacking the receptors for the hallmark Th1 cytokine interferon-γ (IFN-γ), respectively. Surprisingly, allografts in IFN-γ receptor deficient (GRKO) hosts developed severe AAA formation associated with markedly increased levels of matrix metalloproteinases (MMPs)-9 and –12, whereas allografts in WT recipients developed intimal hyperplasia as we expected. Allografts in GRKO recipients treated with anti-interleukin-4 (IL-4) antibody to block the characteristic IL-4 Th2 cytokine or allografts in GRKO hosts also

congenitally deficient in IL-4 did not develop AAA and also exhibited attenuated collagenolytic and elastolytic activities. These observations demonstrate an important dichotomy between cellular immune responses that induce IFN-γ or IL-4 dominated cytokine environments. The findings establish important regulatory roles for a Th1/Th2 cytokine balance in modulating matrix remodeling and have important implications for the pathophysiology of AAA and arteriosclerosis[4] (Fig. 1).

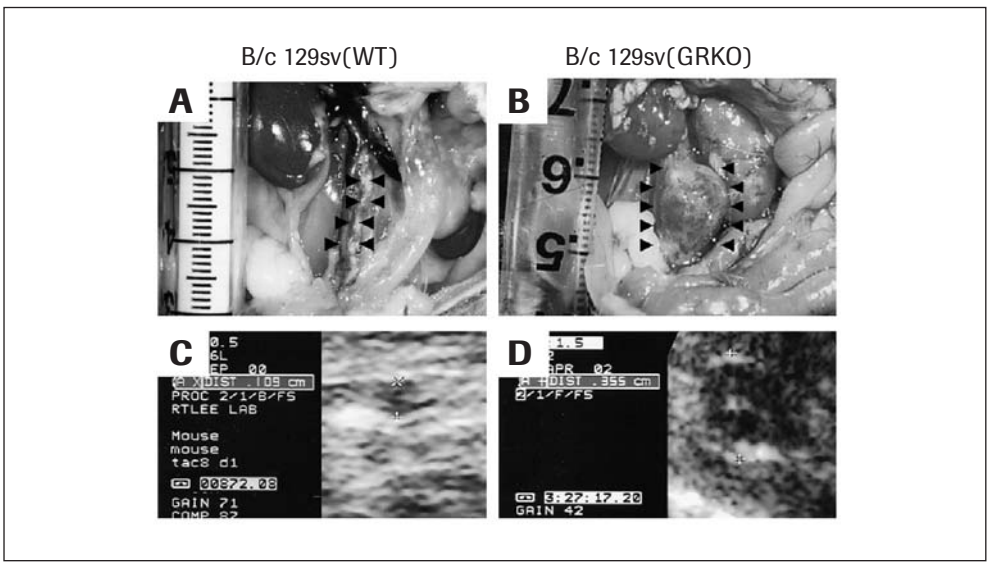

Figure 1. Aortic allografts from WT (A, C) or GRKO (B, D) recipients 12 weeks after transplantation. ***A, B.*** *Representative gross appearance of aortas;* ***C, D.*** *Echoaortograms. Analysis of 9-10 aortic transplants from different donors yielded similar results. Control (WT) experiments, shown in panel (**A, C**), did not reveal measurable aneurysm formation or elastic tissue degradation. Arrowheads indicate elastic lamellae.*

Specific Aim 2: We hypothesize that intimal SMLC in GAD derive from both host bone marrow (BM) as well as host non-BM precursor cells

Although we have demonstrated that intimal SMLC are essentially all host-derived, it is not yet known whether the SMLC of BM origin derive from hematopoietic or mesenchymal stem cell precursors. Moreover, BM origin of such cells is responsible for at most 20% of the total, and ingrowth from the edges of anastomses likewise does not account for the presence of the host cells in GAD lesions. Therefore, the exact anatomical origin of the majority of the apparently non-BM-derived SMLC is unclear. Since the various precursors may utilize

distinct pathways to access grafts and have different growth requirements, identifying all of the potential precursor cells is critical to effectively develop strategies to prevent their recruitment and activation.

For the final year of the study, we will fractionate BM-derived cell populations into hematopoietic and stromal cell groups to identify which is the precursor BM population for the intimal SMLC in GAD using green fluorescence protein (GFP) transgenic stem-cell donors in adoptive transfer experiments. Similarly, we will use the GFP animals as sources of circulating stem cells, and for generating cultured medial SMC and intimal SMLC for adoptive transfer into wild-type (WT) recipients of allografts, to identify which cell populations can contribute to GAD intimal lesions.

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Using the Immunosuppressive IDO Gene for Prevention of Allograft Rejection

Nature has developed a basic model of immunological tolerance against allogeneic tissues – the acceptance of the fetus during pregnancy. Recently, a novel mechanism of tolerance induction in pregnancy has been identified based on expression of the immunosuppressive IDO gene in placental antigen-presenting cells and trophoblasts. We wanted to apply to an allogeneic organ transplant model what nature has developed for suppression of fetal rejection during pregnancy. First, we cloned the IDO gene into a replication-defective adenovirus. Thereafter, dendritic cells (DCs), which are known for their strong stimulatory capacity, were transduced with recombinant IDO-adenoviruses and coincubated *in vitro* with allogeneic T cells. In contrast to normal DCs, IDO-expressing DCs suppressed the T cell response. Because IDO destroys tryptophan, it has been speculated that its suppressive action may be due to a lack of tryptophan – an amino acid required for cell proliferation. In comprehensive studies we demonstrated that not low tryptophan concentrations, but the metabolites resulting from its catabolism mediate suppression[1]. Our findings showed that kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, but no other IDO-induced tryptophan metabolites, strongly suppress the T cell response at concentrations which can be expected at local sites of IDO production *in vivo*. The effect of suppressive metabolites was additive. As shown by our studies, T cells once stopped in their proliferation by the action of tryptophan metabolites, can not be restimulated. This effect may result either from lack of T cell reactivity or cell death. In a series of FACS analyses we demonstrated that tryptophan metabolites induce T cell death and this action preferentially affects activated T cells. Another interesting finding was that the cytotoxic action of these substances increased gradually with exposure time. Our studies showed that not only T cells but also B and NK cells are affected. In contrast, DCs – the cells which produce IDO – are resistant to tryptophan metabolite cytotoxicity.

As already mentioned, IDO is involved in suppression of fetal rejection. According to our observations, this suppression is mediated by metabolites resulting from increased IDO activity. Consequently, high levels of such compounds in the fetal-maternal interface may constitute a “gateway to inferno” which sentences to death all T cells which pass through.

If the immune system "uses" IDO for suppression of "unwanted" immune reactions, the same strategy could be attempted for induction of tolerance in a transplant setting. Theoretically, several ways for prolonging allograft survival are at our disposal. Among them are the use of tryptophan metabolites or the expression of IDO transgene in donor tissues to suppress the local immune reaction against the graft. We examined both strategies.

In a series of rat experiments the effects of metabolites on the immune response towards allogeneic cells were analyzed *in vitro* and *in vivo.* In one experiment T cells were stimulated in cultures with allogeneic DCs in the presence of increasing amounts of tryptophan metabolites. The results showed that the rat T cell suppressive effect appears at the same concentrations as for human T cells. Vital staining and FACS analysis of metabolite-treated T cells demonstrated cell death as the mediator of suppression[2].

In another experiment donor cells were injected into the footpad of an allogeneic recipient. It is well known that this induces an enlargement of the popliteal lymph node. If the recipient was daily injected with 3-hydroxykynurenine, the lymph node reaction was significantly reduced (from 100% to 33%).

In the next *"in vivo"* experiment, LEW rats received a BN skin transplant and daily injections of a tryptophan metabolite mix. Cyclosporin A was used as a positive – and saline as a negative control. The findings showed significant prolongation of allograft survival induced by the metabolites as compared to the negative control ($p=0.002$), but a weaker effect than that observed following cyclosporin A treatment.

Another approach to IDO-mediated suppression of graft rejection is the expression of the gene in donor tissues. We have analyzed the possibility of expressing IDO in a rat heart via adenoviral vector. However, deprivation of tryptophan by IDO expression in the graft not only affects the invading T cells, but heart function as well. Moreover, the adenovirus itself harms the transplanted heart and increases its immunogenicity. In order to avoid the direct adenovirus effect on the graft, a second model has been designed based on generation of suppressive IDO-expressing donor cells. It is well known that DCs stimulate the immune response. Previous experiments in rodents showed that a certain subpopulation of donor DCs (progenitor cells) were able to prolong cardiac and islet allograft survival if injected into the recipient before transplantation. The general mechanism of DC-induced immunosuppression is not well understood but it has been suggested that inhibition in the above model may be due to a lack of costimulatory signals. It is also known that DCs are able to produce – under certain circumstances – molecules which inhibit T cell proliferation and/or induce regulatory T cells. We wanted to generate donor-derived DCs which express IDO. Unfortunately, in contrast to human DCs, expression of the IDO-transgene in rat DCs via adenovirus showed to be difficult and not sufficiently reproducible. We pursued a second way of generating

suppressive donor DCs whose concept was not based on IDO expression. This concept relied on treatment of cells with mitomycin C (MMC), a protein synthesis inhibitor. The use of MMC was based on previous observations showing that pre-treatment of PBMCs with MMC confers these cells T cell suppressive properties.

We treated donor DCs *"in vitro"* with MMC, washed them and injected the cells into the recipient. One week later the animal received a heart transplant from the same donor. The findings showed a significant prolongation of allograft survival in animals pretreated with MMC-DCs as compared to those receiving native donor DCs (30± 0.4 vs 5± 0.2 days) (untreated recipients: 9± 0.5 days). Moreover, the suppressive effect was donor-specific since transplants from third-party donors were immediately rejected (10± 0.3 vs 30± 0.4 days). In extensive studies we analyzed the mechanism of suppression and came to the conclusion that MMC downregulates CD80, CD86 and ICAM-1 on the DC membrane and that this is responsible for induction of T cell suppression towards the donor[3].

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5. Final Reports of ROTRF Grantees

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The Role of Mesenchymal Stem Cells in Transplantation Tolerance

The use of high-dose bone marrow has been an effective strategy to minimize the regimen-related toxicities in host conditioning for the induction of tolerance. In clinical practice, this approach might not be feasible, due to stem cell availability from non-living donors and the potential risk of graft-versus-host disease. Identification of the critical components necessary to the success of high-dose bone marrow infusions may provide the ability to engineer stem cell infusions for optimal effects. Although many theories have been proposed to explain the effectiveness of this approach, none have considered the possibility of high-dose bone marrow to contain higher doses of bone marrow mesenchymal stem cells (MSC). These stromal cells share features with thymic stromal cells and have been observed to suppress T cell proliferation to allogeneic stimuli *in vitro*. When infused *in vivo,* we have observed these cells to modestly prolong skin graft survival in baboons. Our observation that they also home to the bone marrow suggests that these immunoregulatory cells might provide a beneficial and immunoprotective microenvironment for transplanted allogeneic stem cells and thereby be instrumental in the subsequent induction of tolerance. In this one-year proof-of-concept study, we focused on the hypothesis that MSC could facilitate bone marrow engraftment across both major and minor histocompatibility barriers.

Studies and results

In murine hematopoietic transplantation, the use of purified hematopoietic stem cells (HSCs) can eliminate the incidence of graft-versus-host disease. This purified sub-population (in general c-Kit^{+}Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+}) can engraft across major histocompatibility barriers, however, greater numbers of purified HSCs are required for radioprotection and engraftment as the degree of MHC disparity increases. To test whether MSC facilitate tolerance indirectly through enhanced allogeneic bone marrow engraftment, we performed three sets of experiments. In the first experiment, we tested whether Balb/c MSC could facilitate engraftment of purified Balb/c stem cells (c-Kit^{+}Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+}) in lethally irradiated B6 recipients. In this strain combination, it has been reported that 20 x 10^{6} allogeneic whole bone marrow cells can easily reconstitute lethally irradiated recipients. Thus, the purified stem cell component of such a graft, observed to comprise 1-2% of the whole bone marrow graft, is likely to engraft such a recipient. We were surprised to observe that recipients of 0.2, 0.4, and 0.8 x 10^{6} lineage negative

stem cells failed to engraft across this barrier with or without MSC. We hypothesized that MSC might require an additional lineage positive cell for maximal beneficial results, thus the next group of experiments were performed using whole bone marrow.

A bone marrow transplantation model was established in which recipients were administered either the number of bone marrow cells necessary for engraftment (20 x 10^6 bone marrow cells) or a sub-optimal dose which would be insufficient for survival (1 or 10 x 10^6). In recipients of 1 x 10^6 BMC, the addition of MSC extended survival from a mean of 8 to 50 days (data not shown). In recipients of 10 x 10^6 BMC, the addition of 0.1 x 10^6 or 1.0 x 10^6 Balb/c MSC significantly increased 220 day survival to 50% (p=0.0004) and 66% ($p<$ 0.0001), respectively, (data not shown). In converting survival from 0% to 66%, the addition of 1.0 x 10^6 MSC to a sub-optimal bone marrow graft of 10 x 10^6 BMC established a survival rate similar to recipients of optimal BMC grafts comprised of 20 x 10^6 BMC (no statisitical difference was observed between survival of recipients of MSC augmented grafts and optimal grafts). Since whole bone marrow was used, the argument could be proposed that the MSC were contaminated with additional stem cells and these additional contaminating cells were responsible for the facilitating effect. Although flow cytometric analysis revealed a homogeneous extremely large cell population which was $CD29^+CD44^+$ and CD45-CDIIb, consistent with reports of murine MSC, there was still the possibility that trypsin treatments could have theoretically removed cell-surface markers thereby enabling hematopoietic stem cells to remain undetected within the preparation. To determine whether this could be a possible explanation, MSC were administered either 2 hours before or after administration of a sub-optimal BMC graft (10 x 10^6 BMC). We reasoned that if the MSC provided additional stem cells, allogeneic engraftment would still occur. Significantly, no animals in this treatment group survived 14 days (data not showm), indicating that the effect was not likely due to additional hematopoietic stem cells present in the MSC preparation. Further, the MSC effect did not appear to be dependent on systemic immune suppression, since administration of MSC without HSC coculture was not effective. Instead, this observation indicated that the facilitating effect of MSC was cell contact dependent.

These series of experiments have served as the basis for several other areas of study and for the successful award of an NIH grant. We have begun work on: 1) the initial MSC contact with BMC, the molecules and cell structure changes required, 2) identification of the additional cell subtypes present for maximal effect, 3) using proteomic analysis, the identification of soluble factors induced following coculture and the identification of those most critical to engraftment through blocking experiments, 4) the extent of immunological protection MSC offer by testing whether or not NK cell, B cell and alloantibody responses can be subdued, 5) the type and kinetics of regulatory T cells induced as a consequence of MSC administration, and last, 6) whether MSC can inhibit graft-versus-host disease.

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Mechanisms of Graft Destruction in the Absence of Cognate Recognition between the Graft and T Cells

We have recently validated the indirect pathway of T cell allorecognition in a mouse TcR transgenic model[1]. The transgenic TcR specifically recognizes the peptide NAGFNSNRANSSRSS, derived from the male antigen encoded by gene *Dby*, presented in an I-A^b-restricted fashion. Unlike most MHC class II-restricted TcR-transgenic mouse lines, $RAG2^{-/-}$ Marilyn mice do not contain any detectable $CD8^+$ T cells in thymus and periphery. Moreover, as shown by T cell expression of memory markers such as CD44 and CD62L, only naive T cells are present in lymphoid organs of unprimed Marilyn mice. The T cells are not alloreactive to the H-2^k haplotype, because the transgene was originally isolated from a T cell clone derived from a H-2^{bxk} F1 female. Moreover, they are not activated when adoptively transferred into $RAG2^{-/-}$ $\gamma c^{-/-}$ double-mutant H-2^k male or female mice. However, skin from H-2^k males, but not from H-2^k females, is acutely rejected by $RAG2^{-/-}$ TcR-transgenic female recipients. Taken together, these results show that the indirect recognition that triggers rejection in this model is due to the recognition of H-Y antigens shed from H-2^k male allograft and presented by the recipient's own I-A^b APC to transgenic CD4 T cells. The purpose of this project is to understand the mechanisms that ensure the specificity of tissue destruction when rejection occurs in the complete absence of direct physical recognition of the graft by T cells.

Endothelial cells (EC) are known to express MHC class II molecules upon activation and are considered by many as the prime element targeted by alloreactive $CD4^+$ T cells in rejected organs. Until recently, it was believed that the pathogenesis of transplant arteriosclerosis was initiated by immune damage to the endothelium followed by smooth muscle cell migration and proliferation in the intima. According to this concept, endothelium of the graft remained of donor origin and vascular rejection was considered to originate from grafted tissue. However, this conventional hypothesis is challenged by recent studies that have addressed the question of EC replacement in solid-organ transplantation. It has been shown in recent years, that bone marrow (BM)–derived precursor elements have the potential to induce vasculogenesis of ischemic tissues, including transplanted organs[2-4], and to participate in the neoendothelium and neointima in post-angioplasty arteries[5]. EC precursors are present in adult BM and have properties of hemangioblasts. In human transplantation, parts of EC in the

blood vessels of transplanted organs were found to be of recipient origin[6-8]. These cells were observed in poorly functioning and severely damaged grafts and were particularly detected among patients whose graft suffered chronic vascular rejection. It was postulated that, in vascularized allografts, EC of the recipient can replace those of the donor and that this replacement was associated with rejection.

In the present study, we investigated whether recipient-derived EC that have replaced donor cells within the graft could represent the main cell target recognized by indirect pathway alloreactive $CD4^+$ T cells for mediating the rejection of vascularized allografts.

We first wished to determine whether immune recognition of graft EC by recipient $CD4^+$ T cells was required for graft rejection. Using our highly antigen-specific model, we first set up an experimental protocol where presentation of transplantation antigen was carried out exclusively by the allograft itself. Highly purified $CD4^+$ T cells (> 95 %) from RAG2-deficient Marilyn mice were isolated and then injected into immunodeficient $RAG2^{-/-}$ $\gamma c^{-/-}$ double-deficient female mice expressing $H\text{-}2^k$ molecules. Reconstituted animals were then observed for their capacity to reject $H\text{-}2^b$ male skin grafts. All the reconstituted $H\text{-}2^k$ recipients were able to reject acutely $H\text{-}2^b$ male skin (Table 1). In this setting, since i) mouse T cells do not express MHC class II molecules and ii) Marilyn T cells do not recognize $H\text{-}2^k$ molecules, rejection was caused by T cell recognition of graft-derived cells alone and could not be the result of T cell stimulation by recipient antigen presenting cells (APC) infiltrating the graft.

n	Recipients	Donors	Survival (days)
11	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^k$	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^b$ male	11, 11, 12, 12, 12, 12, 12, 14, 14, 18, 18
8	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^k$	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^k$ male	47, 53, 69, >100, >100, >100, >100, >100
2	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^b$	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^b$ male	11, 11
2	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^k$	$RAG2^{-/-}$ $\gamma c^{-/-}$ $H\text{-}2^b$ female	>100, >100

Table 1. Survival of male $H\text{-}2^b$ $RAG2^{-/-}$ $\gamma c^{-/-}$ male skins grafted on $H\text{-}2^k$ $RAG2^{-/-}$ $\gamma c^{-/-}$ female recipients reconstituted with purified Marylin $CD4^+$ T cells.

Graft rejection depended on the expression of H-2^b molecules by the graft since H-2^k male skin was not rejected by T cell-reconstituted H-2^k recipients. Moreover, failure to reject female H-2^b skin demonstrated that rejection was antigen-specific. To identify the cell population responsible for the antigen-specific immune recognition of H-2^b grafts rejected by reconstituted H-2^k recipients, we analyzed the expression of donor MHC class II molecules I-A^b on skin graft sections. In H-2^b grafts rejected by H-2^k recipients, I-A^b expression was restricted to the vascular endothelium (Fig. 1). Numerous graft-infiltrating cells also expressed MHC class II molecules. However, all of them expressed I-E^k and, thus, were derived from H-2^k recipients and could not be recognized by Marilyn T cells (Fig. 1). Taken together these results supported the concept that rejection resulted from the antigen-specific immune recognition of graft endothelium by T cells.

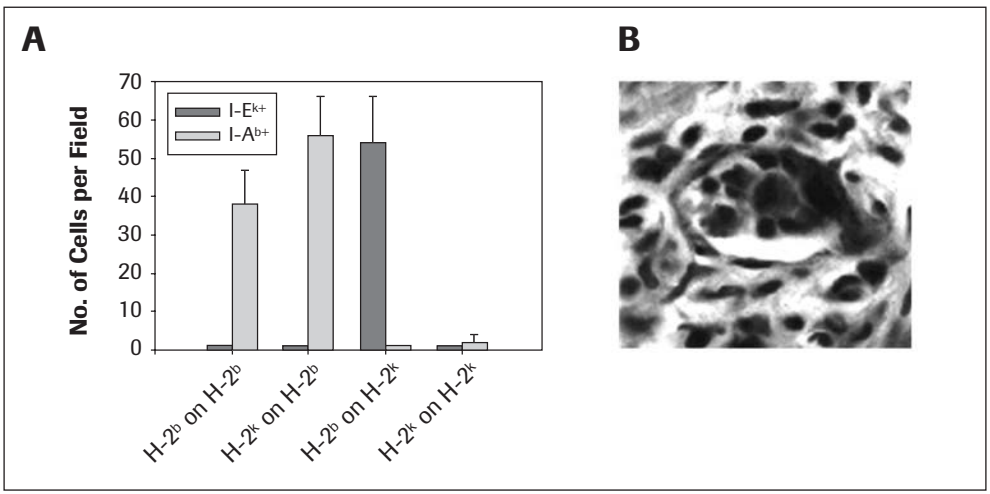

Figure 1. Restricted expression of I-A^b on endothelial cells of H-2^b male graft rejected by H-2^k RAG2$^{-/-}$ γc$^{-/-}$ female recipients reconstituted with H-Y-specific-TcR-transgenic T cells. A. *H-2^b or H-2^k RAG2$^{-/-}$ γc$^{-/-}$ recipient mice were reconstituted with 7x10^6 RAG2$^{-/-}$ Tg T cells, then transplanted with skin from H-2^b or H-2^k RAG2$^{-/-}$ γc$^{-/-}$ donor mice. Eleven days later, grafts were taken and tissue sections were immuno-stained with anti-mouse I-A^b- (clone 25-9-17) or anti-mouse I-E^k- (clone 14-4-4S) specific monoclonal antibodies. Graft infiltrating mononuclear cells expressing I-A^b or I-E^k were then counted. Results are expressed as the mean +/- SD of counts from three different sections.* ***B.*** *Expression of I-A^b on vascular structures from H-2^b RAG2$^{-/-}$ γc$^{-/-}$ skin rejected by H-2^k RAG2$^{-/-}$ γc$^{-/-}$ recipient reconstituted with Tg T cells.*

There is conflicting evidence about the capacity of EC to act as competent APC to stimulate T cells. A few studies suggest that EC are unable to stimulate $CD4^+$ T cells and may not be a target for $CD4^+$ T cell-mediated alloimmune responses. In our model, though expression of targeted MHC class II molecules could be detected at the surface of graft EC, evidence for

direct recognition of EC by Marilyn T cells was lacking. We therefore analyzed *in vitro* the immune recognition of mouse EC by Marilyn T cells. EC were isolated from thoracic aorta and expanded in EC growth factor-containing medium. Mouse vascular EC isolated from thoracic aorta expressed the EC marker CD105 (endoglin) and had low levels of cell surface MHC class II molecules (Fig. 2). Because EC lack cell surface expression of key costimulatory molecules such as CD86 and CD40 and are poor stimulators for T cell priming, *in vitro* experiments addressing the capacity of EC to stimulate T cells were carried out with previously primed T cells. Marilyn T cells were then purified from the spleen of mice pre-immunized with male I-A^b DC. In mixed cultures with primed Marilyn T cells, male H-2^b EC specifically stimulated T cell proliferation and secretion of IFN-γ (Fig. 2), demonstrating the specific immune recognition of male I-A^b EC by Marilyn T cells.

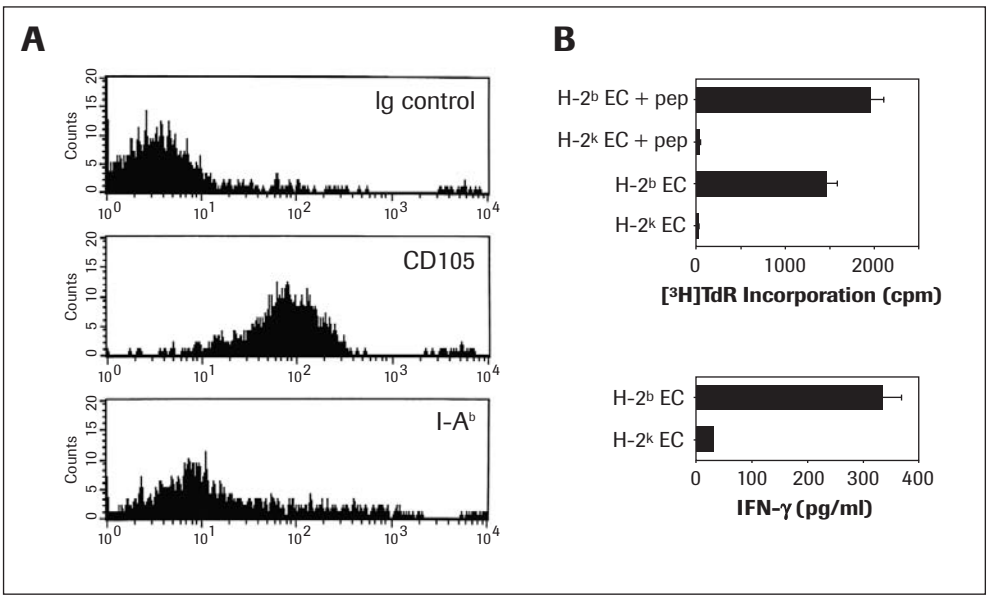

Figure 2. Mouse EC isolated from thoracic aorta express MHC class II molecules and can stimulate antigen-primed T cells for proliferation and production of IFN-γ. A. *Isolated mouse EC express cell surface marker CD105 and MHC class II molecules (I-A^b).* ***B.*** *EC isolated from H-2^b, not H-2^k, male mice stimulated cell proliferation (upper panel) and IFN-γ production (lower panel) in Marilyn CD4$^+$ T cells. Adding H-Y peptide to the coculture increased T cell proliferation in response to EC stimulation (upper panel).*

Recent studies have established that BM-derived cells, including endothelial progenitor cells (EPC), are mobilized in response to trauma or ischemia and are able to contribute to tissue repair and new blood vessel formation. The development of blood vessels from blood-borne

endothelial precursors, termed vasculogenesis, was previously thought to be restricted to embryonic development, but is now accepted to play a role in post-natal processes including tissue repair. Our observation that immune recognition of skin graft EC by $CD4^+$ T cells promotes rejection raised the possibility that recipient-derived EC could represent the APC recognized by indirect pathway $CD4^+$ T cells within the graft and thereby promote rejection. To test this hypothesis, we analyzed Marilyn recipients for their capacity to reject H-2^k grafts. We have previously shown that Marilyn mice reject acutely H-2^k skin. On the contrary, hearts from H-2^k males, but not from H-2^k females, were rejected by unprimed Marilyn mice in a chronic way [median survival time (mst) >50 days]. Weigert staining of heart-beating grafts by day 50 showed typical signs of cardiac vasculopathy including arterial wall thickening and obliteration (Fig. 3). Immunostaining of rejected grafts with anti-I-A^b monoclonal antibodies (clone 25-9-17) demonstrated the presence of numerous recipient I-A^b-expressing cells within the capillary vessels (Fig. 3). Remarkably, infiltrated Marilyn T cells, identified by cell surface expression of CD3, were found in close contact with I-A^b-expressing capillaries (Fig. 3). Taken together, these results suggested that replacement of H-2^k graft endothelium by recipient-derived I-A^b EC rendered heart graft susceptible to rejection mediated by Marilyn T cells.

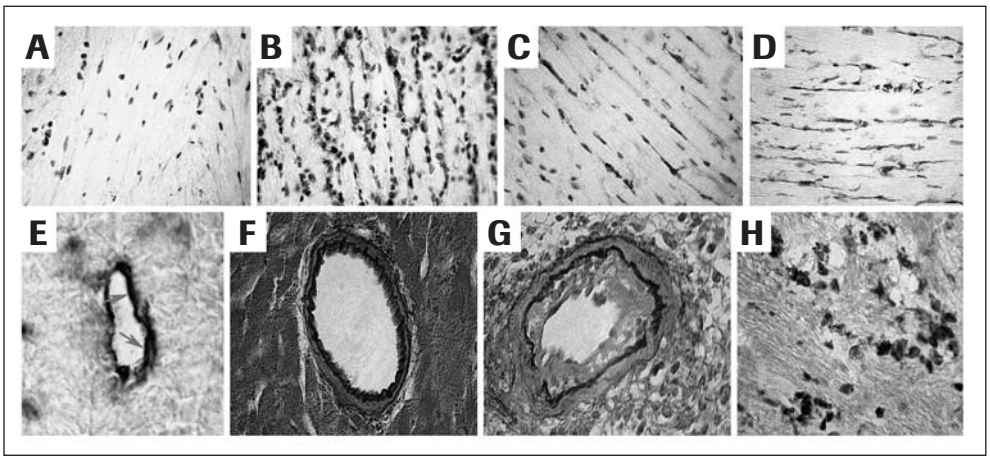

Figure 3. H-2^k male hearts are chronically rejected by Marilyn recipients. *The expression of MHC class II molecule I-A^b was analyzed by immunolabelling with specific antibodies (clone 25-9-17) in non-transplanted H-2^b hearts* ***(A, E),*** *in H-2^k hearts that survived more than 50 days in Marilyn recipients* ***(B),*** *and in non-transplanted H-2^k hearts* ***(C).*** *I-A^b expression colocalized with that of CD105 on cardiac EC* ***(D).*** *Extensive vasculopathy was observed on H-2^k hearts that survived more than 50 days in Marilyn recipients* ***(G).*** *This pathology was absent in non-transplanted H-2^k hearts* ***(F).*** *Immunostaining with anti-mouse CD3 antibodies revealed numerous TcR-transgenic T cells infiltrating H-2^k male hearts chronically rejected by Marilyn recipients* ***(H).***

Total body irradiation has been shown to induce microvascular endothelial apoptosis in tumors and normal tissues[9-11]. To investigate whether endothelium replacement by recipient EC precursors could trigger rejection by indirect pathway T cells, we created chimeric hearts by reconstituting lethally irradiated (15 Gray) male H-2^k $RAG^{-/-}$ $\gamma c^{-/-}$ mice with BM isolated from female H-2^b $RAG^{-/-}$ $\gamma c^{-/-}$ donors. Double immunostaining of frozen heart sections with anti-I-A^b MHC class II molecule and anti-CD105 monoclonal antibodies showed BM-derived I-A^b-positive EC within myocardial capillaries of chimeric mice. I-A^b EC were detected from day 10 after BM grafting and their number increased with time thereafter (Fig. 4). To demonstrate the essential role played by recipient endothelium in grafts recognized by indirect pathway T cells, we then tested whether I-A^b endothelium-containing H-2^k chimeric hearts were susceptible to rejection once transplanted in Marilyn recipients. In order to exclude the possibility that failure to reject acutely H-2^k male grafts could be due to the lack of T cell priming, Marilyn recipients were immunized with BM-derived I-A^b male DC prior to transplantation as described by Buonocore *et al*[12]. One day after immunization, Marilyn recipients were transplanted with normal or chimeric organs and observed for sign of rejection. Immunization by I-A^b male DC induced the acute rejection of H-2^b $RAG^{-/-}$ $\gamma c^{-/-}$ male cardiac grafts but not that of H-2^k $RAG^{-/-}$ $\gamma c^{-/-}$ male hearts (Fig. 4). These observations demonstrated that, in our system, failure to reject H-2^k hearts could not be overcome solely by T cell immunization. They also suggested that lack of rejection could result from the absence of T cell immune recognition of the graft. On the contrary, chimeric H-2^b-into-H-2^k male cardiac grafts were acutely rejected by immunized Marilyn mice (mst=19 days). Taken together, these results suggested that the presence of EC expressing I-A^b within H-2^k male cardiac allograft allowed the immune recognition of the graft by indirect pathway H-Y-specific I-A^b-restricted $CD4^+$ T cells and conferred susceptibility to rejection.

One of our main findings is that graft endothelial expression of MHC class II molecules targeted by recipient's T cells appears to be by itself sufficient to bring about rejection. This was initially shown in a highly antigen-specific TcR transgenic transfer system where transferred T cells could not recognize recipient's MHC class II molecules. In this context, T cell recognition of target MHC class II molecules on skin allograft EC alone appeared sufficient to promote rejection. Subsequently, we also observed, in a transplantation model of vascularized cardiac graft, that hearts containing EC expressing MHC class II molecules targeted by T cells suffered rejection. Finally, the observation that, in normal non-rejected heart, most cardiac EC expressing MHC class II molecules are located within the microvascular structures of the myocardium supports the idea that these cells indeed constitute the prime target for immune recognition of the graft by T cells.

The fact that rejection appears to depend on T cell immune recognition of graft EC raises the question of how $CD4^+$ T cells sensitized by indirect recognition of graft antigens could alone promote allograft rejection. Serial BM transplantation studies have revealed that adult

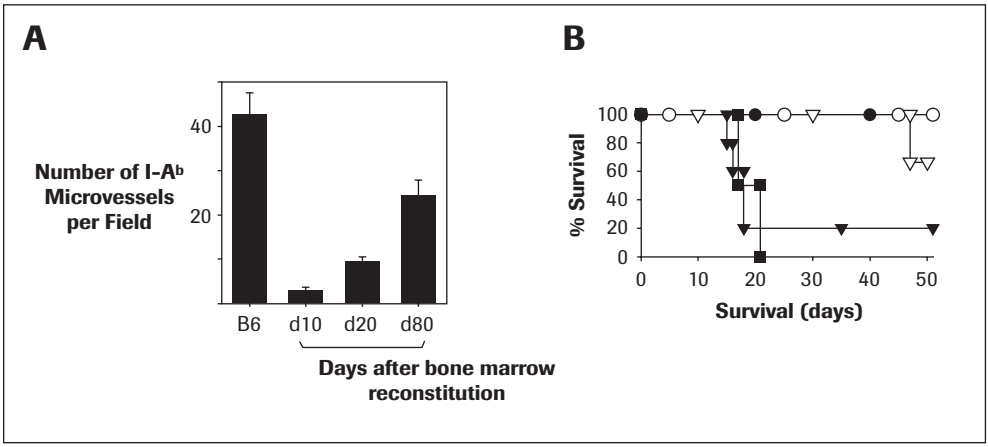

Figure 4. The presence of recipient-derived I-A^b-positive EC within cardiac allograft triggers acute rejection of H-2^k male cardiac graft in Marilyn recipients. A. *Double immunostaining with anti-CD105 and anti-I-A^b (clone 25-9-17) antibodies revealed that CD105-positive EC in hearts isolated from H-2^b-into-H-2^k BM chimera expressed I-A^b molecules. In hearts of H-2^b-into-H-2^k BM chimera, the number of capillaries containing I-A^b-positive cells increased with time after BM transplantation.* ***B.*** *The presence of capillaries containing I-A^b-positive cells induced, in Marilyn recipients, the acute rejection of heart graft isolated from H-2^b-into-H-2^k BM chimera. H-2^b (filled circles) and H-2^k (open circles) male heart grafts survived indefinitely in unprimed Marilyn recipients. Preimmunization with H-2^b-positive male DC induced the acute rejection of H-2^b (filled triangles), not H-2^k (open triangles), male cardiac grafts in Marilyn mice. Cardiac grafts from H-2^b-into-H-2^k BM male chimera (taken 60 days post-BM transplantation) are acutely rejected by immunized Marilyn recipients (closed squares).*

BM-derived stem cells give rise to functional EC that rapidly integrate into blood vessels of several organs, including heart. Our results indicate that recipient-derived EC, integrated into the blood vessels of the graft, could well represent the main targets recognized by indirect pathway CD4$^+$ T cells within transplanted tissues. Marilyn T cells were indeed found in close contact with microvessel cells that expressed recipient's I-A^b molecules in rejected H-2^k male cardiac grafts. Moreover, induction of acute rejection of chimeric hearts containing recipient-derived I-A^b EC also provided strong evidence in support of this concept. These results have an important implication in the pathogenesis of chronic rejection. They indeed suggest that the participation of indirect pathway alloreactive T cells in the process is conditioned by the presence of recipient-derived EC within the transplanted tissues.

It is important to mention that our results do not exclude the possibility that recipient's MHC class II-expressing leukocytes infiltrating the graft do participate in the process of rejection and interact in an antigen-dependent fashion with T cells within the graft, as demonstrated by others[13]. However, these interactions do not seem sufficient to promote acute rejection since

non-chimeric H-2^k hearts are not rejected acutely by Marylin T cells. Again, our results show that acute rejection is primarily linked to the presence of recipient-derived EC in rejected H-2^k organs.

In conclusion, the outcome of transplantation of grafts that contain recipient-derived EC in our model supports the importance of the indirect pathway of allorecognition in chronic vascularized organ rejection. The development of animals exhibiting endothelium-specific deficiency of MHC class II molecule expression will be of great value to ascertain this process. Vascular rejection is a severe complication of transplantation leading to a very poor prognosis that warrants aggressive treatment—even then a high percentage of grafts are lost. Our findings suggesting the role of EC replacement in triggering chronic rejection by indirect pathway CD4$^+$ T cells offer some insight into this complication and appeal new therapeutic inhibitors targeting specifically neovasculogenesis of long-term accepted grafts.

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SCID-Mouse/Human Transplant Model for Gamma Herpesvirus Infection

The aim of this project has been to investigate the effect of immunosuppressive therapy on Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr-Virus (EBV) pathogenesis in transplant patients. KSHV and EBV are both associated with significant mortality and morbidity in transplant patients. This group initially showed that IFN-γ could reactivate KSHV from latency in cultured primary effusion lymphoma cells (PEL)[1]. However, we were unable to study the contribution of IFN-γ or other paracrine factors in cell culture. Therefore, we proposed to establish an animal model for KSHV and EBV-associated lymphoma and to determine the impact of anti-virals, cytokines and immunosuppressive drugs on tumorigenesis in this model. We have established a novel xenograft model for KSHV-associated lymphoma[2].

Embedded in matrigel™, PEL cells formed rapid, well organized and angiogenic tumors after subcutaneous implantation of C.B.17 SCID mice. Without matrigel™ we did not observe comparable tumors, which implies that extra-cellular support and/or signaling aids PEL. All tumors maintained the KSHV genome and the KSHV latent protein LANA/orf73 was uniformly expressed [Fig. 1 adapted from (2)]. However, the expression profile for key lytic mRNAs, as well as LANA-2/vIRF3, differed between tissue culture and sites of implantation. We did not observe a net effect of ganciclovir on PEL growth in culture or as xenograft. These findings underscore the importance of the microenvironment for PEL tumorigenesis, and simplify the pre-clinical evaluation of potential anti-cancer agents.

To measure viral transcription in the lesions, we developed a real-time quantitative RT-PCR to measure KSHV viral load and virus specific-transcripts[3,4]. This put us in a position to quantify mRNA for every single one of the 80 genes that make up the KSHV genome. This is, in effect, a KSHV-specific DNA-chip, except that rather than solid support, we are using solution polymerase chain reaction (PCR). Using this assay we determined that in intraperitoneal grown PEL tumors KSHV viral lytic mRNAs were induced relative to growth in tissue culture[2]. This suggests that a much wider group of viral proteins can be developed into therapy targets. Using this assay we determined that, in contrast to ganciclovir, azidothymidine (AZT)-treated PEL cell lines resulted in a transient induction and subsequent reduction of KSHV lytic mRNAs (data not shown). In the long-term, we plan to pursue our query of transcriptional effects of antiviral drugs on PEL in culture and in our new mouse model.

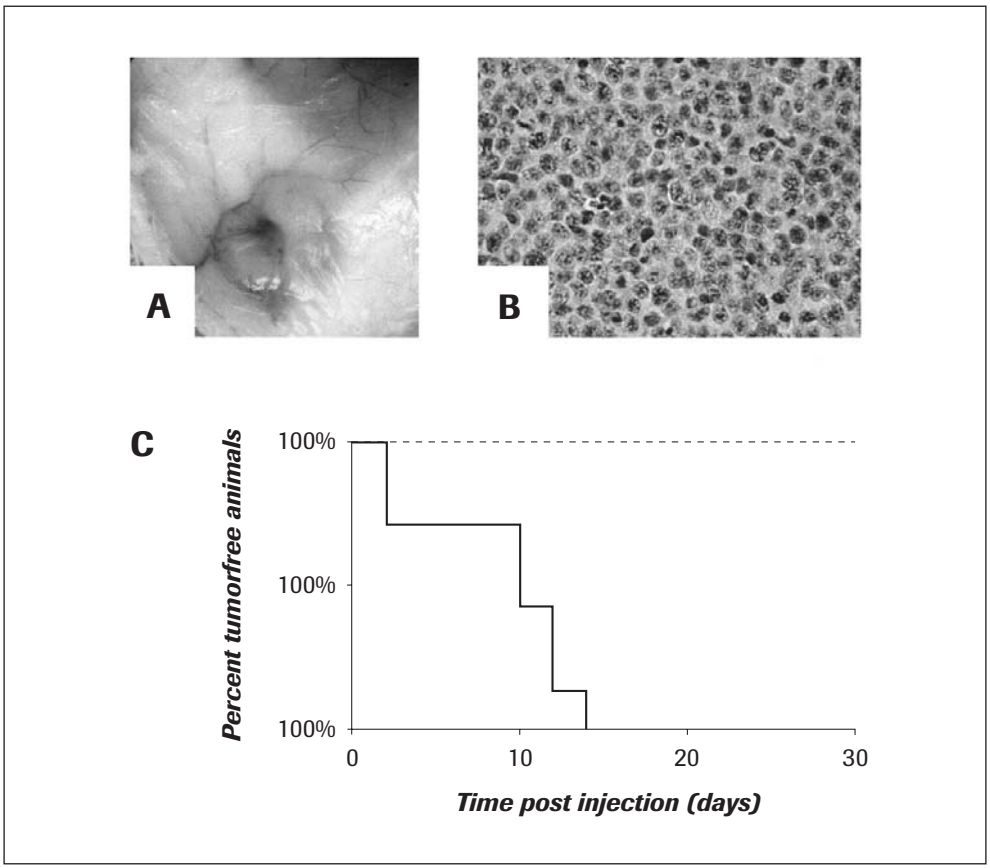

Figure 1 [adapted from (2)]. BCBL-1 cells form subcutaneous tumors in the presence of matrigel™. ***A.*** *Gross anatomy of a representative BCBL-1 tumor with clear evidence of neo-angiogenesis.* ***B.*** *BCBL-1 tumors stained with a monoclonal anti-LANA antibody and counterstained with hematoxylin at 400x. All tumor cells express the latent viral antigen.* ***C.*** *Kaplan Meier plot for s.c. tumor formation in the absence (dashed line) or presence (full line) of matrigel™.*

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Protective Effect of A20 against Transplant-Associated Vasculopathy

We hypothesized that A20 is an ideal gene therapy candidate for the prevention and treatment of transplant-associated vasculopathy (TAV), the prime feature of chronic allograft rejection. A20 is anti-inflammatory and anti-apoptotic in endothelial cells (EC) and anti-proliferative, pro-apoptotic in smooth muscle cells (SMC). This proposal was aimed at studying the function of A20 in SMC and establishing an *in vivo* proof of its efficacy for the prevention of TAV.

We had 3 specific aims:

Specific Aim 1: Evaluate *in vitro,* the inhibitory effect of A20 on NF-κB activation in SMC and determine how this modulates SMC activation and proliferation.
Specific Aim 2: Determine the role of A20 upon SMC apoptosis *in vitro.*
Specific Aim 3: Determine whether expression of A20 protects from TAV *in vivo.*

Specific Aim 1. Evaluate *in vitro,* the inhibitory effect of A20 on NF-κB activation in SMC and determine how this modulates SMC activation and proliferation

We have now completely addressed Specific Aim 1.

1. We have shown that A20 is part of the physiological response of SMC to injury.
2. We have demonstrated that A20 inhibits inflammation via blockade of NF-κB activation and NF-κB dependent pro-atherogenic proteins ICAM-1 and MCP-1.
3. We have established that A20 inhibits SMC proliferation via increased expression of p53 and cyclin-dependent kinase inhibitors (CDKI) $p21^{waf1}$ and $p27^{kip1}$.

To our knowledge, this is the first demonstration that A20 is part of the anti-inflammatory and anti-proliferative response of SMC to injury and promotes vascular healing. *A20 fulfills three requirements for proposing it as a gene therapy candidate to protect from TAV:*

i) A20 is part of the physiological response of SMC to injury;
ii) A20 is a potent anti-inflammatory protein in SMC via blockade of NF-κB and NF-κB dependent proinflammatory and pro-atherogenic proteins such as ICAM-1 and MCP-1;
iii) A20 has a novel anti-proliferative function in SMC via blockade of the CDKI $p21^{waf1}$ and $p27^{kip1}$.

These results have been finalized in a manuscript submitted to *Nature Medicine*[1].

In addition, we have demonstrated in collaboration with Dr. W. Aird that the transcription factor forkhead is also involved in SMC proliferation and its overexpression prevents neointimal hyperplasia[2].

Specific Aim 2. Determine the role of A20 SMC apoptosis *in vitro*

1. Overexpression of A20 sensitizes SMC to cytokine and FAS-mediated apoptosis in a nitric oxide (NO)-dependent manner.

Most importantly, we have confirmed that A20 SMC increases their sensitivity to cytokine and Fas-mediated apoptosis (Fig. 1). This novel function is independent from inhibition of NF-κB activation. Indeed, the pro-apoptotic effect of A20 in SMC was twice as much of that achieved in SMC expressing the specific inhibitor of NF-κB: IκBα. Further, coexpression of the anti-apoptotic bcl family member A1 reverted apoptosis in IκBα but not A20 expressing SMC further indicating that this novel pro-apoptotic effect of A20 in SMC is independent from blockade of NF-κB (Fig. 2).

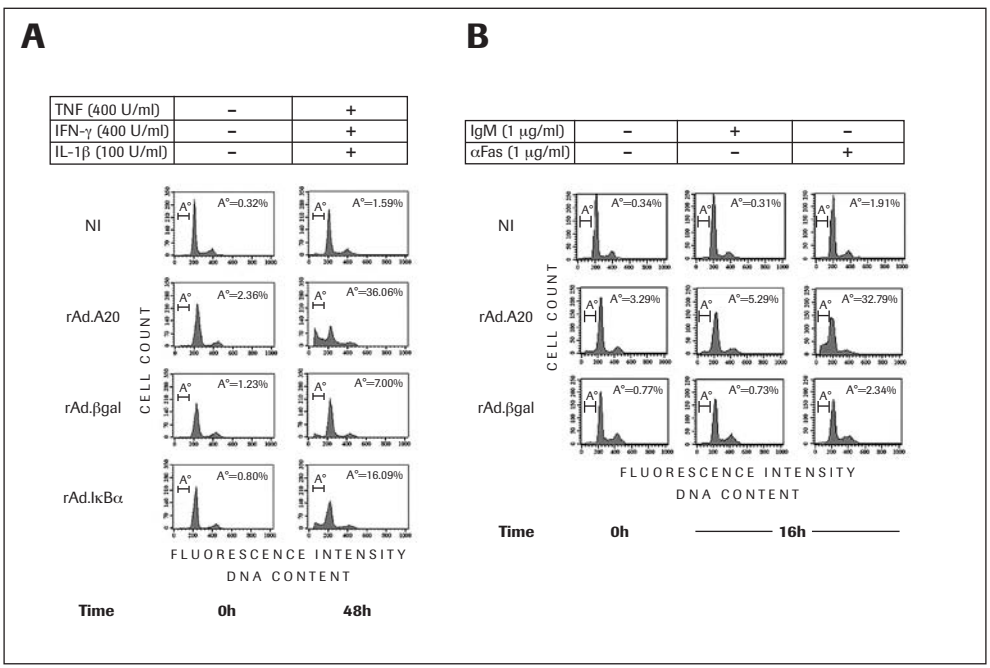

Figure 1. A20 SMC increases sensitivity to cytokine and Fas-mediated apoptosis.

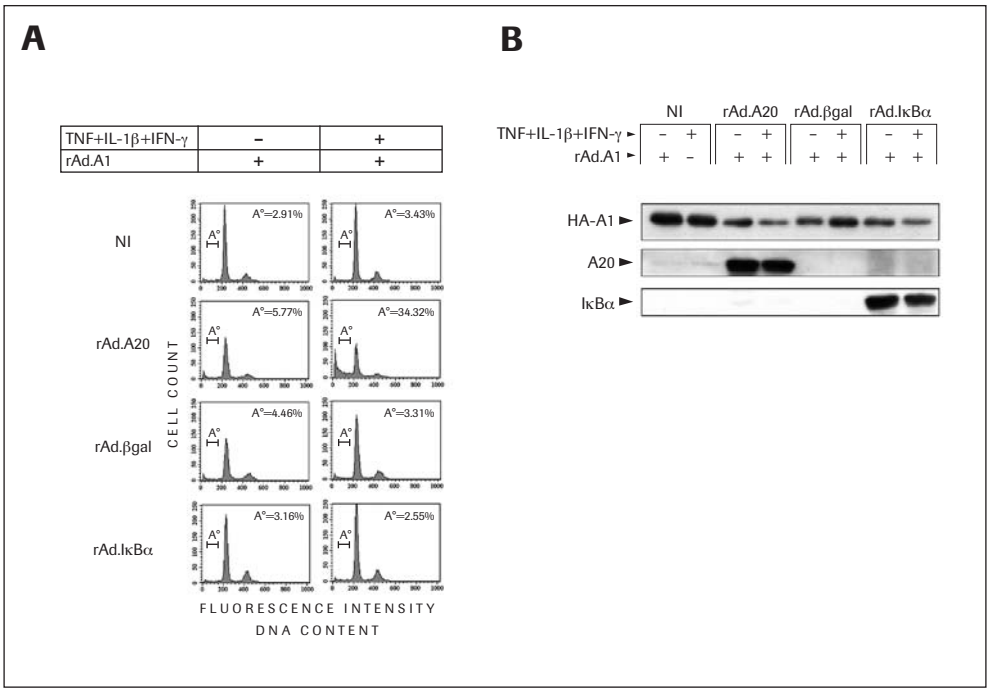

Figure 2. The pro-apoptotic effect of A20 in SMC is independent from blockade of NF-κB.

Rather, we demonstrate that the pro-apoptotic function of A20 in SMC relates to increased nitric oxide (NO) production in cytokine-activated SMC leading to a direct DNA damaging effect. SMC over-expressing A20 demonstrated prototypical DNA fragmentation following cytokine treatment as assessed by TUNEL assay (data not shown). Pre-treatment of SMC expressing A20 with the NO synthase inhibitor L-NAME prior to the addition of TNF, IFN-γ and IL-1β led to a dose-dependent reversal of the pro-apoptotic effect of A20 (Fig. 3). *These results demonstrate that the novel pro-apoptotic function of A20 in SMC is NO-dependent.*

In contrast, the anti-apoptotic effect of A20 in EC was not altered by blockade of NO. The percentage of apoptotic EC was only half that detected in the control groups and was not abrogated in EC pre-treated with L-NAME (Fig. 3).

These results demonstrate for the first time a novel mechanism of action of A20 in SMC: potentiation of apoptosis in an NO-dependent manner which adds a therapeutic dimension to the protective effect of A20 against TAV. These results have been finalized in a manuscript submitted to *Nature Medicine*[3].

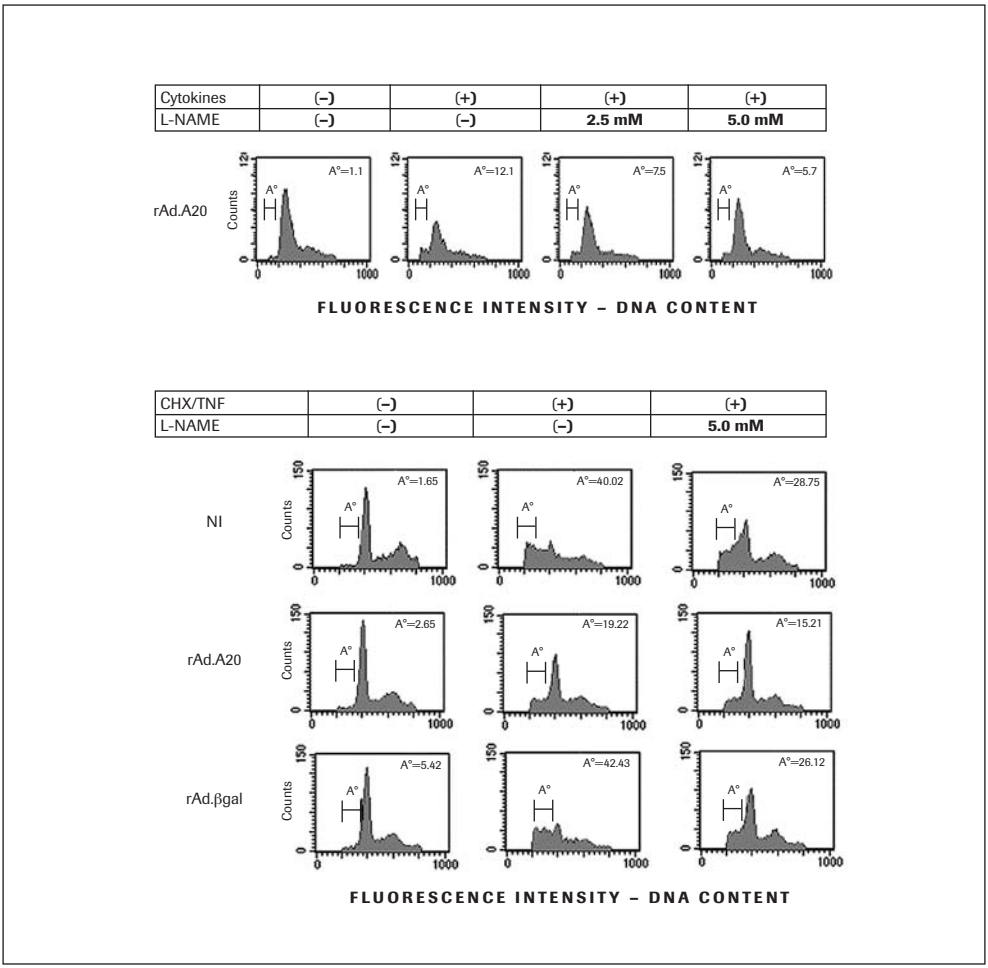

Figure 3. The anti-apoptotic effect of A20 in EC is not altered by blockade of NO.

Specific Aim 3. Determine whether expression of A20 protects from TAV *in vivo*

The work for specific aim 3 has successfully started but is not completed. We have set up the model of rat aortic allograft with the help of three surgical residents who are working in the laboratory: Dr. Virendra I. Patel, Gautam Shrikhande and Salvatore T. Scali.

Because of the possibility that EC and SMC of the recipient can replace those of the donor and contribute to chronic rejection, we have slightly modified this aim to using as recipients rats that were reconstituted with GFP-tagged bone marrow, which will allow us to evaluate

whether bone marrow-derived EC and SMC stem cells home to the site of the transplant and contribute to TAV. In this model, we will be able to address whether A20 expressing grafts are protected from immune-mediated injury and hence will not be replaced by donor EC/SMC or, even if replacement occurs, whether the anti-inflammatory environment provided by A20 will keep these cells in a quiescent environment that is not permissive for the development of TAV. Unfortunately, we encountered some delay with the transfer of these rats kindly provided by our collaborators Drs. H. Wada and K. Sata from Japan. The rats are now readily available and the work is currently being finalized.

While awaiting for the GFP rats, we tested the atheroprotective function of A20 *in vivo* using a carotid balloon angioplasty model in rats which develop lesions whose components are closely related to TAV, i.e. formation of an occlusive neointima with activated, proliferating and apoptosis-resistant neointimal SMC. We injured carotid arteries and treated the vessels with normal saline, rAd.A20 or control rAd.β-gal. We demonstrated high levels of the X-gal transgene expression in medial SMC (Fig. 4). Vascular A20 expression was confirmed by RT-PCR. We observed significant neointimal hyperplasia in NI and rAd.β-gal infected carotids with an I/M ratio reaching 0.79 ±0.15 and 0.84 ±0.13, respectively. A20 expression significantly inhibited neointima formation with an I/M ratio of 0.30 ±0.04 ($n=7$; $p=0.022$ vs. NI and $p=0.001$ vs. rAd.β-gal; Fig. 4). This preventive effect related to decreased SMC proliferation as depicted by significantly lower numbers of SMC expressing the proliferation marker KI67 (Fig. 4).

We then evaluated the impact of A20 expression upon established disease. Rat carotid arteries were subjected to balloon angioplasty and allowed to develop neointimal disease for 28 days. Injured vessels were then revisited and infused with normal saline, rAd.A20 or rAd.β-gal. Rats were sacrificed 14 days later and carotids recovered. The I/M ratio at day 28+14 in saline or β-gal treated carotids reached 1.06 ±0.10 ($n=8$) and 1.04 ±0.16 ($n=6$). In contrast, injured vessels treated with A20 showed a significant reduction of their I/M ratio to 0.49 ±0.06 ($n=7$; $p=0.0003$ vs. NI and $p=0.008$ vs. rAd.β-gal; Fig. 5A). Expression of the transgene 3 to 5 days after gene transfer was detected up to a depth of 3-4 cells within the neointima as demonstrated by X-gal staining and was confirmed by semiquantitative RT-PCR for A20 (Fig. 5A).

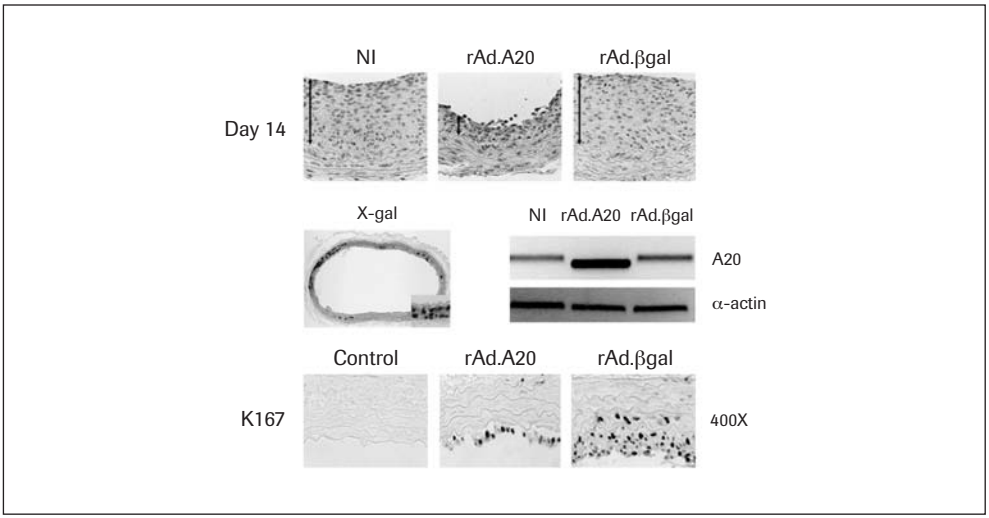

Figure 4. Atheroprotective function of A20 in vivo using a carotid balloon angioplasty model in rats.

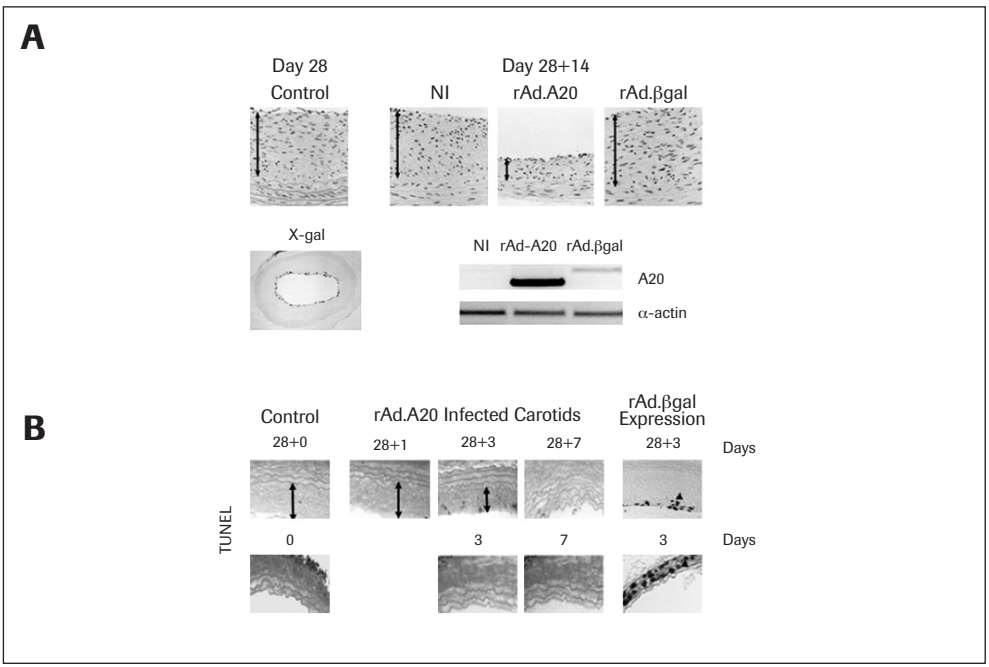

Figure 5. Evaluation of the impact of A20 expression upon established disease.

This effect related to increased apoptosis of neointimal SMC 3-5 days following gene transfer as evaluated by TUNEL staining (Fig. 5B). This novel pro-apoptotic effect of A20 was specific to neointimal SMC. Medial SMC expressing A20 at the time of balloon angioplasty did not undergo apoptosis (Fig. 5B). *These results indicate that, in EC and SMC, A20 fulfills the requirements of an atheroprotective gene therapy candidate: (i) A20 is induced in EC and SMC in response to injury; (ii) in EC, A20 is anti-inflammatory and anti-apoptotic; (iii) in SMC, A20 is anti-inflammatory, antiproliferative and uniquely promotes apoptosis of neointimal SMC. A20-based therapies could be beneficial to both prevent and cure transplant arteriosclerosis.* These exciting and provocative findings have been submitted to Nature Medicine as part of the two manuscripts cited above by V. Patel *et al*.

Six publications have resulted from this grant and where contribution of the ROTRF has been acknowledged including 3 published manuscripts in highly ranked peer-reviewed journals, 1 manuscript in revision for *Circulation* and 2 manuscripts recently submitted to *Nature Medicine*. In addition, data from this grant proposal were used as the basis for another grant proposal submitted as an RO1 to the National Institutes of Health (NIH).

Publications

1. Patel VI, Arvelo MB, Longo CR, Shrikhande G, Scali ST, Csizmadia E, Groft C, Shukri T, Grey ST, Daniel S, Ferran C. A20 prevents neointimal hyperplasia by inhibiting smooth muscle cell activation and proliferation and promoting reendothelialization. *Nat Med, Submitted for publication.*
2. Guo S, Abid MR, Patel VI, Shrikhande G, Ferran C, Aird WC. Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circulation, Submitted for publication.*
3. Patel VI, Daniel S, Longo CR, Shrikhande G, Scali ST, Csizmadia E, Groft C, Shukri T, Grey ST, Arvelo MB, Ferran C. A20 promotes regression of neontimal hyperplasia via a novel NO-dependent pro-apoptotic function in neointimal smooth muscle cells. *Nat Med, Submitted for publication.*

Additional publications

1. Longo CR, Arvelo MB, Patel VI, Daniel S, Mahiou J, Grey ST, Ferran C. A20 protects from CD40/CD40L mediated endothelial cell activation and apoptosis. *Circulation* 2003; 108:1113-8.
2. Kunter U, Floege J, von Jurgenson AS, Stojanovic T, Merkel S, Grone HJ, Ferran C. Expression of A20 in the vessel wall of rat kidney allografts correlates with protection from transplant arteriosclerosis. *Transplantation* 2003; 75:3-9.
3. Daniel S, Arvelo MB, Patel VI, Longo CR, Shrikhande G, Sukti T, Mahiou J, Sun DW, Mothley C, Grey ST, Ferran C. A20 protects endothelial cells from TNF, Fas and NK mediated cell death by inhibiting caspase 8 activation. *Blood* 2004; 104:2376-84.

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

The ROTRF has given us the opportunity to gather a large amount of data to support the concept that mTOR inhibitors have real promise to both prevent allograft rejection and to simultaneously inhibit cancer. We suggest this rather unusual dual effect will provide new hope for transplant patients at high risk for cancer, and for patients that already have developed cancer. Not long before our study was initiated, this basic concept seemed remote, since the thought of tumor regression being mediated by an immunosuppressive substance goes against the dogma that the immune system protects us against cancer development. We have learned many important lessons from our ROTRF study, and before reviewing our results in more detail, we would like to list our most important findings:

1. Rapamycin is a potent anti-angiogenic substance that can reduce the growth of a variety of human and mouse tumors in animal models. In contrast, cyclosporine use at immunosuppressive doses generally promotes the growth of tumors[1,2].
2. Rapamycin not only reduces tumor angiogenesis, it affects blood vessel function (e.g. promotes thrombosis by causing endothelial cell death and coagulation dysregulation)[3].
3. To achieve an optimal antitumor effect, rapamycin should be applied via “metronomic”-type therapy, which coincides with dosing and scheduling used in organ transplant patients. In other words, the way the drug is presently used in organ transplant recipients (every-day dosing) provides for a potent antitumor effect[4].
4. Combination of rapamycin with cytotoxic agents clearly provides for the best antitumor activity[3,5]. However, contrary to our hypothesis, we did not find that rapamycin in combination with paclitaxel was an effective combination.
5. Rapamycin’s combined immunosuppressive and antitumor activity is not diminished in mice that have both an allogeneic heart transplant and an autologous tumor – immunosuppression and anticancer activities can coexist[6].
6. The tumor growth-promoting effects of cyclosporine immunosuppression are blocked with concurrent rapamycin use[2].

Project summary

Anti-angiogenic effects of rapamycin

Early dorsal skin-fold chamber experiments (*in vivo* angiogenesis model) showed that tumor angiogenesis was markedly inhibited by daily treatment of mice with rapamycin[1]. We were able to show that this effect is likely mediated in part by inhibition of VEGF production but the inhibition only reduced VEGF production in tumor cells by $< 50\%$. We interpreted these data to mean that, although VEGF levels were decreased, tumor cells continued to produce significant amounts of the angiogenic factor. Therefore, considering that the primary VEGF intracellular signaling pathway (PI3K – mTOR – p70S6K) is controlled by mTOR, we predicted that even in the presence of VEGF, endothelial cell stimulation would be decreased. Indeed, *in vitro* tubular formation assays with HUVEC showed a complete blockage by rapamycin of *in vitro* angiogenesis in the presence of exogenously added VEGF. In the grant, we also proposed to test whether rapamycin affects the expression of TGF-β and HIF-1α, however, our initial mRNA quantitation studies by real-time PCR did not show any effect of rapamycin on these potential regulatory molecules in either HUVECs or in various types of tumor cells. We have also tested TGF-β protein secretion in the same cells and found no significant effect of rapamycin. However, very recent work from our laboratory does show that when we examine the HIF-1α protein translocation to the nucleus, there is a profound inhibitory effect on HIF-1α. Therefore, rapamycin appears to regulate this critical hypoxia-inducible molecule at the protein level, but not at the mRNA level. We are currently investigating this mechanism further, and feel mTOR inhibition by rapamycin could critically reduce hypoxia-driven tumor expansion, thus revealing a novel mechanism for its effectiveness. The importance of mTOR in this intracellular signaling pathway is presently being explored in tumor cell lines which are rapamycin-resistant (mTOR mutation making this molecule resistant to the negative effect of rapamycin; collaboration with Dr. George Thomas; Basel, Switzerland – now in Cincinnati, Ohio). These experiments will identify any non-mTOR-mediated effects of rapamycin on tumor formation.

An additional observation we have made is that blood vessels within rapamycin-treated CT-26 adenocarcinomas show an abnormal pooling effect after tumor cells are implanted into the dorsal skin-fold chambers. Tumor vessels in controls show typical chaotic interconnections but the vessels appear to function normally as can be seen by the consistent blood flow throughout the tumor in the dorsal skin-fold chamber. In contrast, tumor vessels in rapamycin-treated mice show an inconsistent pattern of growth, where interconnections are less and vessels are highly variable in width. Further analysis of blood flow dynamics has revealed that while $81.1 \pm 11.5\%$ of developing tumor vessels in control mice showed a positive flow, only $42.8 \pm 13.3\%$ of tumor vessels had a positive flow with rapamycin treatment ($n=3$ mice for each group). These results suggest rapamycin can have an effect on the

quality of the circulation within a tumor, as we have reported[7]. The mechanism behind this "blood flow" effect could be related in part to hemostasis. This and other potential vascular effects of rapamycin will be discussed in relation to other experiments below.

Effect of rapamycin on tumors when used in combination with cytotoxic agents

We have examined the effects of paclitaxel on the growth of McA-RH7777 hepatoma cells and on liver allograft rejection. In initial experiments we found that low concentrations (33-99 ng/ml) of paclitaxel strongly inhibit hepatoma cell proliferation *in vitro*. Furthermore, paclitaxel reduces liver allograft rejection when used at doses as low as 0.5 mg/kg/d for 14 days[8]. We have also shown paclitaxel to be effective against allograft rejection in a heterotopic rat heart transplant model[9], with activity against antidonor CTL and the humoral response[10]. Our next objective was to test whether the combination of rapamycin and paclitaxel could be effective at preventing recurrence of liver tumors after liver transplantation. Unfortunately, we were not able to fully develop the appropriate model system. The problem we encountered was that implanted McA-RH7777 tumors simply did not recur after the tumor-containing liver was removed. Therefore, we could not establish the appropriate model to test if rapamycin and paclitaxel are effective towards preventing liver tumor recurrence. To address this problem, we have most recently been working with different hepatic tumors that we believe may be more aggressive. However, more time will be needed to get this type of model operational.

In the meantime, we have looked at the effects of paclitaxel on tumor growth in mice but with disappointing results. Essentially, when we combined rapamycin with paclitaxel in preliminary experiments, no synergistic or additive effect on tumor reduction was observed (CT-26 colon adenocarcinoma or B16 melanoma). In fact, we found that the mice didn't tolerate the combination therapy well, with substantial weight loss. Different dosing and scheduling of the paclitaxel did not seem to improve the anti-tumor effect. At that point we decided to test the effect of rapamycin in combination with other cytotoxic agents, and found that indeed rapamycin synergized very well with gemcitabine, and with 5-FU, against tumor growth. Importantly, these experiments revealed interesting mechanisms of action which are described below.

First, when we combined rapamycin use with gemcitabine against human pancreatic cancer (nude mice), we observed a marked effect. Briefly, gemcitabine (standard therapy against pancreatic cancer) application alone reduced tumor growth, but rapamycin had a greater effect on tumor growth, and moreover, the drug combination produced long-term tumor control. Interestingly, we could show that the effect of rapamycin on tumor growth was related to tumor-specific blood vessel thrombosis. The mechanism of the effect could be correlated *in vitro* and *in vivo* with induction of endothelial cell apoptosis. For details of these experiments,

and discussion of potential mechanisms, please see our recently published manuscript[5]. Of course, these results have implications for rapamycin use in organ transplant recipients, since thrombosis has been observed with its use in bone marrow transplantation (peripheral microvascular thrombosis), and in liver transplantation (hepatic artery thrombosis). Importantly, however, what our experiments suggest is that only damaged (e.g. post-surgery) or newly formed vessels (e.g. tumor vessels) are susceptible to thrombosis with rapamycin treatment; normal vessels appear not to be affected. Nonetheless, these experiments reveal a novel mechanism of action against growing tumors. Furthermore, more recent experiments from our group suggest that rapamycin causes coagulation dysregulation by increasing tissue factor production via combined activities of VEGF and Ang-1 on the MAPK and PI3K intracellular signaling pathways – these results are being prepared for publication. Besides the obvious implications for transplant recipients, these results have provided the basis for a pilot study in pancreatic cancer patients (in Regensburg) – no organ transplant – using the strategy suggested by our experiments (use of daily rapamycin + regular dosing of low-dose gemcitabine). This study is well underway, with very encouraging results, and is leading to a second phase of the study.

We also combined rapamycin with 5-FU use for the treatment of colon adenocarcinoma. Once again, we observed a very strong synergistic effect of the two substances. The combination effect was strong against orthotopic colon adenocarcinomas in mice, and against the growth of colon adenocarcinoma metastases seeded into the liver. In this situation, however, we proposed an angiogenesis-related mechanism of action that involves the expression of thymidine phosphorylase (TP). Tumors expressing TP are thought to promote the cytotoxic activity of 5-FU (by enzymatic conversion to a more active form, FdUMP), but on the negative side, TP has been shown to promote tumor angiogenesis via conversion of thymidine into 2-deoxy-D-ribose (dRib), thus potentially encouraging tumor expansion. We have shown that the angiogenic activity of dRib is through the PI3K signaling pathway, and that rapamycin blocks this pro-angiogenic signaling. Therefore, rapamycin combination with 5-FU in a TP-expressing tumor can theoretically allow for optimal 5-FU cytotoxicity, while simultaneously negating the pro-angiogenic activity of TP-generated dRib (Fig. 1). Numerous other tumor types express TP, suggesting a broader application of this principle in oncology. More details of our findings and experiments can be found in a recently published article[3].

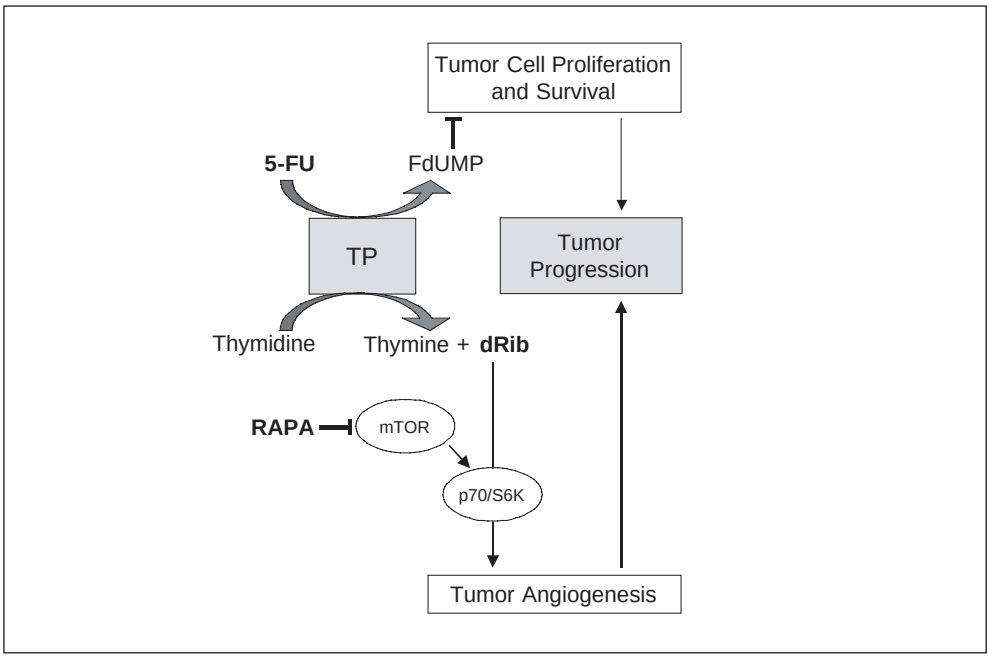

Figure 1.

Rapamycin dose for treating cancer – relationship to immunosuppressive doses

We also studied the doses of rapamycin necessary for achieving an optimal anti-tumor effect. This was critical to determine if rapamycin treatment routinely used in organ transplantation could be expected to have an anti-cancer action. What we could show in this work was that phosphorylation of P70S6 kinase could be strongly inhibited in HUVEC at as little as 0.1 ng/ml, and was completely blocked at 1 ng/ml. Because this pathway is thought to be critical for endothelial cell signaling, we hypothesized that low concentrations of drugs should be effective at reducing tumor growth. Growth of established CT-26 colon adenocarcinoma tumors was measured in Balb/c mice treated with total equivalent rapamycin doses (1.5 mg/kg/day) given once a day, once every 3 days, or by continuous infusion (osmotic pump). Results showed that tumors were most inhibited with continuous rapamycin infusion, and daily dosing, but high doses given every 3 days produced the poorest anti-tumor effect (and the greatest side-effects). Therefore, applying rapamycin less often, in higher bolus doses, reduces its inhibitory effect on tumor growth. A classic bell-shaped curve of dose effectiveness is indicated (peak is between 5-10 ng/ml), which has been a consistent finding with other antiangiogenic agents. Most importantly, we predict that daily immunosuppressive doses of rapamycin given to organ transplant patients will exert a potent anti-cancer action.

With the tumors we tested there did not appear to be a beneficial effect of using rapamycin's cytotoxic properties (high bolus doses) to control tumor growth. These experiments are explained in detail in a paper[4]. To summarize, our data suggest that "metronomic" use of rapamycin is the most effective means of its application for oncologic purposes. Moreover, these experiments suggest that clinically recommended immunosuppressive doses of rapamycin coincide with doses required for optimal tumor inhibition (at least in mice). Therefore, it is reasonable to expect that rapamycin use as an immunosuppressant in transplant patients could provide some protective effect against cancer.

Effectiveness of rapamycin in a tumor/organ transplant situation

One of our goals in this project was to determine whether rapamycin could be simultaneously effective as an immunosuppressive and anticancer agent. We spent a significant amount of effort developing models where recipient mice received an allogeneic organ transplant and were implanted with an *autologous* tumor – thus, mimicking the situation encountered in human transplant recipients. In these experiments we showed that rapamycin could simultaneously protect a heart allograft from rejection, while attacking an established tumor. The concentrations of rapamycin used were standard for immunosuppression in this model. In direct contrast, cyclosporine immunosuppression caused tumors to grow more rapidly than in control mice, and importantly, the mice lost their allografts not due to rejection, but to tumor effects that required mice to be euthanized. These experiments illustrate the point that rapamycin simultaneously protects allografts from rejection and attacks tumors in a complex transplant-tumor situation; cyclosporine protects allografts from rejection, but transplant recipients fall victim to cancer[2].

We also examined the question of whether rapamycin's anti-tumor effect would continue to be as potent in the presence of cyclosporine. When we used rapamycin and cyclosporine in combination, the anti-tumor effect of rapamycin was completely maintained, conversely, the pro-tumor effect of cyclosporine was nullified. In this study we show that at least part of the cyclosporine tumor growth-promoting effects could be related to an increase in angiogenesis, potentially mediated via TGF-beta. Although our analysis is not complete, we now have molecular evidence that the pro-angiogenic effect of cyclosporine relates to a signaling loop involving TGF-beta, ID-1/2 and thrombospondin. We are continuing to work on the details of this mechanism. Nonetheless, these general findings have significant implications for physicians hesitant to take calcineurin inhibitors completely away (and replace it with mTOR inhibitor-based therapy) from their transplant recipients who have developed cancer.

An incidental observation from this same group of experiments was that a tumor can have an effect on the immune reaction to an allograft. More specifically, in one of the tumor/transplant mouse models we used, the presence of a colon adenocarcinoma dramatically prolonged

allograft survival. In our recent publication that describes most of the work presented in this section[2], we give potential explanations for this effect but as of yet, we have no solid data to back any particular mechanism. At this point, this observation only highlights the complicated nature of examining tumor growth and transplant rejection in a setting where transplant recipients have developed cancer. From this perspective, there needs to be a continuing effort to better understand the nature of the problem we face in transplant patients with cancer.

Overview of the problem of cancer in transplant recipients

Finally, I would like to mention that the support from the ROTRF has enabled me to write two recent review articles on the topic of cancer in organ transplantation[6,11]. Many of my theories on this emerging topic were formulated as a result of this grant, and I am grateful for your support. I welcome you to read these review articles to gain an impression of my most current thoughts about how we can reduce the problem of cancer in transplant recipients. I am certain that the work we were able to do on this ROTRF project has moved us towards a better understanding of the problem and the actions we can begin to take to design appropriate clinical trials. In this respect we, at the University of Regensburg, have organized a new prospective, randomized, clinical trial in hepatocellular carcinoma patients after liver transplantation – with the purpose of testing whether mTOR inhibitors can reduce tumor recurrence problems after allografting. I believe this represents a tangible step forward. The phase I pancreatic cancer study that has spun-off from this work is another example of our intent to do translational research.

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LAT, a Molecule Critical for T Cell Activation and Function, is a Potential Substrate for Calcineurin

Results up-to-date

Isolation of LAT

We initially had a hard time in purifying LAT in a sufficient and pure form that could be analyzed by mass spectrometry. We tried several different approaches, including changing buffer compositions, solubilization procedures, immunoprecipitation procedure and time, and using up to 10^9 Jurkat T cells. We reasoned our inability to purify LAT was due to the low affinity of the antibodies. We have recently used a new batch of anti-LAT polyclonoal antibodies that seems to precipitate enough amount of LAT to be visualized by Coomassie Blue staining (Fig. 1). Furthermore, the gel indicates that LAT was precipitated in a pure form.

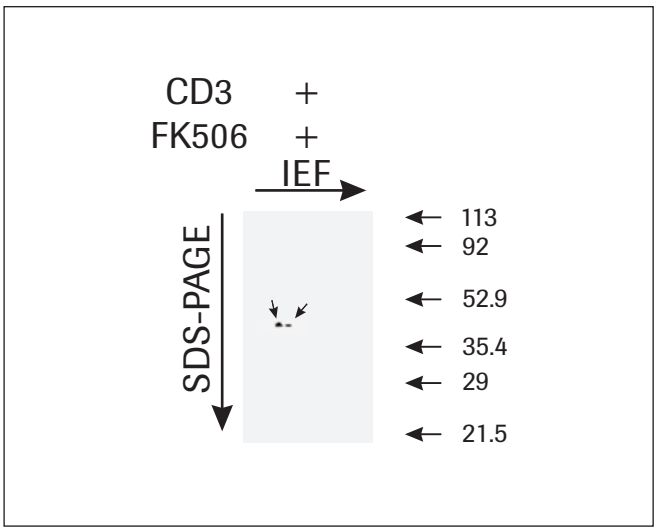

Figure 1. TCR ligation in the presence of calcineurin inhibitors induces a shift in the mobility of LAT in SDS-PAGE. *Cell lysates from 10^9 Jurkat T cells treated with the combination of anti-CD3 mAb and FK506 were subjected to isoelectric focusing (IEF) followed by SDS-PAGE. Gel was stained with Coomassie Blue.*

Inhibitors of PP1 and PP2A do not increase the expression of LAT nor do they modulate its mobility in SDS-PAGE

To determine whether the change in the migration of LAT can be brought about by inhibitors of serine/threonine phosphatases other than calcineurin, Jurkat T cells were activated through TCR ligation in the presence of PP1 and PP2A inhibitors calyculin A, cantharidic acid, and okadaic acid. These agents were used at concentrations that have previously been shown to effectively block PP1 and PP2A activation. These inhibitors failed to induce any detectable change in the migration of LAT (Fig. 2). These results further confirm that the calcineurin inhibitors-induced migration of LAT is due to the inhibition of calcineurin and not due a general inhibition of phosphatases.

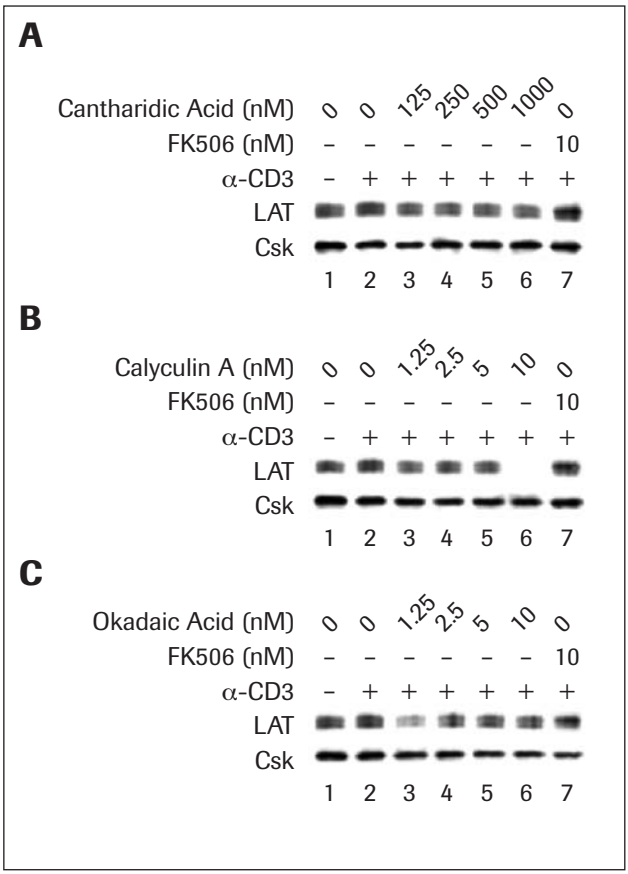

Figure 2. Inhibitors of PP1 and PP2A do not modulate the mobility of LAT in SDS-PAGE. *Jurkat T cells were left untreated (**A**, **B**, **C**; lane 1) or were treated with the indicated concentrations of cantharidic acid (**A**; lanes 3-6), calyculin A (**B**; lanes 3-6), okadaic acid (**C**; lanes 3-6), or FK506 (**A**, **B**, and **C**; lane 7). After a 16 h incubation at 37°C, the cells were left untreated (**A**, **B**, **C**; lane 1) or were stimulated for 60 min at 37°C with anti-CD3 mAb (**A**, **B**, **C**; lanes 2-7). After incubation, the cells were immediately lysed and proteins in WCL were separated by SDS-PAGE, transferred to membranes and blotted with anti-LAT mAb and as a control with anti-Csk Ab.*

Rapamycin blocks activation-induced expression of LAT in T cells

The structurally-related drug, rapamycin, binds the same intracellular receptor as FK506 but exerts different effects on T cells. To determine the effect of rapamycin on TCR-induced LAT expression, Jurkat and normal peripheral blood T cells were incubated with anti-CD3 monoclonal antibody (mAb) in the presence of the indicated concentrations of FK506 or rapamycin. These treatments did not affect the number or viability of T cells, as determined by trypan blue dye exclusion assay (data not shown). After a 16-hour incubation, the cells were lysed with the addition of boiling SDS-PAGE sample buffer. The resulting WCL were separated by 10% SDS-PAGE, electrotransferred to PVDF membrane, and blotted with anti-LAT Ab or, for control purposes, with anti-Csk Ab. Initial studies had demonstrated that regulation of Csk expression was fairly resistant to the various treatments described in this report. In agreement with our previously published results, FK506 reproducibly potentiated activation-induced expression of LAT in a dose-dependent manner (Fig. 3A). In contrast, rapamycin reproducibly inhibited activation-induced expression of LAT in a dose-dependent manner (Fig. 3B). The inhibitory effect of rapamycin on activation-induced LAT expression was also observed in normal T cells drawn from peripheral blood (Fig. 3C). Time course experiments using Jurkat T cells demonstrated no evidence of increased LAT expression at any time with rapamycin treatment, whereas FK506 and CsA strongly potentiated LAT expression within 4 hours of treatment (Fig. 4).

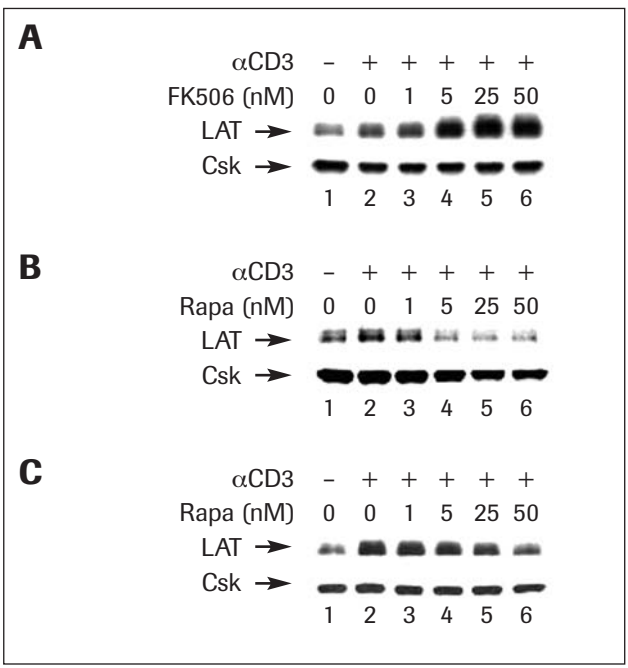

Figure 3. Rapamycin blocks activation-induced expression of LAT in T cells. A. *2 x 10^5 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-6) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) with varying concentrations of FK506 (lanes 3-6) at 37°C for 16 hours. Cells were then lysed, and proteins were separated by 10% SDS-PAGE. Gels were electrotransferred to PVDF membranes and immunoblotted with anti-LAT or anti-Csk Ab as shown.* ***B.*** *Similar experiments were conducted using Jurkat T cells with varying concentrations of rapamycin (Rapa; lanes 3-6).* ***C.*** *The same reaction conditions were then repeated using 4 x 10^5 normal T cells collected from peripheral blood. These experiments were performed three times with similar results.*

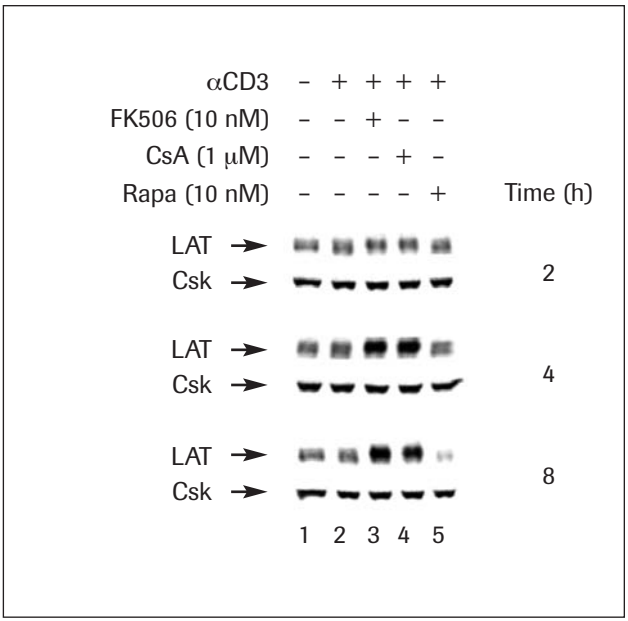

Figure 4. Time-course studies for rapamycin-induced inhibition of LAT expression. *2 x 10^5 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-5) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) in the presence of 10 nM FK506 (lane 3), 1 nM CsA (lane 4) or 10 nM rapamycin (Rapa; lane 5) at 37°C. After 2, 4 or 8 hours, cells were lysed, and proteins were separated by 10% SDS-PAGE. Gels were electrotransferred to PVDF membranes and immunoblotted with anti-LAT or anti-Csk Ab as shown. This experiment was performed three times with similar results.*

A downstream result of TCR engagement is the activation of protein kinase C, a serine/threonine kinase that has been shown to regulate protein expression in T cells. We have previously shown that T cell stimulation by phorbol myristate acetate (PMA), a phorbol ester which directly binds and activates PKC, also upregulates expression of LAT. To determine if rapamycin inhibited activation-induced LAT expression by blocking signals downstream of PKC, we examined the effect of rapamycin on T cells activated with PMA. Rapamycin inhibited PMA-induced expression of LAT in a dose-dependent manner, suggesting that rapamycin is exerting its inhibitory effect downstream of PKC activation (Fig. 5). These treatments did not affect the number or viability of T cells, as determined by trypan blue dye exclusion assay (data not shown). Together, these results strongly suggest that, although FK506 and rapamycin bind the same intracellular immunophilin receptor (FKBP), the distinct signals initiated by their interactions result in opposite regulatory effects on LAT expression.

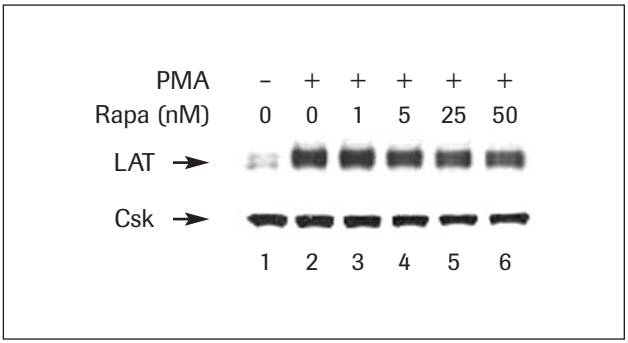

Figure 5. Rapamycin inhibits PMA-induced expression of LAT. *2 x 10^5 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-6) of 1 ng/ml PMA with varying concentrations of rapamycin (Rapa, lanes 3-6) at 37°C for 16 hours. Western blot analysis was then performed as described above. This experiment was performed three times with similar results.*

Rapamycin and FK506 induce mutually antagonistic effects on activation-induced expression of LAT

Because FK506 and rapamycin share the same intracellular binding protein species (FKBP), it is conceivable that they may function as pharmacological antagonists vis-à-vis their effects on LAT expression. To explore this possibility, mixing experiments were performed by stimulating Jurkat T cells with plate-immobilized anti-CD3 mAb in the presence of suboptimal concentrations of FK506 (5 nM) and varying concentrations of rapamycin. Rapamycin inhibited the ability of FK506 to enhance activation-induced LAT expression in a dose-dependent manner (Fig. 6A). Conversely, Jurkat T cells were stimulated with plate-immobilized anti-CD3 mAb in the presence of suboptimal concentrations of rapamycin (5 nM) and varying concentrations of FK506. FK506 overcame the ability of rapamycin to inhibit activation-induced LAT expression in a dose-dependent manner (Fig. 6B). These observations of drug antagonism suggest that FK506 and rapamycin induce their stimulatory and inhibitory effects on activation-induced LAT expression in part using a common pathway.

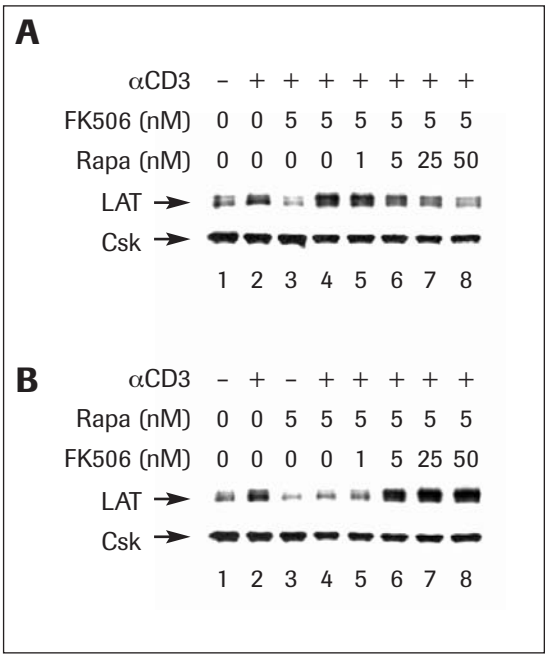

Figure 6. Rapamycin and FK506 induce mutually antagonistic effects on activation-induced expression of LAT.
A. *2 x 10^5 Jurkat T cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4-8) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) with 5 nM FK506 (lanes 3-8) and varying concentrations of rapamycin (Rapa, lanes 5-8) at 37°C for 16 hours. Cells were then lysed, and proteins were separated by 10% SDS-PAGE. Gels were transferred to PVDF membranes and immunoblotted with anti-LAT or anti-Csk Ab as shown.*
B. *2 x 10^5 Jurkat T cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4-8) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) with 5 nM rapamycin (Rapa, lanes 3-8) and varying concentrations of FK506 (lanes 5-8) at 37°C for 16 hours. Western blot analysis was then performed as described above. These experiments were performed three times with similar results.*

Rapamycin and CsA induce mutually antagonistic effects on activation-induced expression of LAT

Unlike FK506, CsA binds the intracellular receptor protein cyclophilin and not FKBP. Therefore, CsA and rapamycin do not share the same intracellular binding target. To examine if the antagonistic effects observed between rapamycin and FK506 were due strictly to competition for FKBP association, mixing experiments were performed by stimulating Jurkat T cells with plate-immobilized anti-CD3 mAb in the presence of suboptimal concentrations of CsA (0.5 μM) and varying concentrations of rapamycin. Rapamycin inhibited the ability of CsA to enhance activation-induced LAT expression in a dose-dependent manner (Fig. 7A). When Jurkat T cells were stimulated with plate-immobilized anti-CD3 mAb in the presence of suboptimal concentrations of rapamycin (5 nM) and varying concentrations of CsA, the inhibitory effect of rapamycin was overcome by CsA in a dose-dependent fashion (Fig. 7B). This suggests that the antagonism between the CsA, FK506, and rapamycin in their influence on activation-induced LAT expression is not simply due to competition for intracellular immunophilin binding.

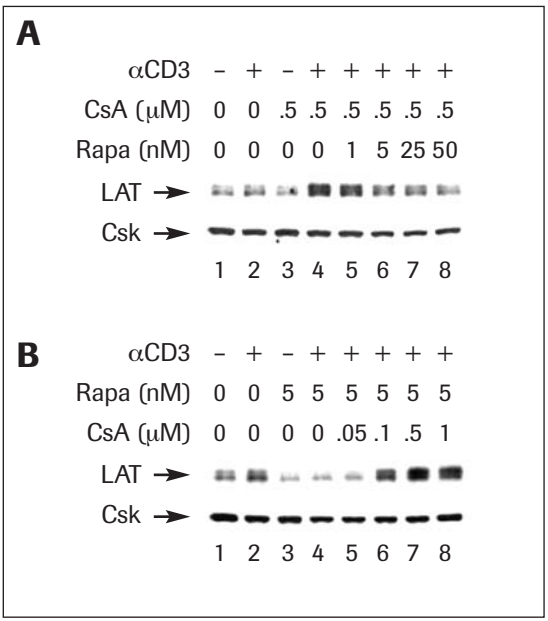

Figure 7. Rapamycin and CsA induce mutually antagonistic effects on activation-induced expression of LAT. ***A.*** *2 x 10^5 Jurkat T cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4-8) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) with 0.5 μM CsA (lanes 3-8) and varying concentrations of rapamycin (Rapa, lanes 5-8) at 37°C for 16 hours. Western blot analysis was then performed as described above.* ***B.*** *2 x 10^5 Jurkat T cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4-8) of 3 μg/ml plate-immobilized anti-CD3 mAb (αCD3) with 5 nM rapamycin (Rapa, lanes 3-8) and varying concentrations of CsA (lanes 5-8) at 37°C for 16 hours. Western blot analysis was then performed as described above. These experiments were performed three times with similar results.*

Rapamycin-induced inhibition of LAT protein expression is not associated with a decrease in LAT mRNA

To determine if the inhibitory effect of rapamycin on activation-induced LAT expression is associated with a decrease in the level of LAT mRNA, Northern blot analysis was performed on mRNA collected from Jurkat T cells stimulated for 16 hours with PMA in the presence of varying concentrations of rapamycin. Levels of LAT mRNA were increased in PMA-stimulated T cells, as we have reported previously (Fig. 8A). However, stimulation in the presence of the indicated doses of rapamycin did not decrease LAT mRNA levels. Importantly, these doses of rapamycin were sufficient to inhibit activation-induced upregulation of LAT protein expression. This observation was supported using RT-PCR analysis of RNA prepared from Jurkat T cells stimulated for 16 hours with PMA in the presence of varying concentrations of rapamycin. Again, rapamycin did not increase the level of LAT mRNA as measured by RT-PCR of LAT-specific sequences (Fig. 8B). Similarly, amplification of LAT cDNA using PCR demonstrated an increase in LAT mRNA levels in response to PMA stimulation, but these levels were not affected by the addition of various concentrations of rapamycin (Fig. 8C). These data suggest the inhibitory effect of rapamycin does not involve downregulation of LAT mRNA.

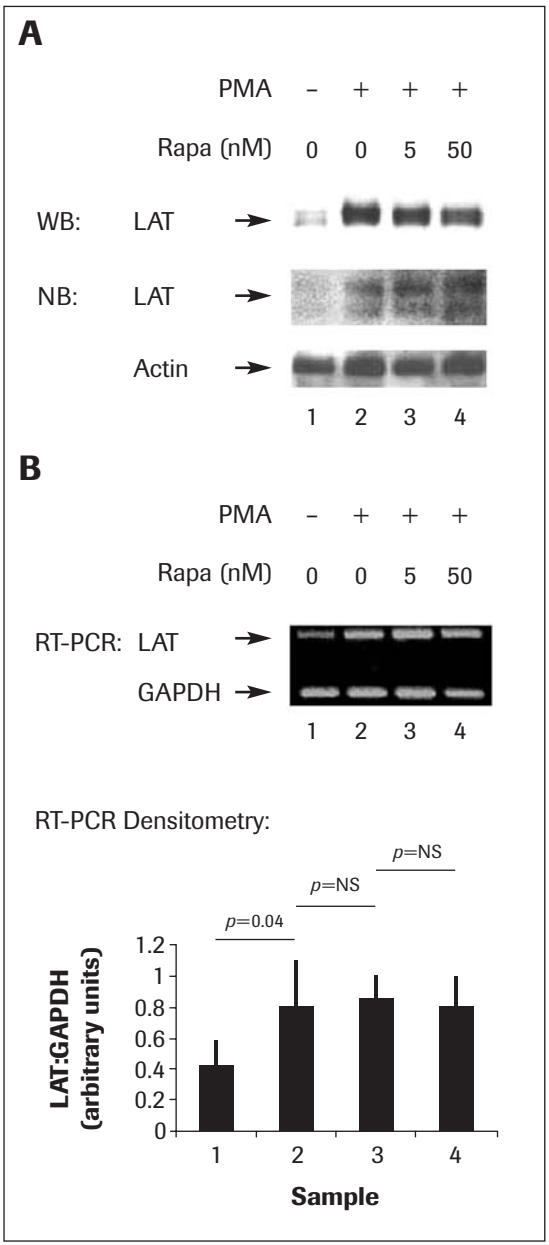

Figure 8. Rapamycin-induced inhibition of LAT protein expression is not associated with a decrease in LAT mRNA. A. *2 x 10^7 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-4) of 1 ng/ml PMA with 0 nM (lanes 1 and 2), 5 nM (lane 3), or 50 nM of rapamycin (Rapa; lane 4) at 37°C for 16 hours. An aliquot was used for cell lysis and Western blot analysis as described above. Poly A^+ RNA was isolated from the remainder of the cells, and separated by 1% agarose gel electrophoresis. The gel was transferred to a positively charged nylon membrane overnight. RNA was then cross-linked onto the membrane at 80°C for 30 minutes. The membrane was probed with biotinylated antisense oligonucleotides specific for LAT and actin mRNA as shown. This experiment was performed three times with similar results. WB (western blotting), NB (northern blotting).* ***B.*** *2 x 10^7 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-4) of 1 ng/ml PMA with 0 nM (lanes 1 and 2), 5 nM (lane 3) or 50 nM of rapamycin (Rapa; lane 4) at 37°C for 16 hours. Poly A^+ RNA was isolated and reverse transcribed to cDNA. The resultant cDNA was ampified by PCR using oligonucleotide primers specific for LAT and GAPDH cDNA. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Densitometric analysis of LAT PCR products normalized to GAPDH PCR products from three experiments are also represented histographically. Error bars denote standard deviations.*

Rapamycin does not induce post-translational degradation of LAT protein

Given that the level of LAT mRNA did not decrease by rapamycin treatment, we further sought to determine if the inhibitory effect of rapamycin was due to a post-translational instability of the LAT protein. Jurkat T cells were stimulated with PMA for 16 hours in test tubes. After this incubation, the cells were washed in RPMI-5% FCS free of PMA. Actinomycin-D was added to a final concentration of 3 μg/ml to block all new transcriptional activity along with, or without, rapamycin to a final concentration of 50 nM. Cells were then lysed at 4, 8 and 12 hours after removal of PMA and inhibition of transcription. The lysates were evaluated by immunoblotting analysis. At 4, 8 and 12 hours after introduction of rapamycin, no decrease in LAT protein levels was seen (Fig. 9), suggesting that protein stability was unaffected by the addition of rapamycin. This observation suggests that the inhibitory effect of rapamycin on activation-induced LAT expression does not involve protein breakdown.

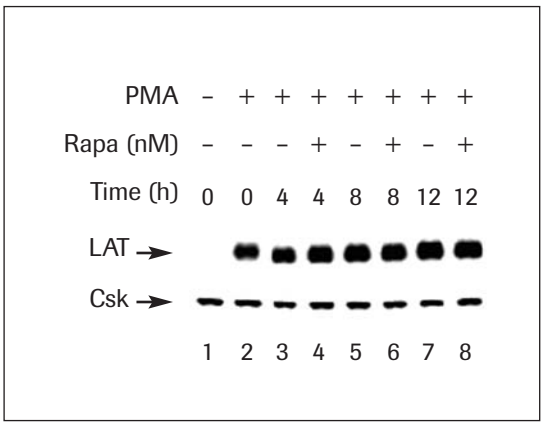

Figure 9. Rapamycin does not induce post-translational degradation of LAT protein. *2 x 10^5 Jurkat T cells were incubated in the absence (lane 1) or presence (lanes 2-8) of 1 ng/ml PMA at 37°C for 16 hours. Cells were then washed twice and actinomycin-D was added to a final concentration of 3 μg/ml to all samples. Cells were then incubated at 37°C for the indicated time in the absence (lanes 1-3 and 5 and 7) or presence (lanes 4 and 6 and 8) of 50 nM rapamycin (Rapa). Western blot analysis was then performed as described above. This experiment was performed three times with similar results.*

Current studies

We are now in the process of sending LAT to be analyzed by spectrometry to determine what residues become phosphorylated in response to TCR stimulation in the presence of FK506 and CsA. We have submitted a grant to the National Science Foundation (USA) to obtain funding to continue the project.

Publications

1. Cho CS, Elkahwaji J, Manthei ER, Chang Z, Scheunemann TL, Hamawy MM. Modulation of the electrophoretic mobility of the linker for activation of T cells (LAT) by the calcineurin inhibitors CsA and FK506: LAT is a potential substrate for PKC and calcineurin signaling pathways. *Cell Signal* 2003; 15:85-93.
2. Cho CS, Manthei ER, Elkahwaji J, Scheunemann TL, Knechtle SJ, Hamawy MM. Rapamycin antagonizes cyclosporin A- and FK506-mediated augmentation of linker for activation of T cells (LAT) expression in T cells. *Int Immunol* 2003; 15:1369-78.

Presentations

1. Manthei ER, Alam T, Knechtle SJ, Hamawy MM. The linker for activation of T cells (LAT) is involved in calcineurin signaling pathways: LAT is a substrate for calcineurin. *Exp Biol* 2001. (Abstract)
2. Cho CS, Manthei ER, Elkahwaji J, Knechtle SJ, Hamawy MM. Downregulation of LAT expression in T lymphocytes by the mTOR inhibitor rapamycin. *Exp Biol* 2001. (Abstract)
3. Cho SC, Menthei E, Knechtle S, Hamawy MM. Calcineurin inhibitors and rapamycin have opposing effects on activation-induced T cell expression of LAT, a molecule that is critical for T cell activation and development. Oral presentation, *AST Meeting* 2001. (Abstract)
4. Hamawy MM, Manthei E, Elkahwaji J, Alam T, Knechtle SJ. The linker for activation of T cells (LAT) is a target for modulation by cyclosporin A and FK506: LAT is a potential substrate for calcineurin. Oral presentation, *AST Meeting* 2001. (Abstract)
5. Cho SC, Menthe E, Knechtle S, Hamawy MM. Rapamycin and calcineurin inhibitors exert opposing influences on activation-induced expression of LAT. Oral presentation, *Society of University Surgeons Meeting* 2001. (Abstract)
6. Cho SC, Menthei E, Knechtle S, Hamawy MM. Upregulated expression of the signaling protein LAT: a potential mediator of activation-induced cell death in T lymphocytes. Oral Presentation, American College of Surgeons Forum 2001: Extended abstract in *Owen H. Wangensteen Surgical Forum.* Vol LII:2001. (Abstract)
7. Cho SC, Scheunemann TL, Hamawy MM, Knechtle SJ. Rapamycin and tacrolimus respectively potentiate and inhibit T lymphocyte activation-induced cell death in a Fas ligand-independent manner. Oral presentation, *Novartis Research Fellow Program Meeting* 2001. (Abstract)

Prof. Ian V. Hutchinson, Principal Investigator
Dr. Vera Pravica, Co-Investigator

University of Manchester, Manchester, UK

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

This study was carried out by Mrs Mojgan Mohammadi.

Background

Vascular endothelial growth factor (VEGF) is a potent pro-inflammatory cytokine that is involved in acute transplant rejection. It is also involved in wound repair and contributes to the process of fibrosis and arteriosclerosis known as chronic transplant rejection. We have shown that the VEGF gene is polymorphic and that two of these variants or alleles, -2578*G and -1154*C, are (a) associated with greater VEGF production, and (b) associated with both acute and chronic rejection of kidney, heart and lung transplants.

The biology of VEGF is interesting. It is released as splice isoforms of different size, including 121, 145, 165 and 206 amino acids. These have different biological activities, the smaller isoforms being angiogenic and pro-inflammatory while the larger ones have fibrogenic activity. Furthermore, the production of VEGF is affected by the presence of pro- and anti-inflammatory cytokines, for example being increased by tumour necrosis factor alpha (TNF-α) and decreased by interleukin-4 (IL-4).

Research objectives

The question then arose as to whether the association between genotype and graft rejection is due simply to the amount of VEGF produced or whether genetic variation changes the isoform profile and modulation by TNF-α and IL-4. The expression of VEGF isoforms in transplant biopsies has never been examined, and the possibility that this may be influenced by the TNF-α and IL-4 genotype of the recipient was unexplored. In addition, nothing was known of the influence of immunosuppressive agents on VEGF production and whether this was related to VEGF genotype.

Results

In this study we have confirmed the relationship between VEGF production and genotype (Fig. 1).

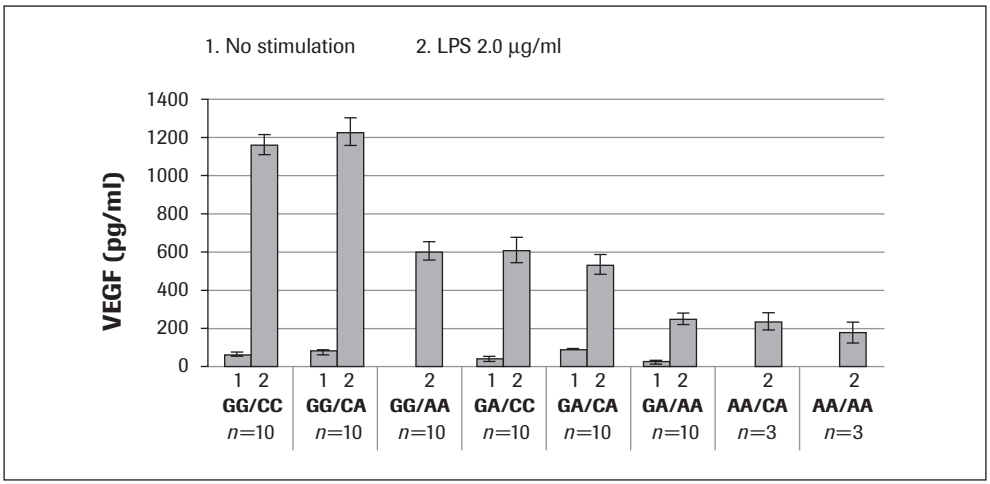

Figure 1. In vitro production of VEGF in healthy Caucasian individuals is associated with VEGF -2578/-1154 haplotype. *Peripheral blood leukocytes were incubated with or without lipopolysaccharide (LPS) for 96 hours. The culture supernate was then harvested and the VEGF level measured using a commercial VEGF-specific ELISA.*

TNF-α slightly increased the production of VEGF by cells from individuals regardless of VEGF genotype (Fig. 2).

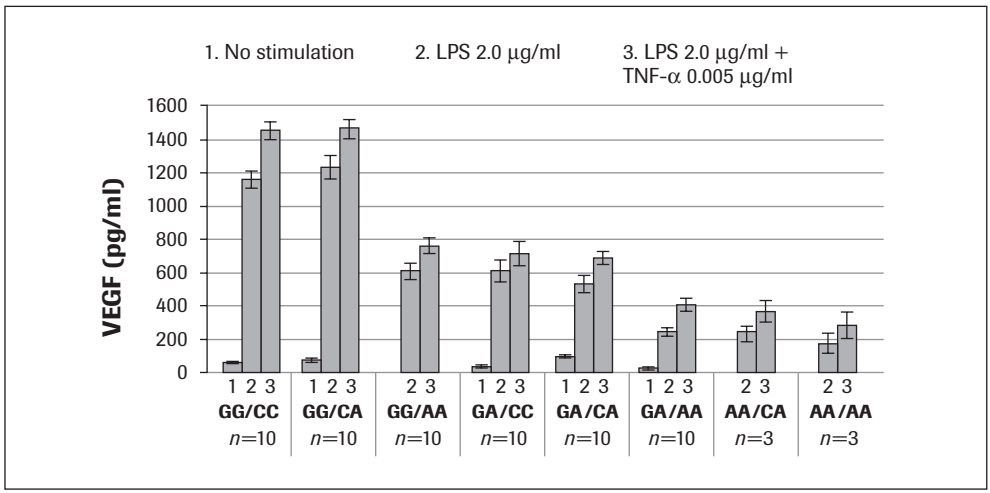

Figure 2. Influence of TNF-α on VEGF production by PBMCs from healthy individuals according to VEGF haplotype. *Peripheral blood mononuclear cells (PBMCs) from healthy Caucasian donors were stimulated with LPS in the presence or absence of recombinant TNF-α. VEGF production was measured using a commercial VEGF-specific ELISA.*

Similarly, IL-4 dramatically decreased VEGF production by PBMCs from individuals of all VEGF genotypes (Fig. 3).

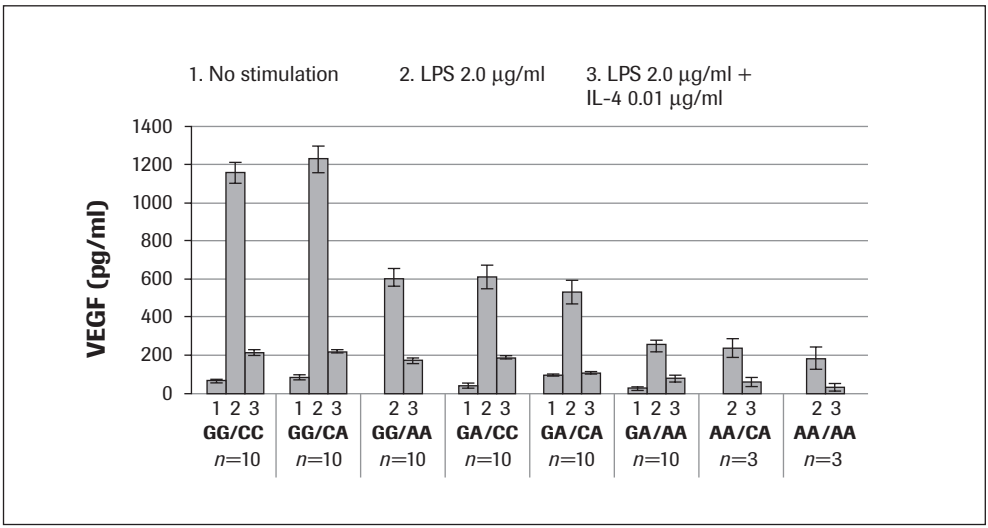

Figure 3. Influence of IL-4 on VEGF production by PBMCs from healthy individuals according to VEGF haplotype. *PBMCs from healthy Caucasian donors were stimulated with LPS in the presence or absence of recombinant IL-4. VEGF production was measured using a commercial VEGF-specific ELISA.*

Of the immunosuppressive agents tested, cyclosporine (Fig. 4) and steroids (Fig. 5) were very effective in reducing VEGF production *in vitro* and an influence of genotype was not evident. Mycophenolic acid and rapamycin also suppressed VEGF production, but to a lesser extent and, again, regardless of VEGF genotype (Figs 4, 5, respectively).

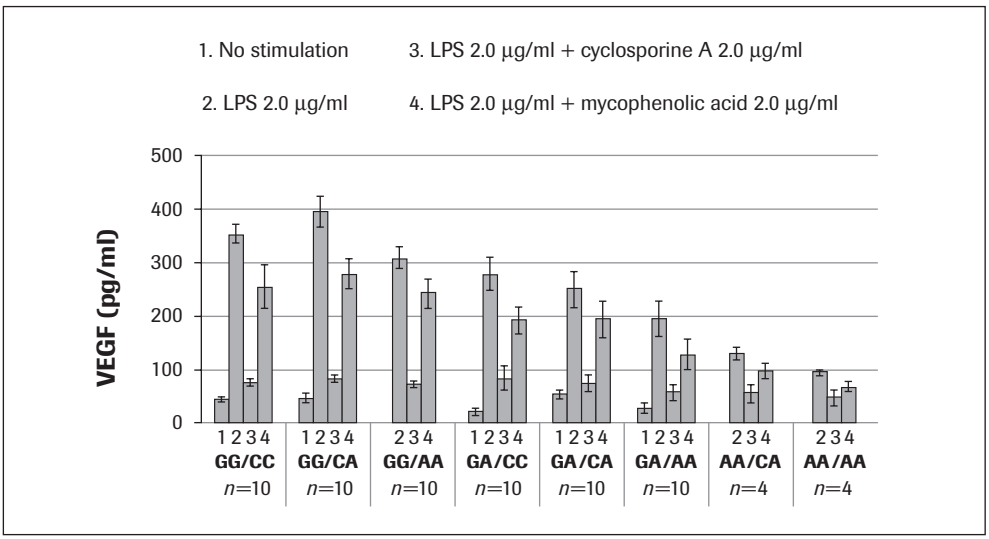

Figure 4. Suppressive effect of cyclosporine A and mycophenolic acid on VEGF production by PBMCs from healthy controls with different VEGF haplotypes. *PBMCs from healthy Caucasian donors were stimulated with LPS in the presence or absence of cyclosporine A or mycophenolic acid. VEGF production was measured using a commercial VEGF-specific ELISA.*

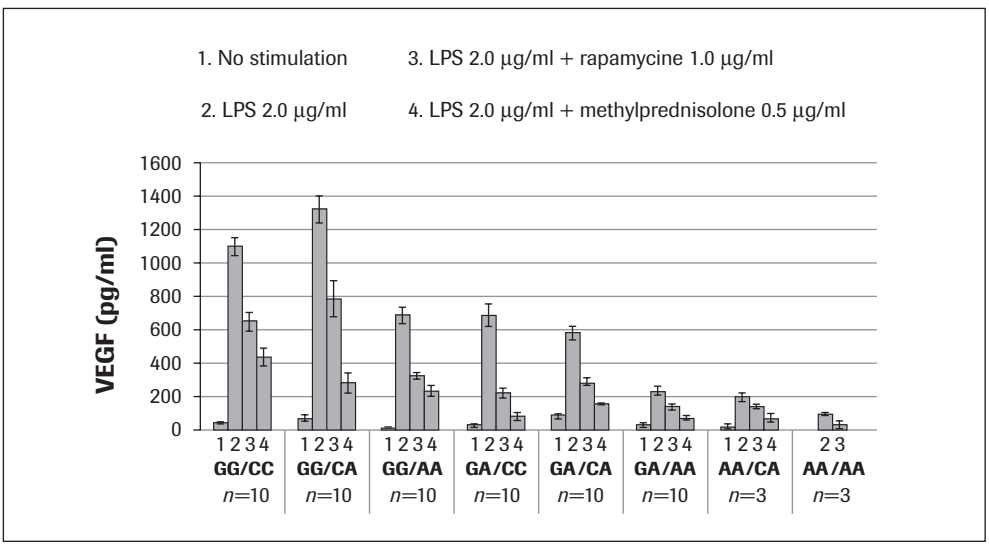

Figure 5. Suppressive effects of rapamycin and methylprednisolone on VEGF production by PBMCs of healthy individuals with different VEGF haplotype. *PBMCs from healthy Caucasian donors were stimulated with LPS in the presence or absence of cyclosporine A or methylprednisolone. VEGF production was measured using a commercial VEGF-specific ELISA.*

The mRNA encoding the VEGF isoforms are different in heart transplant biopsies and PBMCs, detected using RT-PCR and TaqMan (Fig. 6).

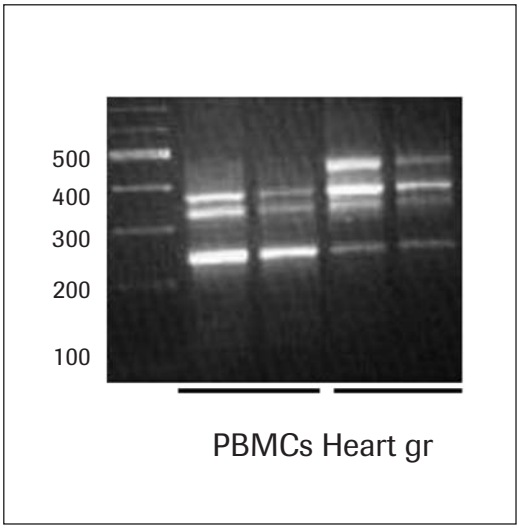

Figure 6. VEGF isoforms expressed in PBMCs from healthy volunteers and in endomyocardial biopsies from two heart transplant recipients who have had a functioning graft for more than 5 years. *mRNA was isolated and reverse transcribed into cDNA using Touch Down RT-PCR. An analysis of the isoforms expressed demonstrated that the bands correspond to protein isoforms with 121, 165, 189 and 206 amino acids. The 206 isoform is expressed only in heart biopsies. A more sensitive and quantitative TaqMan© Real Time RT-PCR method was established subsequently to examine VEGF isoform expression in heart biopsies. This analysis revealed no differences between patients in VEGF isoform expression profile, regardless of VEGF genotype, other cytokine genotypes or immunosuppression (data not shown).*

An extensive analysis of heart transplant biopsies has shown no differences in VEGF isoform profile in grafts regardless of genotype, immunosuppression and polymorphisms in other cytokines (data not shown).

Conclusion

Thus, we can conclude that the association between graft outcome and VEGF genotype is purely a matter of the amount of VEGF produced and is not related to any change in the biology of VEGF splice variation or to the influence of pro- and anti-inflammatory cytokines or immunosuppressive drugs.

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Cellular Mechanisms of Cardiac Allograft Vasculopathy (CAV) in Tolerant and Immunodeficient Mice

We have continued to pursue our experiments designed mainly to elucidate the probable involvement of innate immune responses in the production of coronary vascular disease in hearts transplanted between selected strains of inbred mice. Our results support the likelihood of participation of natural killer (NK) cells in this important pathological process. Our overall strategy has centered upon the use of recipient mice that are incapable of mounting a conventional adaptive immune response to the heart donors used but retain innate immune reactivity, including that of NK cells.

The experiments fall into several categories as follows:

1. Transplants from parental strain donors to F1 recipients

These experiments were selected because of previous reports that bone marrow transplanted in the same parental to F1 setting will not survive, a phenomenon termed “hybrid resistance”. This has been traced to the activity of NK cells that act in this circumstance in an isolated mode as ordinary adaptive immunity is not generated when parental cells are presented to a hybrid recipient containing the genes of that parent in combination with those of another strain (Table 1).

Donor	Recipient	No. of animals	Hearts with CAV
C57BL/6	(C57BL/6 x BALB/c)F1	22	19/22
C57BL/6	(C57BL/6 x B10.D2)F1	4	3/4
C57BL/6	(C57BL/6 x C3H/HeJ)	9	3/9
C57BL/6	C57BL/6	7	0/7
(C57BL/6 x BALB/c)F1	(C57BL/6 x BALB/c)F1	9	0/9
(C57BL/6 x B10.D2)F1	(C57BL/6 x B10.D2)F1	3	0/3

Table 1. Chronic cardiac allograft vasculopathy (CAV) in parental to F1 heart transplants.

The results summarized in Table 1 show that vascular disease occurs in several parental to F1 combinations but is entirely absent from hearts transplanted to syngeneic recipients.

2. Treatment of heart transplant recipients with monoclonal antibodies to inactivate various cell populations

Clearing NK cells from the spleens of recipients, as confirmed by accompanying *in vitro* tests of NK cell numbers and reactivity, with an antibody directed to them (NK1.1) does not, by itself, eliminate CAV. Suppression of T lymphocytes by itself with appropriate antibodies (antiCD4 and CD8) is also incapable of eliminating CAV. Treatment with both agents together, however, has a strong inhibitory effect. This suggests, of course, that T lymphocytes are involved in some way in the activation or effectiveness of NK cells (Table 2).

Donor/recipient combination	Treatment	No. of animals	Hearts with CAV
BL/6 to CB6F1	none	22	19/22
	anti-CD4/CD8	5	5/5
	anti-NK1.1	5	5/5
	anti-CD4/CD8 + anti-NK1.1	9	2/9

Table 2. Treatment with anti-CD4, CD8 and NK1.1 mAbs in parental to F1 combination.

These results are not altogether surprising and suggest further work on the cell interactions and mechanisms involved on which we are now embarking.

3. Possible involvement of virus infection in the activation of NK cells and in the production of CAV

We have encountered a particularly interesting finding using heart transplants to immunologically inert recipients ($RAG1^{-/-}$). When hearts were transplanted into 14 recipients housed in our standard animal facility, which maintains healthy-appearing mice according to institutionally approved standards, the transplants develop advanced CAV during our standard observation period of 56 days. When similar transplants are performed to mice housed in extremely clean "pathogen-free" conditions these lesions fail to appear (Table 3). A high proportion of mice maintained in the former facility show evidence of mouse hepatitis virus exposure by the presence of specific antibodies to this virus even though they remain ostensibly healthy throughout. This observation, which we expect to pursue further, suggests that virally infected cells may attract the action of NK cells more than non-infected cells. This would be consistent with the clinical observation that patients affected by viruses, especially cytomegalovirus, are more likely to develop CAV than non-infected patients.

Donor to recipient combination	Facility	No. of animals	Hearts with CAV
B6.$RAG1^{-/-}$ to B6CF1.$RAG1^{-/-}$	standard	14	12/14
B6.$RAG1^{-/-}$ to B6CF1.$RAG1^{-/-}$	pathogen free	9	0/9

Table 3. Development of CAV in $RAG1^{-/-}$ heart transplant recipients housed in "standard" vs. "pathogen free" facility.

4. The importance of incompatibilities determined by genes outside the major histocompatibility complex (MHC) on the production of CAV

The special opportunities offered by highly inbred mice to select strain combinations in which incompatibilities are confined to single genetic differences make possible the determination of effects by selected genes between strains bred for this purpose as "congenic" pairs. Working in collaboration with Dr. Derry Roopenian of the Jackson Laboratory in Bar Harbor, ME we are exploring some of these combinations. Initially we examined the most available example of non-MHC determined incompatibility, the male to female combination (C57BL/6 male to female). We found that this incompatibility is fully capable of generating CAV which is, of course, attributable to ordinary adaptive immunity, in contradistinction to the combinations studied above. In all examples of non-MHC incompatibility the immune response is produced by what is termed the "indirect pathway" of activation through antigen presentation by recipient antigen presenting cells. We have also begun to examine the effects of H60 antigen incompatibility. This incompatibility has been shown by Dr. Roopenian to be a "dominant"

minor antigen in the sense that it appears to produce immune responses exclusively even in the presence of other non-MHC incompatibilities. An interesting feature of this antigen is that it is represented only on lymphohematopoietic cells and not on endothelium or cardiac myocytes. Heart transplants in this combination are spared from developing CAV. A modified strain pair being produced by Dr. Roopenian in which endothelium expresses this antigen will soon be tested. Another single non-MHC determined antigen, H4, which is represented widely on various tissues, has also been tested, and this combination does result in the production of CAV (Table 4).

Donor	Recipient	No. of animals	Hearts with CAV
BL/6 male	BL/6 female	7	7/7
B6.H60 female	BL/6 female	5	1/5
B6.H4 female	BL/6 female	5	4/5

Table 4. Development of CAV in non-MHC incompatible heart transplants.

Publications

1. Uehara S, Chase CM, Rose H, Colvin RB, Russell PS, Madsen JC. Evidence that NK cells mediate a third pathway of allograft vasculopathy. *Manuscript in preparation*
2. Uehara S, Chase CM, Colvin RB, Russell PS, Madsen JC. Further evidence that NK cells may contribute to the development of cardiac allograft vasculopathy. *Transplant Proc, in press*

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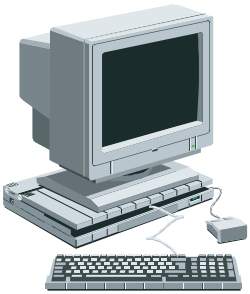

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