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

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

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

7. Board of Trustees (BT)

8. Scientific Advisory Committee (SAC)
We are proud to announce that the first five years of activity of the Roche Organ Transplantation Research Foundation (ROTRF) have been completed. At the beginning of the latest application cycle (Cycle XI) in October 2003, the second 5 years have commenced. In the first five years of its existence, covering 10 application cycles, the Foundation has awarded 105 grants to scientists around the world; the total amount of funding awarded for grants has been over 20 million Swiss Francs (CHF).

This funding has helped scientists working in the area of solid organ transplantation. The excellent work carried out by the researchers has been demonstrated by the high-quality papers published in peer-reviewed journals and the presentations of the scientists at international meetings.

The activities of the ROTRF were highlighted at the Congress of the European Society for Organ Transplantation (ESOT) in Venice in September 2003. During the congress the Foundation organised a symposium where five young investigators supported by ROTRF grants were given the opportunity to present the results achieved during their ROTRF-funded research. The talks presented were of outstanding scientific quality. We are looking forward to repeat this experience in September 2004 when, during the International Congress of the Transplantation Society in Vienna, the ROTRF will organise a series of State-of-the-Art Lectures.

All the ROTRF activities could never have taken place without the constant support of various people. We would like to thank F. Hoffmann-La Roche Ltd for its uninterrupted support to the Foundation. We would also like to thank all the scientists and clinicians involved: the members of the Board of Trustees; the Scientific Advisory Committee; and, most of all, the applicants.

On the behalf of the Board of Trustees

Philip Halloran
2. Facts and Figures

Funding Cycle X – Letter of Intent Submission in April 2003

The Roche Organ Transplantation Research Foundation (ROTRF) is pleased to announce that in the last cycle of the first five-year period, 2.1 million Swiss Francs (CHF) have been distributed to eleven applicants who were awarded a ROTRF grant. The Board of Trustees and the Scientific Advisory Committee (SAC) of the ROTRF were once again pleased with the high quality and originality demonstrated by the applicants.

During the last funding cycle (Cycle X) the ROTRF received 82 Letters of Intent from scientists around the world. Almost half of the applications (45.2%) came from European scientists, where the majority came from UK (17%), Germany (9.8%) and France (3.7%). 42.7% of the Letters of Intent were submitted by scientists in the USA, 7.3% by Canadian and the remainder by Australian and Egyptian scientists.

All the Letters of Intent were reviewed by the ROTRF SAC for relevance to organ transplantation, scientific excellence and originality. Based on the evaluations and comments of the SAC, the Board of Trustees invited 22 applicants to submit a Full Paper Application.

Based on the recommendations of the SAC, who reviewed the Full Paper Applications, the Board of Trustees awarded eleven full or partial grants of up to 300’000 CHF to five applicants from USA, two from Canada, two from Germany and one each from Belgium and Australia.

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 improvement of long-term graft survival, the prevention of chronic organ dysfunction, the induction of tolerance and the problem of rejection in xenotransplantation.
Statistics on Applications to the ROTRF

Figure 1. Geographical distribution of the applicants who submitted Letters of Intent (LOI) during the first ten 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 ten ROTRF funding cycles.
The Global View of Applications to the ROTRF

Distribution of the ROTRF applications worldwide

North America: 48% of applications

Europe (incl. former Soviet Union and Israel): 43% of applications

South America: <2% of applications

Africa: <1% of applications
Cycle I
Grantees

Cycle II
Grantees

Cycle III
Grantees

Cycle IV
Grantees

Cycle V
Grantees

Cycle VI
Grantees

Cycle VII
Grantees

Cycle VIII
Grantees

Cycle IX
Grantees

Cycle X
Grantees

Berlin, Germany
Bordeaux, France
Boston, USA
Cincinnati, USA
Edmonton, Canada

Boston, USA
Helsinki, Finland
London, Canada
Madison, USA
Montreal, Canada

Atlanta, USA
Birmingham, UK
Cagliari, Italy
Houston, USA
Houston, USA

Boston, USA
Boston, USA
Chicago, USA
Dundee, UK
Laval, Canada
Madison, USA
Montreal, Canada

Bergamo, Italy
Boston, USA
Chicago, USA
Edgbaston, UK
Hanover, Germany
Heidelberg, Germany
Madison, USA

Augusta, USA
Boston, USA
Boston, USA
Brussels, Belgium
Chapel Hill, USA

Boston, USA
Boston, USA
Boston, USA
Brussels, Belgium
Cagliari, Italy
Chicago, USA
Columbus, USA

Baltimore, USA
Bergamo, Italy
College Station, USA
Heidelberg, Australia
London, Canada
London, UK

Birmingham, USA
Boston, USA
Columbus, USA
Edmonton, Canada
Heidelberg, Germany

Boston, USA
Brussels, Belgium
Charlestown, USA
Heidelberg, Germany
Heidelberg, Germany
Herston, Australia

Madison, USA
Melbourne, Australia
New Haven, USA
Pittsburgh, USA
San Francisco, USA

Nantes, France
New York, USA
Oxford, UK
Pittsburgh, USA

Madison, USA
Nijmegen, The Netherlands
Portland, USA
Winnipeg, Canada

Nantes, France
Newcastle-upon-Tyne, UK
Oxford, UK
Philadelphia, USA
Rehovot, Israel
Warsaw, Poland

Minneapolis, USA
Munich, Germany
Nantes, France
Oklahoma City, USA
Toronto, Canada

Durham, USA
Madison, USA
Manchester, UK
Regensburg, Germany
Vienna, Austria

Milwaukee, USA
Melbourne, Australia
Nantes, France
New York, USA
Pittsburgh, USA

Madison, USA
Montreal, Canada

London, Canada
Newcastle-upon-Tyne, UK
New Haven, USA
Sydney, Australia

Iowa City, USA
Montreal, Canada
New Haven, USA
Sainte-Foy, Canada
Stanford, USA

Asia and The Middle East:
< 3% of applications

Australia:
< 5% of applications

at least one application ever received
no application received
Immunological Properties and Therapeutic Potential of Endothelial Precursor Cells in Transplantation

Alloimmune endothelial injury appears to be the initial lesion of transplant vasculopathy. Circulating endothelial progenitor cells (EPC) have recently been identified as capable of neovascularisation and vascular protection through regeneration. Recent studies have documented cellular chimerism in allograft vessels, suggesting a physiological repair mechanism involving such EPCs. We hypothesise that the prevention of developing vasculopathy depends on efficient repair of endothelial cell loss in graft vessels.

Rapid recovery from endothelial defects will inhibit the inflammatory cascade initiating neointima formation, and may facilitate graft adaptation through immune-modulating effects. The current project investigates the antigen-presenting and immuno-modulatory capacity of autologous EPC. It also tests if exogenous application of EPC can achieve cellular chimerism in the endothelium, and confer protection against transplant-related vascular injury. Different protocols for adhesion and flow cytometry sorting will be compared. Cells will be characterised by expression analysis of endothelial-cell-specific antigens, T cell co-stimulators and antigen uptake receptors, and by examination of angiogenic potential. The allogenic T-cell-stimulatory capacity of EPC to differentiate human umbilical vein endothelial cells (EC) will be compared in several read-out systems. Differences in T cell stimulation will be investigated for anergy or tolerogenic effects. Antigen presentation and activation of autologous T cells by EPC will subsequently be investigated in the same systems. Two hybrid vascular models (human EC in SCID mouse) will be used to study the replacement of resident endothelium by EPC: implantation of gels with 3-dimensional EC tubes and aortic interposition of human artery segments. In both models, endothelial chimerism will be assessed for native conditions and states of endothelial damage. In the EC gel model, damage and EC loss are induced by antibody-mediated complement action or alloimmune CTL attack. For the aortic interposition graft, ischaemia/reperfusion injury and CTL attack will be studied.

The project aims to define the therapeutic role of human endothelial precursor cells in the prevention of transplant-related vascular damage and to elucidate the T-cell-stimulatory and potential tolerogenic properties of EPC. Finally, potential therapy-enhancing effects of genetic modification of EPC will be studied. Beyond transplant immunology, our expected findings will contribute to the understanding of vascular pathogenesis and vascular protection (vasculitis, atherosclerosis).
New Approaches to Prevent Transplant Rejection

Current protocols for the transplantation of solid organs require long-term immunosuppression with drugs that have significant failure rates and side effects. Transplantation of haematopoietic stem cells, however, is associated with eventual development of permanent tolerance without this requirement for long-term immunosuppressive therapy. Graft rejection and Graft-versus-Host Disease (GVHD) are the consequence of immunological recognition of foreign donor or host antigens following solid organ and stem cell transplantation respectively. The induction of permanent antigen-specific tolerance remains the ultimate goal of both solid organ and cellular transplantation in order to prevent these reactions. Efforts to prevent and treat graft rejection and GVHD have predominantly focused on suppression or removal of effector T cells.

We have recently described two novel types of antigen-presenting cells (APC) of both donor and recipient type that are capable of inducing regulatory T cells and tolerance. This tolerance is an active process and is antigen-specific. In this proposal, we aim to optimise an approach utilising these regulatory APCs to induce a state of permanent donor-host chimerism and tolerance that will permit the transplantation of solid organ and cellular grafts without permanent immunosuppression.
NK Cells in Transplantation

Although there have been great advances in the field of organ transplantation, most transplant recipients are still dependent upon immunosuppressive drugs for long-term graft survival. The goal of having the immune system simply “ignore” the foreign graft has yet to be achieved. In recent studies, our group has determined that NK cells of host origin infiltrate liver allografts very early after transplantation. These NK cells are a source of both proinflammatory cytokines and death-inducing molecules.

Currently, little is known about the activation of NK cells, especially within the context of transplantation. We have cloned a novel NK cell activation receptor, rat NKp30 (rNKp30), from infiltrating lymphocytes of a rejecting rat liver allograft. The goal of this research is to examine the role of NK cells and the receptors that activate NK cells in solid organ transplantation. Knowledge of NK cell activation receptors and effector pathways will clarify the role of NK cells in the innate immune system and in solid organ transplantation. Successful completion of these studies may lead to novel strategies to induce tolerance to foreign grafts.
Long-Term Acceptance of Transplanted Organs

Indefinite acceptance of a transplanted organ without continuous intake of immunosuppressive medicines is a desirable but elusive goal for patients with end-stage kidney, liver, heart, or lung disease. Attempts to achieve this goal have traditionally focused on methods to alter the patient’s immune response so that it does not respond to and reject the foreign organ, a phenomenon referred to as immunologic tolerance. Safe and effective induction of transplantation tolerance, however, has proven to be very difficult in humans. Here, we suggest an alternative and possibly complementary approach to achieving long-term acceptance of transplanted organs by a mechanism known as immunologic ignorance. Immunologic ignorance occurs if communications between the transplanted organ and the patient’s immune system are interrupted so that the immune reaction that leads to rejection is not initiated.

In order to begin to understand how to achieve immunologic ignorance, we propose to first determine why foreign organs almost always initiate immune responses even after they have had a chance to recover from the trauma of the transplantation procedure. Specifically, we wish to explore whether specialised cell aggregates, known as tertiary lymphoid tissues, form within the transplanted organ and allow for direct communication between the cells of the organs and those of the patient’s immune system leading to either rapid (acute) or slow (chronic) rejection. This type of analysis would allow us to identify therapeutic strategies to block tertiary lymphoid tissue formation and help patients extend the survival of their transplanted organs.
Dr. Alain Le Moine, Principal Investigator

Université Libre de Bruxelles, Brussels, Belgium

Suppression of Allograft Rejection and Tolerance Induction by Self-Regulatory T Cells

Self-reacting regulatory CD4+CD25+ T cells play a critical role in the suppression of autoreactive T cells as well as in the control of innate immunity. They are physiological tools that prevent excesses of immune responses against pathogens and secure homeostasis of the immune system. Nevertheless, their antigen specificity still remains unknown. They can suppress other naïve T cells and make them tolerant to the relevant antigen. Allograft rejection depends on the balance between effector T cells and regulatory T cells. In unmanipulated allograft recipients, alloreactive effector T cell precursors exceed regulatory T cells, automatically tipping the balance toward allograft rejection. Transplanted tissues from allogeneic donors actually share far more identities than disparities with the host. So far, the roles played by these identities in the allograft outcome remain unknown and only little evidence suggests their active role in the allograft acceptance. Sharing identical MHC class II molecules between donor and recipient allows donor cells to present self-peptides that are identical in the host and the recipient. In this way, self-reacting regulatory T cells could mediate linked suppression of anti-donor-specific T cells.

We have recently observed an array of evidence showing that recipient stimulation with self-antigens modulates alloreactive response and suppresses allograft rejection in the absence of any immunosuppressive drug. Self-antigen stimulation was performed either through adjacent syngeneic transplants, the use of semi-allogeneic (donor x recipient) F1 allografts, or injections of lipopolysaccharides (LPS)-activated autologous dendritic cells (DC).

In the present project, we will examine whether or not self-reacting regulatory T cells are involved in these regulation processes, their critical mechanisms as well as their antigen-restriction. We will investigate the promoting or inhibiting effect of classical immunosuppressive drugs. As their rationale is based on homeostatic highly conserved mechanisms, they should be easily adapted to non-human and human primates. They might lead to feasible cell or molecular therapy through recipient DC manipulations and/or repeated heat-shock protein administrations.
Massachusetts General Hospital, Charlestown, USA

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

For patients with severe lung disease, lung transplantation offers the opportunity for a better quality of life and longer survival. However, despite improvements in surgical techniques and immunosuppressive drugs, overall median survival after lung transplantation is only 3.7 years. The major obstacle in lung transplantation is the development of severe scarring of the airways, so called bronchiolitis obliterans (BO). This scarring results from repeated injuries to the airways. Rejection, a severe inflammatory reaction mediated by the immune system against the transplanted organ, is the major source of this injury. It is dependent upon the movement of lymphocytes from the blood into the transplanted lung. The chemokines are proteins that control lymphocyte movement into organs. These proteins are expressed at high levels in transplanted lungs with rejection and BO.

Our hypothesis is that the chemokines contribute to the development of rejection and BO, and that inhibition of chemokine function increases the survival of lung transplants. In our grant, we propose to determine the expression profile of chemokines and their receptors in transplanted human lungs. In addition, we will use mouse models of lung transplantation to further delineate the precise role of a number of chemokines as well as the factors that control their expression.

We feel that our proposal will expand our understanding of the mechanisms underlying rejection and BO. The chemokines may control crucial steps in the development of these processes, and thus, may be ideal targets for therapeutic intervention. Our research is the first step towards the development of novel anti-chemokine therapies that could extend the survival of people with lung transplants.
The survival of transplanted kidneys is shorter than the expected survival in the organ donor. This is due to many stresses that occur during the transplantation process (e.g. duration of ischaemia, rejection episodes). The histological picture of such a damaged organ resembles the features of a normally-aged old kidney. This disease of the transplanted kidney is called allograft nephropathy (AN). Because of the similarities of AN to aging, we hypothesised that AN could be a state of accelerated aging of the kidney.

In our previous work, we found that p16, a protein regulating cell replication, is strongly associated with kidney aging and is also induced in transplanted, rejected kidneys. The aim of this project is to prove that p16 is a key player in the development of AN. To do this we will use mice deficient in the functional p16 gene (p16 KO) in a transplantation model. The outcome will be compared with wild-type mice (wt) that carry a functional p16 gene. We expect that p16 KO mice will show less severe histological changes and better graft function than wt mice. The material from these mice will be used to explore the p16 regulation in detail by using specific methods for detection of gene and protein expression in the whole kidney and in different cell types within the kidney. This part of the project will provide new insights in the so far poorly understood regulation of p16 expression in vivo. Finally, we will transfer the animal data to the human situation by investigating p16 in human renal biopsies from transplanted kidneys. We will examine whether p16 is a predictor of graft survival. Thus, p16 could emerge as an important marker to estimate the real potential of transplanted kidneys. This is particularly important as the actual organ shortage forces the use of older and diseased kidneys.
Our long-term aim is to develop a treatment to prevent the progressive muscle weakness in Duchenne muscular dystrophy (DMD) patients. Our research group has already demonstrated that the transplantation of muscle precursor cells, called myoblasts, can restore the expression of the protein, called dystrophin, whose absence is responsible for DMD. Following the transplantation of normal myoblasts, dystrophin was expressed in 90% of the muscle fibers of dystrophic mice, who received a drug (tacrolimus) to prevent the rejection of these cells. Similar results have also been obtained in monkeys. We have also recently obtained encouraging results in a new phase I clinical trial of myoblast transplantation in DMD patients also treated with tacrolimus to prevent rejection. However, tacrolimus may increase the risk of cancer.

My collaborator, Dr. Rothstein, has already shown that an antibody (anti-CD45RB) against a protein present on the surface of some cells from the immune system prevents the rejection of skin transplants. We have also recently obtained preliminary results indicating that this antibody also prevents the rejection of myoblasts in mice.

The first aim of this project is, therefore, to improve these preliminary results by performing myoblast transplantation after bone marrow transplantation. The protocol will use two different antibodies against proteins on the cells of the immune system (anti-CD45RB and anti-CD154).

The second aim is to determine the mechanism involved in preventing the rejection of the transplanted cells so that we can improve the treatment.

We hope that this research project will permit the development of a treatment for Duchenne muscular dystrophy, based on the transplantation of the cells involved in the formation of the muscle fibres, into the muscles of the patients. The present research project will enable us to prevent the rejection of these cells without using an anti-rejection drug.
Use of a Death Decoy Protein to Protect Organ Graft from Rejection

Organ transplantation is an effective way to treat various end-stage organ failures. Organ grafts will be rejected by recipients because their immune system will recognise the grafts as foreign entities. Even with administration of immunosuppressive drugs, many grafts are eventually lost due to chronic rejection, which is caused by long-term, low-level immune response and inflammation. During such processes, endothelial cells (EC) and vascular smooth muscle cells (VSMC) around blood vessels are killed, and their death in turn causes a cascade of events that leads to chronic rejection.

A molecule known as death decoy receptor 3 (DcR3/TR6) belongs to the tumour necrosis factor family. Many tumours secrete this molecule to gain survival advantage. DcR3/TR6 can interfere with immune responses and protect many types of cells from death. In this project, we will use the molecule for a good cause. We will investigate whether DcR3/TR6 can ameliorate acute as well as chronic graft rejection. Through genetic engineering, we will let recipient mice secrete high levels of DcR3/TR6 into their blood, or let donor organs produce a large amount of DcR3/TR6 locally. We will then assess whether such manipulation can inhibit activation of the host immune system, reduce death of EC and VSMC, and subsequently reduce acute as well as chronic rejection. The results, if as expected, will prove in principle that this strategy can be used to improve outcome of human organ transplantation.
Dr. Yong-Guang Yang, Principal Investigator  
Prof. Megan Sykes, Collaborator  
Dr. Noriko Tonomura, Research Associate  
Shumei Wang, Research Associate  

Massachusetts General Hospital, Boston, USA  

Approach to Preventing Xenograft Rejection

The severe shortage of organ donors currently limits the number of transplants performed. As an example, in the United States, 82,149 patients (updated on July 4, 2003) are currently waiting for a life-saving organ and approximately 17 patients on the waiting list die each day (reports from United Network for Organ Sharing). The ultimate goal of our proposal is to solve the problem of this supply-demand disparity by xenotransplantation (xenografts) from pigs, the most suitable donor species for humans. As xenografts are subject to vigorous rejection, the amount of immunosuppressive drugs for overcoming xenograft rejection is likely to destroy the host immune system that the body needs to fight viruses/bacteria and eradicate cancer cells, thus rendering the recipient at a high risk for infections and cancers. It would then be highly desirable to control only the immune responses that are responsible for the rejection while preserving host immunocompetence.

The focus of this project is to understand the mechanisms of xenograft rejection by macrophages. Macrophages, a major component of the immune system, are activated first when the body is exposed to foreign antigens. Normally, macrophages are restrained from activation by the patient’s own tissues because self-tissues express a number of molecules that deliver inhibitory signalling by engaging specific receptors on macrophages. In this proposal, we intend to identify the counterpart molecules of pigs, which are essential to maintain the inactive status of macrophages, but fail to function in humans with respect to inhibiting human macrophage activation. The ultimate aim is to genetically modify pigs to overcome macrophage-mediated rejection. Information obtained from these studies may guide the development of effective strategies for preventing porcine xenograft rejection in patients, so that xenotransplantation from pigs can be used clinically to save the lives of patients who are waiting for a life-saving organ.
Application of Embryonic Stem Cells to Avoid Rejection of Transplanted Organs

Organ transplantation has been ongoing now for some 50 years. However, despite improvement in immunosuppression, more than 50% of the organs are lost within 10 years. These poor results re-enforce the need for establishing tolerance models that will in the future prevent or drastically reduce organ loss.

Here, we propose to study mouse embryonic stem cells and their efficacy to prevent organ rejection. Our hypothesis is that embryonic stem cells are tolerated by recipient animals due to their low immunogenicity. Once stable mixed chimerism has been achieved, we anticipate that transplantation of organs will subsequently be successful. We will use skin grafting as a model and study the mechanisms by which embryonic stem cells induce a state of mixed chimerism. For example, we plan to examine whether FasL, naturally expressed by embryonic stem cells, is a requirement for the engraftment of embryonic stem cells. The lpr/lpr mouse that expresses a Fas mutant molecule will be used for these studies. Achievement of tolerance by the use of embryonic stem cells will facilitate their practical application in clinical medicine and biomedicine overall. The data obtained here will not only be useful and critical in organ transplantation, but also in the use of embryonic stem cells in the treatment of autoimmune and degenerative diseases.
4. Progress Reports of ROTRF Grantees

Dr. Dirk P. Dittmer, Principal Investigator

The University of Oklahoma Health Sciences Center, Oklahoma City, USA

SCID-Mouse/Human Transplant Model for Gamma Herpesvirus Infection

The aim of this project is to investigate the effect of immunosuppressive therapy on Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) and Epstein-Barr-virus (EBV) pathogenesis in transplant patients. KSHV and EBV are both associated with significant mortality and morbidity in transplant patients. We initially showed that Interferon (IFN)-gamma could reactivate KSHV from latency in cultured primary effusion lymphoma cells (PEL)\(^1\). Yet, the contribution of IFN-gamma and other paracrine factors cannot be adequately studied in cell culture. Therefore, we proposed to establish an animal model for KSHV-associated and EBV-associated lymphoma and to determine the impact of anti-virals, cytokines and immunosuppressive drugs on tumorigenesis in this model. We have now established a tumorigenesis model for KSHV-associated lymphoma.

Having established a colony of immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice at OUHSC in year one, we then established a tumour transplantation model. Our colony is located outside the common animal facility and this physical separation allowed us to pursue the experiments as planned.

We injected KSHV-positive lymphoma cells (BCBL-1) directly into SCID and SCID/NOD mice. The BCBL-1 cell line was obtained from a pleural effusion and was previously believed to grow

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only in the peritoneal cavity of mice. However, we were able to show that, given proper extracellular support (matrigel™, Beckman Inc.), we could grow subcutaneous tumours within two weeks. Without matrigel, we did not obtain any tumours. This demonstrates the strict requirement of extracellular or paracrine factors for the growth of KSHV-associated lymphoma. The lesions are highly angiogenic and all cells express at least the KSHV latent antigen LANA, as evidenced by staining with a monoclonal antibody directed against this protein (Fig. 1).

To measure viral transcription in the lesions, we developed a real-time quantitative RT-PCR to measure KSHV viral load and virus-specific transcripts. We are now in a position to quantify mRNA for every 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 use solution PCR. This design offers two advantages: due to the power of PCR, we can use minimal input samples, and we can look specifically for the various spliced mRNAs. Using a subset of these probes, we determined that, in the tumour, all cells transcribe the viral latent mRNAs and that in intraperitoneal grown tumours, in particular KSHV, viral lytic mRNAs were transcribed. This finding establishes (a) a new set of targets for potential anti-viral therapy, and (b) the absolute dependence on host factors for PEL tumours.

For the final year, we will continue our work as proposed. Now that we have characterised an in vivo model, we will investigate the response to antiviral drugs (AZT, ganciclovir) and IFN-gamma. By implanting KSHV-positive tumour cells directly, we circumvent the problems that are associated with the notoriously weak infectivity of this human tumour virus.

Publications

Dr. Christiane Ferran, Principal Investigator

Beth Israel Deaconess Medical Center, Boston, USA

Protective Effect of A20 against Transplant-Associated Vasculopathy

We hypothesised 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 acts anti-inflammatory and anti-apoptotic in endothelial cells (EC) and anti-proliferative, pro-apoptotic in smooth muscle cells (SMC). This proposal is aimed at studying the function of A20 in SMC and establishing 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 nuclear factor-κB (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.

Progress report:

We have now addressed Specific Aim 1 completely.
1. We have shown that A20 is part of the physiological response of SMC to injury and inhibits inflammation and proliferation via blockade of NF-κB activation. A20 was induced, with mRNA levels peaking at 1–2 hours, following stimulation of primary human aortic SMC (Clonetics) with tumour-necrosis factor (TNF) (100 U/ml; Fig.1). Recombinant-adenovirus- (rAd)-mediated gene transfer, using a multiplicity of infection (MOI) of 500, led to high expression of A20 in >95% of cultured SMC. Overexpression of A20-inhibited recombinant human TNF-mediated IkBα degradation (Western blot) and prevented NF-κB nuclear translocation (electrophoretic mobility shift assay, EMSA), correlating with inhibition of intracellular adhesion molecule 1 (ICAM-1) and monocyte-chemoattractant protein 1 (MCP-1) upregulation (Northern blot; Fig. 2A-D). Non-infected and rAd.β-galactosidase-(rAd.β-gal)-infected SMC were used as controls. Having established that A20 inhibits NF-κB activation, we questioned whether such inhibition would affect the rate of SMC proliferation. Human SMC were cultured in 0.5% fetal-calf serum (FCS) medium for 48 hours to synchronise them in the G0 phase of the cell

Figure 1.
cycle, then infected with rAd.A20, rAd.IκBα or the control rAd.β-gal. Recombinant IκBα adenovirus was used as an alternative approach to achieve NF-κB inhibition. Twenty-four hours after infection, 10% FCS was added to trigger cell-cycle progression and cells were collected 24 hours and 48 hours later for cell-cycle analysis. DNA content was analysed by FACSscan using Cellquest acquisition software. Analysis of DNA ploidy allows discrimination of cells in G0/G1, S and G2/M phases of the cell cycle. Non-infected or rAd.β-gal-infected SMC proliferate in response to serum addition and progress to the S+G2/M phases of the cell cycle (47%) (Fig. 3). In contrast, the amount of A20-expressing SMC in the S+G2/M phase is not modified following serum addition (16%; Fig. 3).
SMC expressing the specific inhibitor of NF-κB, IκBα, showed a similar pattern of cell-cycle inhibition in response to FCS (20%; Fig. 3). Since cell-cycle progression is regulated by hyperphosphorylation of the retinoblastoma (Rb) protein, we examined the effects of A20 expression in SMC upon Rb hyperphosphorylation. Rb hyperphosphorylation was determined by Western blot analysis using a monoclonal antibody from PharMingen. Our results indicate that overexpression of A20 in SMC abolishes Rb hyperphosphorylation in response to serum addition (Fig. 4). This effect of A20 is associated with the prevention of serum-mediated down-regulation of the cyclin-dependent kinase inhibitors p21^{waf1} and p27^{kip1} and an increase in p53, as shown by Western blot analysis using specific antibodies from PharMingen (Fig. 4). These results are similar to that seen in IκBα-expressing SMC, whereas a normal pattern of Rb phosphorylation with a decrease in the expression of p21^{waf1}, p27^{kip1} and p53 are detected in non-infected SMC and SMC infected with the control rAd.β-gal (Fig. 4).

These results demonstrate that A20 fulfills three requirements for proposing it as a gene therapy candidate to protect against 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 pro-inflammatory and pro-atherogenic proteins such as ICAM-1 and MCP-1.

(iii) A20 has a novel anti-proliferative function in SMC.

Figure 4.
2. We have also been able to show that expression of A20 in endothelial cells inhibits their activation and protects them from apoptosis triggered by a highly relevant stimulus to graft rejection and TAV, i.e., the cross-linking of CD40/CD40L.

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

1. We have shown that A20 protects endothelial cells from TNF-mediated apoptosis by inhibiting the activation of caspase-8 and maintaining mitochondrial integrity. We have also shown that A20 protects EC from Fas-associated death domain (FADD) and significantly blunts natural-killer-cell-mediated apoptosis by inhibiting caspase-8 activation. In addition to protecting EC from apoptotic stimuli, A20 safeguards EC from complement-mediated necrosis. These data demonstrate, for the first time, that the cytoprotective effect of A20 in EC is not limited to TNF-triggered apoptosis. Rather, A20 grants broad EC-protective functions by effectively shutting-down cell-death pathways initiated by immune offenders associated with graft rejection. Thus, A20 is an ideal gene therapy candidate to protect the graft from rejection.

| A | TNF (400 U/ml) | - | + |
|   | IFN-γ (400 U/ml) | - | + |
|   | IL-1β (100 U/ml) | - | + |

**Figure 5A.**
2. We have also confirmed that A20-SMC display increased sensitivity to cytokine and Fas-mediated apoptosis (Fig. 5A,B). This novel function is independent from inhibition of NF-κB activation. Indeed, the pro-apoptotic effect of A20 in SMC was twice that achieved in SMC expressing the specific inhibitor of NF-κB: IκBa. In addition, coexpression of the anti-apoptotic Bcl family member A1 rescued IκBα but not A20 expressing SMC from apoptosis (Fig. 6). We are currently analysing the impact of alteration of mitochondrial membrane potential, cytochrome c release and cleavage of death-substrates upon activation of caspases.

Specific Aim 3. The work for Specific Aim 3 has been started.
We have set up the model of a rat aortic allograft with the help of two surgical residents who are working in the laboratory of Dr. Virendra I. Patel and Gautam Shrikhande. In light 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 Aim 3 by using, as recipients, rats that were reconstituted with green-fluorescent protein (GFP)-tagged bone marrow. This 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 the question of whether A20-expressing grafts are protected from immune-mediated injury and, hence, are not replaced by donor EC/SMC or, in case replacement occurs, whether the anti-inflammatory
environment provided by A20 will keep these cells in a quiescent state not permitting the development of SMC. We have successfully established the model and all the conditions for adenoviral-mediated gene transfer. Our preliminary data, using the control rAd.β-Gal, showed that 5.108 plaque-forming units resulted in concentric expression of rAd to a depth of 3-4 cells within the vessel wall (Fig. 7).

<table>
<thead>
<tr>
<th></th>
<th>TNF + IL-1β + IFN-γ</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAd. A1</td>
<td>+</td>
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Publications
**Induction of Transplantation Tolerance by Endogenous CD8α⁺ Dendritic Cells**

**Introduction**: Dendritic cells (DC) are rare white blood cells that exist throughout the body and serve as immune sentinels. After encountering a “danger” signal in the form of microbes, inflammatory molecules or allergens, DC ingest fragments of foreign material and transform into potent stimulatory cells. Albeit rare, DC have been ascribed with a large number of highly specialized functions that provide a critical, regulatory link between innate and adaptive immunity. Through intimate cell-to-cell contact DC trigger other cells of the immune system to make vigorous responses, in particular, the activation of killer T cells and the production of neutralizing antibodies by B cells. Importantly, DC also appear to be equally responsible for the seemingly opposite process known as immune tolerance.

Until recently, these functions were thought to stratify over discrete maturation stages of “classical” DC. Immature DC are well positioned and equipped for antigen capture and uptake, while mature DC with their extremely high surface expression of costimulatory molecules are specialized for T cell activation. Similar to other leukocyte families, however, recent studies have identified phenotypically distinct subpopulations of DC in many species including rodents, primates and humans. It is increasingly apparent that these DC subpopulations also exhibit a specialized function that is conserved across species. For example, DC that drive the development of type-1 T cells (IFNγ-producing) necessary for resistance to microbes and anti-tumour immunity, are recognized as a distinct subpopulation from those DC that elicit the differentiation of type-2 T cells (IL-4-producing) needed to fight extracellular parasites. Following exposure to viruses, plasmacytoid DC (circulating DC with an atypical morphology) produce large amounts of the type-1 interferons, IFNα and IFNβ that stimulate anti-viral immune responses.

**Aims**: Recently, we have shown that mouse DC expressing the CD8α homodimer impair host, anti-donor immune responses and promote extended survival of transplanted tissues in the absence of conventional immunosuppression. The goal of the current study is to determine the mechanism(s) via which discrete DC subpopulations differentially initiate and terminate immune reactivity. We hypothesized that CD8α⁺ donor DC specifically impair host immune reactivity to alloantigen and thereby, prolonging allograft survival, either through the activity of a regulatory T cell or the direct deletion of alloreactive T cells.
Results: The aim of our initial work was to determine the cellular targets of CD8α⁺ DC allo-regulatory activity. In order to study the activity of CD8α⁺ DC in an allogeneic model, we imported two T cell receptor (TCR) transgenic (tg) mouse strains, 2C and D10, and established breeding colonies in an exclusion barrier mouse facility. The 2C and D10 mice express TCRs that are specific for the MHC class I protein Ld and the MHC class II protein IAd, respectively. Next, we produced and affinity purified the clonotypic monoclonal antibodies (mAb) 1B2 and 3D3 from hybridomas. 1B2 and 3D3 specifically label the 2C and D10 tg TCRs. The purified clonotypic mAbs were then biotinylated or directly conjugated to the fluorophore, FITC. We have used these TCR tg mice and an adoptive transfer system to examine the capacity of distinct DC subsets to stimulate the activation and proliferation of alloantigen-specific T cells in vivo. Both CD8α⁺ DC and CD8α⁻ DC were purified by flow sorting from BALB/c mice mobilized with Flt3 ligand. DC were then injected s.c. into the footpads of C57Bl/6J recipients who had received 2-5 × 10⁶ highly purified CD8⁺ 2C T cells i.v., 2 days previously. The activation of CD8⁺ 2C T cells from the draining popliteal and inguinal lymph nodes was assessed by labelling with mAbs for CD25 and CD28. Proliferation was measured by stepwise loss of the fluorescent tracer CFSE. Data were collected by multi-colour (4) flow cytometry using a BD FACs Calibur and analysed using Cell Quest (BD Biosciences), Flow Jo (Tree Star) or Modfit (Verity Software House) software. Using this system, we found that both CD8α⁺ DC and CD8α⁻ DC induce strong proliferation of CD8⁺ 2C tg T cells. Comparison of CFSE profiles demonstrated that proliferating CD8⁺ 2C tg T cells undergo a similar number of cell-division cycles, in response to either DC subset. These observations are supported by the finding that the expression profiles of the T cell activation molecules, CD25 and CD28, by proliferating CD8⁺ 2C tg T cells were remarkably similar in recipients of either CD8α⁺ DC or CD8α⁻ DC. Combined, these data indicate that both DC populations are capable of stimulating the equivalent activation and proliferation of alloantigen specific T cells. However, on examining the total number of proliferating T cells, 2-5-fold fewer CD8⁺ 2C tg T cells were detected in recipients of CD8α⁺ DC versus mice that were administered with CD8α⁻ DC. These data support our hypothesis that donor CD8α⁺ DC impair host immune reactivity to alloantigen by the deletion of alloreactive T cells.

Aims for the second year: During the second year, we will extend our findings to examine the molecular mechanism (Aim 2) through which CD8α⁺ DC mediate their immune regulatory activity. First, we will directly confirm our observation that CD8α⁺ DC induce the deletion of alloantigen-specific CD8⁺ T cells. Next, we will consider the mechanism via which CD8α⁺ DC mediate their activity. There are several likely mechanisms which we will consider including the induction of apoptosis by tumour necrosis factor (TNF) family members. Interestingly, recent studies have strongly implicated the tryptophan-degrading enzyme indoleamine 2,3 dioxxygenase (IDO) in the immune regulatory activity of DC and, in particular, the activity of mouse CD8α⁺ DC. In addition, we will broaden our study to consider the outcome of alloantigen-specific CD4⁺ D10 T cells in response to stimulation with CD8α⁺ DC or CD8α⁻ DC.
of donor origin. Last, it is conceivable that by optimizing the administration of CD8α+ DC (Aim 3), we may achieve a more comprehensive deletion of alloantigen-specific T cells, and thereby improving their tolerogenicity.

Publication
Mechanisms of T Cell Modulation of Renal Ischemia Reperfusion Injury

Ischemia reperfusion injury (IRI) to the allograft is a major limiting factor for early transplant success, and by unclear mechanisms, predisposes to an increased incidence of acute rejection and long-term allograft. We had found that T cells are direct mediators of kidney IRI, which provides the theoretical basis for T cell manipulation to treat IRI. However, the role for the T cell in IRI is not explained by classic models of T cell function or adaptive immunity. The aims of the present study were to begin to explore the mechanisms by which T cells mediates IRI. We began to explore what specific T cell would be most important in the pathogenesis of renal IRI. We first hypothesized that the Th1 CD4 cell played a pathophysiologic role. We used signal transducer and activator of transcription (STAT)4-deficient mice which are deficient in Th1 CD4 T cell function. We found by intracellular T cell staining that these mice had deficient interferon-gamma (IFN-γ) production, and had moderate protection from renal IRI. However, for the sake of completeness, we also studied the

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

**Figure 2.** STAT6−/− mice had significantly worse renal function compared with wild-type Balb/c (and STAT4−/−) at 24, 48 and 72 hours post-ischemia. STAT4−/− mice were modestly protected compared with Balb/c, and only at 24 hours post-ischemia. *p < 0.05 versus Balb/c wild type mice (n = 12/each strain). (circle: Balb/c wild-type, triangle: STAT4−/−, square: STAT6−/−)
STAT6-deficient mice. To our surprise, these mice, deficient in interleukin-4 (IL-4) production and Th2 T cells, had worse renal IRI (Fig. 1, 2). Thus, CD4 T cells potentially played an injurious or protective role in IRI depending on their precise phenotype. These results were independently and simultaneously found in liver IRI by the UCLA group, making this conclusion more credible. We then further explored the mechanisms of the CD4 T cell modulation of IRI, and studied mice deficient in IL-4 or IFN-γ. As expected and similar to the STAT4 and STAT6 data, the IL-4-deficient mice had a marked worsening of kidney injury after IRI, though the IFN-γ-deficient mouse had little protection. We evaluated neutrophil trafficking as the potential effector mechanism for the T cell modulation of IRI, but neutrophil infiltration into the injured kidney did not correlate with either T cell modulation, function or structural injury after IRI. Based on this data, we speculate that modification of T cells or their products early during IRI can decrease the course of delayed graft function, and possibly reduce the enhanced allogenicity associated with IRI.

The non-heart-beating deceased donor represents a major opportunity to expand the organ donor pool. However, warm IRI severely limits the number of organs that can be used. We then hypothesized that the same concepts for isolated renal IRI could be extrapolated to whole-body IRI. We developed a novel model of kidney injury after whole body IRI following cardiac arrest in mice (Fig. 3), and evaluated the role for T cells in improving organ quality. We

![Figure 3. Mean arterial blood pressure pre-, during and post-cardiac arrest.](image)

![Figure 4. T-cell-deficient mice are functionally protected from renal injury following cardiac arrest. Creatinine was significantly reduced at 24 hours (*p < 0.05) after cardiac arrest in T-cell-deficient mice (white bars) compared with creatinine in wild-type mice (black bars).](image)

found that T cell deficiency conferred a marked protection from both functional and structural consequences of ischemic injury to the kidney after the non-heart-beating state (Fig. 4). This was associated with a partial reduction in pro-inflammatory cytokine production, and particularly less expression of intracellular adhesion molecule 1 (ICAM-1). Unexpectedly,
T cell deficiency led to enhanced resuscitation after the non-heart-beating state, which implicates T cell products in the systemic consequences of heart stoppage and identifies T cells as a target for improved survival after cardiac arrest. Based on our data, we speculate that T cell modulation of the non-heart-beating human organ donor would increase the number of organs available for transplantation. The ROTRF-sponsored project is ongoing and we are continuing to focus on the mechanisms by which the T cell mediates renal IRI.

Publications
Disordered Thromboregulation in Xenotransplantation

Xenotransplantation, the transplantation of viable cells, organs or tissues between species, has been proposed as a solution to the shortage of human organs for the treatment of end-stage organ failure. Although hyperacute rejection (HAR) in experimental discordant combinations can now be effectively managed by complement inhibition, vascularised discordant xenografts are still subject to acute vascular rejection (AVR), alternatively referred to as delayed xenograft rejection (DXR). This latter mode of rejection is associated with vascular-based inflammation that may be associated with xenoreactive antibody deposition, thrombocytopenia and the consumption of coagulation factors that may ultimately evolve to disseminated intravascular coagulation (DIC).

Molecular incompatibilities have been shown between primate coagulation factors and porcine natural anticoagulants. These incompatibilities would exacerbate the inflammatory and thrombotic state within a rejecting xenograft, e.g. thrombin and thrombomodulin (TM) or factor Xa and tissue factor pathway inhibitor (TFPI), hence contributing to the consumptive coagulopathy. Triple transgenic animals (initially mice) over-expressing human natural anticoagulants (TM and TFPI) and the thromboregulatory factor CD39 (to block excessive platelet sequestration) are to be generated and tested in experimental models of xenograft rejection. This experimental system may show efficacy, in tandem with other recognised avenues of complement-inhibition and deletion of Gal epitopes, in the prevention of rejection, thrombosis and infarction in small-animal models such that we may then proceed to the generation of transgenic pigs for testing in primate systems.

This successful application to ROTRF has provided funding in the first year (2002/2003) for finalising the generation of such mice and further examining defined transplantation and inflammatory models to determine potential toxicity and, in the next 2003/2004 cycle, examining the utility of this approach in correcting disordered vascular thromboregulation under conditions of inflammatory stress. This work has been carried out in close collaboration with Dr. Anthony J.F. d’Apice and Dr. Karen M. Dwyer of the Immunology Research Centre, St. Vincent’s Health, Melbourne, Australia, with Dr. David H. Sachs and Dr. David K.C. Cooper of TBRC, Massachusetts General Hospital, Boston, USA.
Specific Aims (2002/2003):

1) To develop porcine-bone-marrow-derived cell lines over-expressing TM, TFPI and CD39, and to test these molecules for anticoagulant and/or antithrombotic function.

2) To generate transgenic mice expressing TM, TFPI and CD39 in various combinations, to determine any deleterious phenotype and to test these animals in thrombotic inflammatory models, including xenograft rejection. In the light of prior experience by d’Apice and colleagues, the H2-Kb promoter was chosen and despite the possible theoretical disadvantages of widespread expression, transgenic mice and pigs generated to date with this approach were highly stable with good levels of expression. We have also investigated the utility of the promoter of the Tie2 gene, which codes for one of the receptors of the family of regulators of vascular remodelling called angiopoietins, to facilitate vascular specific expression (not shown here).

Progress report:

TFPI: The pcDNA3/TFPI was initially cloned into pCEP4 and transfected cells were used to study the specific anticoagulant activity by inhibition of factor Xa activity in an indirect assay (TF-dependent pathways). Minimal TFPI expression was demonstrated. Given that this anticoagulant may be lost from the cell surface with the shedding of heparan sulphate, TFPI was cloned with a glycosyl-phosphatidylinositol (GPI) anchor, as already done for von Willebrand factor A1 domains. This approach is likely to have more success. The GPI-linked TFPI was then used to generate transgenic mice.

TFPI transgenic mice: One single male mouse has been generated to date and does not have grossly abnormal hemostatic responses, viz there is no excessive hemorrhage after tail biopsies for DNA extraction or following eye bleeding.

Thrombomodulin: The human cDNA was cloned into pCMVpuro for transfection and testing for activated protein C generation, using techniques published previously.

TM transgenic mice: The native human TM was cloned into pEA (H2-Kb promoter) for microinjection. Four transgenic mice have been generated to date. The mice express TM very strongly (Fig. 1). Again, these mice do not have grossly abnormal hemostasis and are being bred to generate sufficient mice for full characterisation of the phenotype.

The absence of an obvious anti-coagulant phenotype in these mice despite high-level human TM expression may suggest that human TM is not fully functional in mice.

Figure 1. Cytofluorometric analysis of murine T cells for human TM expression. Three transgenic mice over-expressing TM are shown (Dywer, unpublished). Histological analyses are still pending.
CD39/Nucleoside triphosphate diphosphohydrolase (NTPDase): Extracellular nucleotides play an important role in inflammation and thrombosis, triggering a range of effects including platelet sequestration, endothelial cell activation and vasoconstriction. CD39, the major vascular NTPDase1, converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), which is further degraded to the anti-inflammatory and anti-thrombotic molecule adenosine. Deletion of \( cd39 \) renders mice exquisitely sensitive to acute vascular injury, and \( cd39 \)-null cardiac xenografts show compromised survival. Conversely, upregulation of CD39 by somatic gene transfer or administration of soluble NTPDases has major benefits in models of transplantation and inflammation\cite{15-17}.

cDNA in pcDNA3/CD39 was cloned into pCMVpuro for transfection into SVAP cells. When transiently transfected into COS cells, CD39 can be detected by fluorescence-activated cell sorter (FACS) analysis with an approximate efficiency of 60\%\cite{16}. Substantial increases in activity have been measured by the generation of free inorganic phosphate from ADP (or ATP) by transfected vs. mock-transfected SVAP cells\cite{18,19}. Physiological functions of CD39 have also been determined by inhibition of ADP-mediated platelet aggregation\cite{18,19}, as measured in a dual-sample platelet aggregometer \textit{in vitro}.

CD39-transgenic mice: Human CD39 was cloned into pEA (H2-Kb promoter) for microinjection and the transgene molecule was over-expressed in several lines (Fig. 2).

CD39-transgenic mice exhibited a modified hemostatic system with impaired platelet aggregation to collagen and ADP \textit{in vitro}, prolonged bleeding times, and resistance to thrombogenic stimuli (Table 1). Importantly, the transgenic mice displayed no overt-bleeding tendencies under normal circumstances, and coagulation profiles, international normalised ratio (INR) and the activated partial thromboplastin time (aPTT) were normal. As demonstrated in Table 1, mice transgenic for CD39 were protected against thromboembolism induced by collagen and ADP injections. Basal plasma ATP, AMP and adenosine levels in transgenic mice were comparable with normal mice, however after intravenous administration of sublethal doses of collagen, the AMP levels were significantly higher in the transgenic mice.
Table 1. CD39 transgenic mice are resistant to systemic thromboembolism. A mixture of collagen and epinephrine was injected into the jugular vein of anaesthetised mice. Incisions in surviving mice were stitched, and the mice allowed to recover. CD39-transgenic mice were protected from systemic thromboembolism at doses lethal to wild-type mice.

Cardiac pseudodiscordant xenograft model. Donor hearts (Gal-positive hCD39⁺⁺) were harvested and grafted into Gal⁻⁻ mouse recipients. At 3 days post-surgery, the recipient mouse was injected with 75 μg anti-Gal IgG1 monoclonal antibody via the tail vein. The transplanted heart was monitored by daily palpation and was deemed rejected by humoral mechanisms when the cardiac beat was absent. Wild-type hearts were rejected within 24 hours in 6/7 cases while 11/13 CD39-transgenic hearts survived beyond 24 hours, with a mean survival of over 4 days. The histopathology of rejected non-transgenic hearts showed inflammatory vascular-type rejection processes involving platelet deposition and intravascular coagulation, while in transgenic hearts there was minimal evidence for vascular injury. Ultimately, rejection in the transgenic hearts appeared to be by cellular mechanisms but type-IV hypersensitivity reactions could also be compromised by CD39 in this system. Importantly, persistent CD39 expression on the endothelium was still observed on these late-rejecting allografts.

We are therefore developing a practical strategy that may be applicable in preventing serious thrombotic events that compromise the survival of pig organ xenografts.

Publications
Gene Expression in Tolerogenic Dendritic Cells

While the exact physiological subset, or relative maturation state, of dendritic cells (DC) responsible for inducing tolerance in vivo remains controversial, a number of pharmacological agents have been reported to modulate the immunostimulatory potential of DC in vitro, apparently ‘forcing’ them to become functionally tolerogenic.

The major goals of this project are:
1. To use serial analysis of gene expression (SAGE) of pharmacologically modified DC to determine diagnostic gene expression patterns and the expression of individual genes associated with tolerance.
2. To establish systems to probe the relevance to DC function, in particular with respect to tolerance of candidate genes identified both from:
   a) SAGE and,
   b) The Literature.

1. Phenotyping pharmacologically modified DC using SAGE

Our aim was to compare gene expression patterns of variously modulated DC, with and without exposure to lipopolysaccharides (LPS), with each other, their unmodulated counterparts and unrelated cell types, and to identify common and/or distinct changes. Substantial progress has been achieved in this area. We have now generated data on DC counter-modulated by interleukin-10 (IL-10), with and without exposure to LPS, the vitamin D3 analogue, 1α,25-dihydroxyvitamin D3 (VD3), and transforming growth factor (TGF)β1. In each case, the concentration of agent and the length of exposure required to drive bone marrow DC (bmDC) to a state in which they are unable to stimulate an allogeneic mixed lymphocyte reaction (MLR) have been experimentally determined, and SAGE libraries generated. LPS do not rescue these cells from becoming immunogenic. A summary of our current library resource is shown in Table I.

The LPS-treated and untreated gene-expression profiles for the IL-10-modulated populations, appear to support a mechanism by which IL-10 actively ‘conditions’ DC while modifying the quality of any subsequent response to stimuli. The phenotype of our cells is consistent
with previous literature with respect to antigen uptake and presentation, cell surface levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules and CCR7 mRNA levels. As a consequence of IL-10 treatment, 31 tags varied, in common with changes seen in the response of bmDC to LPS, suggesting at least some form of partial activation, although 102 tags were also modulated independently. Following LPS treatment of these cells, 41 of 73 changes associated with LPS maturation in the absence of IL-10 were retained, again suggesting that aspects of the normal response to LPS have been preserved, with 71 additional tags modulated. Inspection of individual SAGE tags, in combination with real-time polymerase-chain reaction (PCR) data indicated that, while the ability to initiate local inflammation and bacterial clearance is promoted, accompanied by a co-ordinate, presumably protective, increase in anti-inflammatory agents, the ability to activate and recruit T cells in response to microbial stimuli is constrained. How these observations relate to particular subsets of DCs and biological scenarios in vivo remains to be established, although they do highlight immediate, fundamental considerations for the field of ex vivo manipulated DC-based therapeutics. Global gene changes in response to VD3 and TGFβ also suggest that a process more subtle than mere blockage of maturation is occurring; it will be revealing to observe gene changes in these cells in response to microbial or other stimuli to see if they also display a skew in immune responsiveness.

Table 1. Summary of SAGE libraries, 30 November 2003

<table>
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<tr>
<th>Cell population</th>
<th>Library source</th>
<th>No. Tags (after excl.)</th>
<th>NCBI GEO Accession No.</th>
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<td>bmDC</td>
<td>Waldmann</td>
<td>21 789</td>
<td>GSM3833</td>
</tr>
<tr>
<td>bmDC +LPS</td>
<td>Waldmann</td>
<td>13 085</td>
<td>GSM3832</td>
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<td>GSM3834</td>
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<td>Normal brain granular cells</td>
<td>SAGEMAP</td>
<td>61 526</td>
<td>GSM767</td>
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*Long SAGE libraries
Libraries that have been generated or expanded during the period of ROTRF funding are shaded blue.
Using our accumulated SAGE resource, now constituting over 20 immune-related murine libraries, we have confirmed the reliability of inter-library and inter-laboratory library comparisons, determining that at a depth equivalent to 0.1–1% of the most abundant transcript, artefacts accumulate faster than novel genes\(^1\). For this reason, we have limited our libraries to 30 000 tags. ISAGEclus software, developed in-house, is being used to investigate ‘signatures’ of genes associated with particular phenotypes, using cluster analyses\(^1\), and by inserting ‘seed’ tags displaying idealised expression patterns and searching for tags with similar expression patterns.

We have extended our SAGE protocols to include ‘long SAGE’\(^3\), in which the alternative-tagging enzyme, \(Mm\), is used for direct generation of longer, 21-bp, tags. By retaining the common anchor enzyme, \(Nla\), tags derived from all the libraries remain directly comparable. However, the longer tags provide for ‘deeper’ more informative libraries, by reducing the effect of accumulated sequence artefacts, facilitate mapping of tags directly to the genome while avoiding inconsistencies generated by incomplete cDNA database entries and alternative splicing, and simplify the cloning of novel candidate genes.

\section*{2. Establishing relevance of candidate genes to DC function}

The results of these experiments are illustrated in the following publications\(^2,4\).-\(^6\).

\subsection*{i) Assessing biological function using manipulated embryonic stem (es) DC}

We are continuing to develop the \textit{in vitro} differentiation system, initially described by our laboratory, for generating genetically manipulated DC. Genetic manipulations are achieved in \textit{es} cells that are then differentiated along the DC lineage. These \textit{esDC} exhibit a high proliferative capacity, are phenotypically stable over time and retain the capacity to mature in response to LPS\(^7\). Comparative analysis of SAGE libraries derived from the parent ES line and \textit{esDC} has provided further supportive data for the integrity of this system\(^4\). These SAGE libraries are also now available for subtractive purposes to identify genes responsive to candidate gene expression in modified \textit{esDC}. Enhanced-green-fluorescent protein (EGFP) has been used to illustrate the feasibility of generating stable lines of genetically modified \textit{esDC}. These EGFP\(^+\) \textit{esDC} maintain their capacity for maturation while acquiring a potent immunostimulatory phenotype in response to LPS with no associated loss of transgene expression, as previously described in the ROTRF proposal. Additionally, we have demonstrated that there is no perturbation of induced CCR7 expression (real-time PCR) in these cells, and that genetically modified \textit{esDC} retain their migration patterns \textit{in vivo}\(^4\).

Plasmid vectors, based on the strong, constitutive promoter EF1–\(\alpha\) that maintained expression of EGFP throughout the process of \textit{esDC} differentiation and maturation, have been established for use in the \textit{esDC} culture system. A bicistronic plasmid has been generated with EGFP positioned downstream of an internal ribosome entry site to facilitate sorting and
monitoring of modified esDC. We are also investigating incorporation of tetracycline-inducible plasmids to avoid perturbations during differentiation to esDC.

A protocol was recently described for transient transfection of bmDC using naked RNA interference (RNAi) constructs. It is our aim to incorporate such a system to rapidly screen for constructs generating interesting knockdown phenotypes, prior to establishment of stable lines in esDC. We aim to develop a plasmid-based inducible interference system based on the lentiviral system recently reported by Wiznerowicz and Trono. We are in the process of obtaining constructs from the Trono laboratory to achieve this goal.

ii) Investigation of specific candidate genes
Preliminary inspection of the modulated DC SAGE data indicates a number of tags for which there is no gene currently assigned. Particularly when using long SAGE tags, it should be possible to clone the corresponding transcripts for functional investigation. We have recently published a novel proinflammatory CXCR2 ligand, DCIP-1, identified using such methodology, whose induction in response to LPS is not perturbed by pre-incubation with IL-10.

Influenced by knowledge from the literature, we have been attempting rationale design of tolerogenic esDC. While CD40 signalling is required for DC maturation, previous work from our laboratory has described an alternative-spliced type-III isoform that lacks the cytoplasmic signalling region and interferes with signalling from the full-length type-I isoform. Indeed, esDC over-expressing the type-III isoform are rendered incapable of T cell priming.

The enzyme indoleamine 2,3-dioxygenase (IDO) is responsible for the catabolism of tryptophan and has been implicated in dominant tolerance mediated by regulatory T cells. In order to investigate whether stable expression of IDO by esDC might confer protection from rejection and permit polarisation of T cells to a regulatory phenotype, we are in the process of generating male and female IDO+esDC lines to be assessed in A1xRAG1-/- mice.

We have begun development of an in vivo readout to assess the tolerogenicity of modified esDC. We have shown that T-cell-depleted splenocytes labelled with 5-(6)-carboxy-fluorescein succinimidyl ester (CFSE) can be detected in peripheral blood of syngeneic recipients for up to 40 days, and that by using different concentrations of CSFE, syngeneic and allogeneic populations can be tracked simultaneously. The effect of the administration of the potentially tolerogenic esDC on clearance of allogeneic cells provides a measure of tolerance induction. To avoid complications of alloantigen presentation by the indirect pathway we will make use of mice expressing class II MHC only on the thymic epithelium, permitting positive selection of CD4+ T cells, but preventing their recognition of peptide-MHC complexes in the periphery.
Agonist antibodies against glucocorticoid-induced tumour-necrosis-factor receptor (GITR) neutralise the suppressive activity of CD4⁺CD25⁺ regulatory T cells. Using a GITR-Fc fusion protein we previously demonstrated a significant increase in binding to bmDC conditioned by exposure to IL-10. It was proposed to clone the murine ligand for GITR and investigate its function with respect to dendritic cells and immunity/tolerance. We have recently identified and cloned the murine GITR ligand and confirmed that its interaction with GITR reverses suppression by CD4⁺CD25⁺ T cells. The relevance of GITRL expression on IL-10-modulated DC is, as yet, undetermined, but is increased following exposure to LPS.

Publications
Role of Costimulatory Molecules in Cardiac Allograft Rejection

Specific Aim #1. To determine how antigen recognition and rejection occur in a transplant model of indirect costimulation

Specific Aim #2. To determine the mechanisms of rejection operating in CD28-deficient mice in vivo
We have previously shown that, in contrast to wild-type (wt) mice in which CD4+ but not CD8+ T cells are necessary for cardiac allograft rejection, both T cell subsets play a major role in rejection of cardiac allografts in CD28-deficient mice. It has been reported that natural killer (NK) cells also play a major role in acute allograft rejection in CD28-deficient mice. We are currently investigating the role of NK cell subsets and of NK T cells in this context.

Progress report for 2002-2003
The initial stimulation of T cells by antigen presentation in the context of self-antigen-presenting cells (APCs) is thought to occur in secondary lymphoid organs. Recognition of alloantigen in transplantation settings may be more complex, in that the antigen can be presented both by donor cells, including graft stromal cells and passenger APCs (direct presentation) and, following processing, by host-antigen-presenting cells (indirect presentation). Direct presentation has been shown to play an important role in cardiac transplantation settings for stimulation of both CD4+ (#1) and CD8+ T cells, and is sufficient to promote acute allograft rejection. However, whether direct presentation occurs at the graft site or following migration of donor cells to host secondary lymphoid organs has not been fully elucidated.

Results
Effective skin graft rejection by LTβR−/− mice.
To examine the role of secondary lymphoid organs in the rejection of unvascularized tissue allografts, fully allogenic BALB/c skins were transplanted into intact or splenectomized B6 and LTβR−/− mice. Acute rejection of skin grafts occurred with similar kinetics in intact wt and LTβR−/− recipients (MST 8+1 days, Fig. 1). Surprisingly, splenectomized LTβR−/− mice effectively rejected skin allografts, albeit with slower kinetics than intact mice (MST 17 days, p < 0.05). Rejection was not restricted to this particular strain combination, as C3H/HEJ (H-2') skin
grafts were also effectively rejected in splenectomized LTβR⁻/⁻ mice (MST 11+2, n = 5, data not shown). These data suggest that secondary lymphoid structures are not absolutely required for acute rejection of skin allografts.

**Cardiac allograft rejection occurs in splenectomized LTβR⁻/⁻ and LTαR⁻/⁻ mice.**

To address the role of secondary lymphoid organs in the rejection of a vascularized allograft, BALB/c hearts were transplanted into intact and splenectomized wt and LTβR⁻/⁻ mice. Acute rejection of cardiac allografts occurred rapidly in intact B6 and LTβR⁻/⁻ recipients (MST 8, and 11 days, respectively, Fig. 2A). The surgical removal of the spleen prolonged survival of cardiac allografts in B6 mice (MST 16 days, p < 0.001) and markedly delayed rejection in LTβR⁻/⁻ recipients (p < 0.001). However, between 60 and 87 days post-transplant, 5 out of 6 splenectomized LTβR⁻/⁻ mice eventually rejected their grafts.

**Figure 1.** Splenectomized LTβR-KO mice effectively reject allogeneic skin grafts.

**Figure 2.** Delayed but effective rejection of cardiac allografts by splenectomized LTβR-KO and Ltα-KO mice.
All LTβR−/− transplanted animals were carefully examined at necropsy and we were unable to detect any lymphoid structures. However, as an alternative model, experiments were repeated using LTα−/− mice as recipients. Similar to what was observed in LTβR−/− mice, rejection of cardiac allografts effectively occurred in both intact (MST 26 days) and splenectomized (MST 50 days) LTα−/− recipients (Fig. 2B). Taken together, these results suggest that secondary lymphoid tissues are not absolutely required for rejection of either skin or heart allografts.

Increased numbers of CD4+ cells in cardiac allografts rejected by splenectomized LTβR−/− mice. To compare the cell types infiltrating rejected cardiac allografts, histology and immunohistochemistry were performed on heart transplants harvested from splenectomized B6 and LTβR−/− recipients at different time points. Histological features in all mice were consistent with cellular-mediated acute rejection (scores ∃ 2, data not shown). Greater numbers of CD8+ than CD4+ cells were observed in grafts from intact (data not shown) and splenectomized (Fig. 3A) B6 recipients, confirming our previous findings in this strain combination. Surprisingly, a significant increase in the number of CD4+ cells was observed in grafts from splenectomized LTβR−/− when compared with grafts from splenectomized B6 mice at the same time point (day 10, p < 0.01). Numbers of CD8+ cells were not significantly different. Numbers of infiltrating CD4+ and CD8+ cells both gradually increased over time in splenectomized LTβR−/− mice (Fig. 3A and 3B). To determine if the increase in infiltrating CD4+ cells was a feature of LTβR−/− mice or was secondary to the removal of the spleen, the number of intra-graft CD4+ cells was compared at the time of rejection in intact and splenectomized LTβR−/− mice. Interestingly, significantly increased numbers of CD4+ cells were detected within allografts from splenectomized LTβR−/− mice when compared with intact LTβR−/− (p < 0.001), suggesting that acute allograft rejection in the absence of secondary lymphoid organs may require increased influx of CD4+ cells.

**Figure 3.** Increased numbers of CD4+ T cells in cardiac allografts from splenectomized LTβR-KO mice.
Effective acute allograft rejection in LTβR−/− and LTα−/− mice is probably not due to greater numbers of memory T cells than in wild-type mice. It has been reported that previously activated T cells or memory cells do not require trafficking to secondary lymphoid organs for mediating acute allograft rejection. Thus, it is conceivable that effective allograft rejection by splenectomized LTβR−/− mice in our model is due to the presence of memory T cells. Therefore, we examined the expression of CD44 and CD62L on splenic CD4+ and CD8+ T cells from wt and LTβR−/− mice. Similar amounts of splenic T cells from wt and LTβR−/− displayed the CD44high and CD62Llow phenotypes (Fig. 4, top panels). Transplantation of syngeneic and especially of allogeneic grafts resulted in an increased proportion of T cells expressing activation markers when compared with untreated mice. No difference between the amounts of T cells expressing activation markers (Fig. 4, top panels) or the expression levels (mean fluorescence intensity, data not shown) of surface markers was detected between T cells from wt and LTβR−/− mice.

![Figure 4. Similar number of T cells with surface markers of activation/memory in the spleen and blood of LTβR-KO mice.](image)

To exclude the possibility of an accumulation of an activated population of T cells in splenectomized animals over time, we examined the expression of activation markers on peripheral blood T cells from splenectomized wt and LTα−/− mice. Similar amounts of activated T cells
over the course of the experiment were obtained in both strains of mice (Fig. 4, bottom panels). Together, these results suggest that effective allograft rejection observed in splenectomized LT-deficient mice is unlikely to be due to a higher amount of allospecific memory T cells.

Finally, we compared the degree of alloreactivity in wt and LTα-deficient mice, as a readout of the number of alloantigen-specific T cells in both strains of mice. To this end, splenocytes from B6, LTα-deficient, and B6 mice that had rejected BALB/c hearts were stimulated with irradiated BALB/c or C3H/HEN splenocytes. Similar levels of IL-2 production were found in the supernatants of B6 and LTα-deficient splenocytes in response to both stimulator strains, whereas significantly more IL-2 was found in the cultures of splenocytes from previously transplanted B6 mice re-stimulated with donor but not third party cells (Fig. 5). This result indicates that the degree of alloreactivity in LTα-deficient mice is comparable to unmanipulated rather than to sensitized animals. Taken together, these data suggest that LTα-deficient mice do not have more activated or antigen-experienced T cells than wt mice.

**Figure 5.** Similar alloreactivity in splenocytes from unmanipulated B6 and Ltα-KO mice.

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Secondary lymphoid organs are not required for generation of memory responses. We next investigated whether priming to alloantigens could occur in the absence of secondary lymphoid organs. To address this question, LTβR−/− mice were first splenectomized and transplanted with BALB/c skins. Following skin graft rejection, the animals were challenged with donor cardiac allografts. Splenectomized LTβR−/− mice previously sensitized with skin allografts rejected cardiac allografts significantly faster (MST 41±35 days) than non-sensitized splenectomized LTβR−/− mice (MST 84±17 days, p < 0.01;Fig. 6), suggesting that T cell priming by skin alloantigens did occur in the absence of secondary lymphoid structures. As expected, when skin grafts were first placed onto intact LTβR−/− recipients that were later splenectomized at the time of cardiac allograft challenge, heart rejection occurred even faster (MST 11±3 days, p < 0.001), indicating that the presence of the spleen allowed for more effective T cell priming. Together, these results indicate that the presence of a spleen facilitates, but is not required for, generation or expansion of memory responses to alloantigens.
Summary
These results suggest that, although peripheral lymphoid organs play an important role in allowing allograft responses to occur, they do not appear to be absolutely required for either acute rejection of skin, cardiac allografts, or T cell priming. These results suggest that immunologic events capable of leading to allograft rejection can successfully occur at sites other than classical secondary lymphoid organs.

Publications

Figure 6. Splenectomized LTα-KO mice experience faster cardiac allograft rejection if first primed with donor skin graft.
I. Background and Significance

Corneal Transplantation: Success and Failure

Corneal transplantation is the most common and successful form of solid-tissue transplantation in humans. In the United States alone, nearly 40,000 cases are performed annually. In uncomplicated (so-called ‘low-risk’) cases, the two-year-graft-survival rate is over 90% under cover of topical-steroid immune suppression. However, immune rejection of graft endothelium and possibly stroma, and infiltration of graft stroma with alloreactive immune cells, continues to represent the principal threat to corneal transplants. This immune-based threat is nowhere more acutely felt than in ‘high-risk’ transplants grafted onto inflamed host beds where even standard systemic or local pharmacotherapies (e.g. topical steroids) remain largely ineffective.

Graft Failure & Cell Death

Our evaluation of inflamed corneal specimens, either after corneal transplantation or after other insults to the cornea or ocular surface (unrelated to transplantation), has revealed that there is enhanced apoptosis in such corneas (see below). Indeed, it appears that corneal (in particular endothelial) cell death is the common denominator of most forms of corneal-graft

**Figure 1.** Conceptual figure proposing the role of apoptotic cell death both before and after corneal transplantation (y-axis denotes number of viable endothelial cells; x-axis, time). Corneas require a threshold (minimum) number of endothelial cells (EC) to retain clarity. The number of graft EC normally decreases with time (black curve) in storage as well as after surgery (vertical arrow), with a precipitous fall after immune rejection (dashed curve). We hypothesize that preventing apoptosis (depicted in blue) will have the effect of moving “the curve to the right,” and hence will lower the chance of EC counts falling below the threshold required to maintain corneal clarity.
decompensation since, what is ultimately shared by all failed grafts, regardless of cause, is a loss in the number of viable endothelial cells. Our hypothesis that death of the corneal endothelial cells promotes graft rejection is indirectly supported by the observation that the likelihood of graft survival decreases as the endothelial number decreases. Corneas are susceptible to loss of endothelial-cell viability whilst in storage, prior to surgery. This concept is reflected in the conceptual diagram on the previous page (Fig. 1).

II. Objectives

- Optimize ex vivo adenovirus-mediated gene transfer to corneal-endothelial cells during hypothermic organ preservation.
- Determine the kinetics of marker and anti-apoptotic-gene expression, and any adverse effects of this gene transfer procedure (via adenoviral vector) on syngeneic corneal grafts and preserved donor tissue.
- Compare the incidence of graft failure and cell apoptosis in control, and anti-apoptotic gene expressing, allogeneic corneal transplants.

III. Results

1. Apoptosis in Corneal Transplantation

Transplantation: Low-risk and high-risk variations of our standardized and validated model of orthotopic corneal transplantation in mice was used. In the low-risk version, allogeneic grafts were performed across both major histocompatibility complex (MHC) and minor histocompatibility antigenic barriers between C57BL/6 donors and normal BALB/c recipients; for the high-risk setting C57BL/6 grafts were placed onto inflamed and vascularized (high-risk) BALB/c host beds. Normal corneas were used as controls. Another control group consisted of the placement of three 11-0 nylon intrastromal sutures onto the corneas to induce inflammation and neovascularization, but without accompanying transplantation. Graft success and failure was measured by the standard grading scheme for evaluation and scoring of orthotopic corneal transplants.

Immunohistochemistry: Whole eyes from control and grafted mice were frozen and cross-sections mounted on microscope slides and probed for the presence of fragmented DNA using the TUNEL stain.

Western blotting: Corneal lysates were prepared from freshly excised corneas (including grafts) by homogenizing the tissue in a detergent-containing lysis buffer. Lysates were sonicated to break the tissue further, and probed for caspase-3 expression by Western-blot methods.
Results:
We have found that, in the low-risk setting (Fig. 2A), some apoptotic cells were present, and that their number was greatest in grafts that were rejected. There was a substantial increase in the overall number of cells that stained positive for TUNEL under all conditions in the high-risk setting (Fig. 2B). The idea that apoptosis was occurring in grafts that were failing was supported by a second approach. Western-blot analysis of corneal lysates revealed a dramatic increase in the amount of activated caspase-3 in the failed grafts (Fig. 2C). We have also begun investigating the identity of the apoptotic cells in the corneal stroma by double immunofluorescent staining for TUNEL (or caspase-3) and CD45, which is a pan-leukocyte marker. Our data to date suggest that the overwhelming majority of cells undergoing apoptosis are CD45- and are hence not leukocytes; ongoing experiments are being performed to confirm this finding.

Figure 2. A. TUNEL-stained sections through low-risk corneal allografts at 1 and 3 weeks. In the low-risk model, nearly 50% of grafts are rejected by weeks 3-4; the remainder survive. Results show that there is no demonstrable apoptosis at week 1. Few apoptotic cells are evident in the accepted graft (middle panel). However, there is evidence of apoptosis in all three layers of the rejected allograft, including the endothelial layer. B. In the high-risk setting, all grafts uniformly reject by weeks 2-3. Data show evidence of apoptosis in epithelial and anterior stroma of these grafts, even before rejection (left panel). Rejection is associated first with stromal, then with full-thickness evidence of apoptosis (middle and right panels). Similar data were obtained for active caspase-3 expression (data not shown). C. Western blot for active subunits of caspase-3 performed on homogenized corneas explanted from normal (1st column from left) and inflamed neovascularized (NV) ungrafted eyes (2nd column), as well as low-risk (3rd column) and high-risk (4th column) rejected grafted eyes. Data suggest a significant increase in the expression of active caspase-3 in rejected corneas, in particular those from high-risk transplants, correlating with the in situ data shown above.
Answers:
These preliminary data demonstrate that apoptosis does occur in corneal transplants and that the degree of apoptosis correlates strongly with anti-graft immunity (rather than with non-specific inflammation). We have only occasionally detected apoptotic endothelial cells, which probably reflects the fact that the endothelial cell layer is usually absent from the graft that is undergoing rejection. Finally, our preliminary studies demonstrate our ability to measure apoptosis in the cornea both in situ and in vitro.

2. Overexpression of Anti-Apoptotic Gene Bcl-xL in Transgenics is Associated with Enhanced Corneal Transplant Survival

Methods: Bcl-xL is a potent anti-apoptotic factor and member of the Bcl-2 family. We have primarily focused on this anti-apoptotic gene to create a vector for Bcl-2 expression, which proved to be of some difficulty, as was the access to a large number of Bcl-2 transgenics. As is true of most tissues, we have confirmed by immunohistochemistry that the corneal endothelium constitutively expresses this factor. We hypothesized that corneal grafts that overexpress Bcl-xL have an increased survival rate compared to wild-type controls. To test this hypothesis, we compared the survival of corneal donor tissue derived from either non-transgenics or Bcl-xL-transgenics in an allogeneic transplant setting. Bcl-xL-transgenics (on a C57BL/c background) were obtained from Dr. DongFeng Chen (Harvard Medical School) and their corneal buttons were used as donor tissue for transplants into BALB/c mice. Wild-type C57Bl/6 donor tissue was used as a control. Hosts were left untreated; no local or systemic immunosuppressive medications were used. Graft survival was assessed in a masked fashion using a standard grading scheme for transplant opacification.

Results:
We observed a statistically significant increase in the survival of transgenic grafts compared with the B6 wild-type controls (Fig. 3).

Figure 3. Kaplan-Meier survival curves for Bcl-xL-transgenic or wild-type allogeneic corneal grafts in BALB/c hosts. There is a significant enhanced survival when the corneal donor tissue is derived from Bcl-xL transgenic overexpressors (p = 0.04).
Answer:
These preliminary data strongly suggest that the overexpression of anti-apoptotic genes in the corneal graft can have a significant effect on graft survival even without the use of any immunosuppressives.

An important issue that we will be addressing in the near future is which layers of the cornea overexpress the Bcl-xL protein in the transgenic animal. In these mice, the Bcl-xL gene is under the control of Tα1-α-tubulin, a pan-neuronal promoter. Since both endothelial and stromal corneal cells are thought to be derived from the neural-crest, the anti-apoptotic gene should be overexpressed in both layers. However, further expression studies need to be performed in order to elucidate which of the layers of the cornea is the preferred site for expression of anti-apoptotic genes.

3. In Vitro Assays of Corneal Cell Apoptosis
Our goal here was to develop a reliable in vitro assay system to measure corneal stromal and endothelial cell apoptosis. The ideal system would allow apoptosis to be measured in select corneal cells in response to different apoptotic stimuli. The cornea of the mouse is unfortunately quite small, limiting the possibility of routinely using primary cultures. To circumvent this obstacle, our laboratory obtained an immortalized line of corneal endothelial cells (CEC) from the C57BL/6 mouse (kind gift of Dr. Jerry Neiderkorn, University of Texas Southwestern Medical Center, Dallas). We also obtained an immortalized line of corneal stromal cells from the C57BL/6 mouse (MK/T-1 cells) from the laboratory of Dr. Robert Gendron at Memorial University of Newfoundland, Canada.

Methods:
Cells were incubated with various concentrations of apoptotic factors including tumor-necrosis factor α (TNF-α), interleukin-1-β (IL-1-β), interferon-γ (IFN-γ), and agonistic Fas monoclonal antibody. Treated cells were then lysed in a strongly denaturing lysis buffer and separated on SDS-PAGE. Expression of active isoforms of caspases-3 was determined by Western blot analysis using a commercially available antibody (Fig. 4). The positive control cells for active caspase-3, were incubated with camptothecin or staurosporine, which are known inducers of apoptosis.

Figure 4. Western-blot detection of capase-3 from mouse corneal endothelial cells (left panel) and corneal stromal cells (right panel).
Results:
These preliminary data suggest that (i) there are synergistic effects of pro-apoptotic inflammatory cytokines with Fas in triggering apoptosis, and (ii) stromal and endothelial cells differ in their sensitivity to apoptotic stimuli. In addition to the caspase-3 Western-blot assay, we have developed and optimized a flow-cytometric-(FACS)-based approach to monitor apoptosis. This approach involves measuring the abundance of annexin V, a protein that binds with high affinity to phosphatidylserine (PS), a phospholipid which is normally present only on the intracellular surface of the plasma membrane but which becomes translocated to the extracellular surface during apoptosis. Annexin V is considered to be amongst the best markers for early apoptosis.

4. Use of Adenovirus as an Efficient Vector for Gene Transfer to Corneal Endothelial Cells Ex-Vivo

Question: Can viral vectors be used to transfer genes into corneal cells ex vivo?
We have screened three different types of viruses (retrovirus, adenovirus and adeno-associated virus) for their ability to express GFP in corneal buttons. We found that adenovirus efficiently and selectively delivered GFP to the endothelial layer of the cornea (Fig. 5). Retrovirus and adeno-associated virus were not able to transduce to detectable GFP in our hands. Our initial work revealed that adenoviruses are the most efficient vector for gene delivery to the corneal endothelium, and much of this work has been summarized in the manuscript.

Methods:
BALB/c mouse corneas were incubated with replication-deficient adenovirus encoding-GFP or empty vector ex vivo at a dose of 6 x 10⁷ PFU or 6 x 10⁶ PFU at temperatures of 4°C or 37°C (4°C was chosen to replicate the temperature used by tissue banks for corneal storage in medium). The corneas were then sectioned and GFP detected under a fluorescent microscope (Fig. 5). Once we had optimized the conditions for infection, the infected corneas were transplanted to BALB/c or C57BL/6 recipients. After transplantation, localization of GFP in the grafts was determined in cryosections of enucleated eyes and GFP expression in the grafts was visualized in vivo by using an epifluorescent microscopy over 12 weeks (Fig. 6). All grafts were evaluated clinically by slit-lamp biomicroscopy.

Figure 5. A. Localization of GFP was primarily in the endothelium (arrow) of the cornea. “EP”, “S”, and “EN” refer to the epithelial, stromal and endothelial layers, respectively. B. No GFP expression was observed in empty-adenoviral-vector-infected corneal grafts.
Results:
1. We have successfully established a method for introducing genes into the corneal endothelium using adenoviral-mediated gene therapy.
2. *In vivo* retention of gene expression can be prolonged for weeks without use of any immunosuppressive.

**IV. Summary of Results to Date**
In brief, our data to date have successfully addressed our objectives. These data demonstrate that apoptosis is occurring in the cornea as grafts are being rejected. In addition, we have established and optimized experimental approaches to detect apoptosis in the cornea *in situ*, and in cultured stromal and endothelial cells. Furthermore, graft failure is reduced when the donor corneas are from a Bcl-xL transgenic animal, suggesting that expression of anti-apoptotic genes is a viable strategy to improve graft survival. Finally, we have begun to work out conditions and approaches to transduce genes into the cornea in an *ex vivo* setting so as to extend our work into the translational domain for modification of human corneal graft donor tissues.

**Publications**
Chemokines and their Receptors as Therapeutic Targets to Prevent Allograft Rejection

Rejection of organ allografts proceeds in a stepwise fashion. Initiation of the alloimmune response is thought to occur in secondary lymphoid organs (lymph nodes, spleen), where alloreactive T lymphocytes and antigen-presenting cells convene. Activated alloreactive T cells subsequently migrate to the allograft and exert their detrimental effector functions. Chemokines have been shown to play a central role in the migration of leukocytes during the initiation phase (i.e. homing to secondary lymphoid organs) and during the effector phase (i.e. migration to extralymphoid tissues) of an immune response. Chemokines responsible for migration to secondary lymphoid organs have been termed "lymphoid" chemokines, those governing migration to extralymphoid tissues "inflammatory" chemokines. The aim of the proposed project was to study the role of lymphoid chemokines in allograft rejection using mouse transplantation models. Particular emphasis was placed on the chemokine receptor CCR7 that has an essential role in T cell and dendritic cell migration to secondary lymphoid organs.

Specific aims were:

1. Study rejection of skin and heart allograft responses in CCR7-deficient mice
2. Study migration of lymphocytes and dendritic cells following organ transplantation
3. Study the mechanisms involved in anti-Kb T cell receptor (TCR) transgenic mice
4. Develop therapeutic strategies to block chemokine/chemokine receptor interactions in vivo

Prolonged survival of CCR7-deficient skin and heart allografts

To determine the role of CCR7 in the rejection of primarily vascularised and non-vascularised allografts, fully major histocompatibility complex (MHC)-mismatched C3H (H-2k) hearts and skin were grafted to CCR7-/- mice and to wild-type (WT) littermates (129Sv/BALB/c, H-2b). Whereas WT mice rejected C3H hearts within 7.3 days, allograft survival in CCR7-/- mice was prolonged to 10.7 days ($p < 0.01$). Similarly, rejection of C3H skin grafts was significantly faster in WT (8.9 days) compared to rejection in CCR7-/- recipients (12.3 days, $p < 0.01$). Similar results were observed in mice that were matched at the MHC, but mismatched at non-MHC loci. Since CCR7 has also been shown to critically influence
dendritic cell migration, it was of interest to examine the role of graft-derived dendritic cells in allograft rejection. To this end, CCR7<sup>−/−</sup> hearts and skin were grafted to completely allogeneic C3H recipients. Neither heart- (7.6 days in WT vs. 8.2 days from CCR7<sup>−/−</sup>) nor skin-grafted mice (12.0 days in WT vs 11.0 days from CCR7<sup>−/−</sup>) showed any signs of prolonged survival in this model. These data suggest that unimpaired emigration of skin dendritic cells is not a prerequisite for the initiation of alloimmune responses. Finally, deficiency of CCR7 in both donor and recipient was analysed in CCR7<sup>−/−</sup> recipients of CCR7<sup>−/−</sup> allografts. To this end, minor histocompatibility antigen disparate CCR7<sup>−/−</sup> skin was grafted to WT or CCR7<sup>−/−</sup> recipients. Whereas CCR7<sup>−/−</sup> skin was rejected by WT recipients within 13.5 days, graft survival in CCR7<sup>−/−</sup> recipients was significantly extended to 21.2 days (p < 0.01). There was no significant difference between the survival of WT and CCR7<sup>−/−</sup> skin grafts in WT recipients (11.3 days vs 13.5 days, NS), and between the survival of WT and CCR7<sup>−/−</sup> allografts in CCR7<sup>−/−</sup> recipients (17.5 days vs 21.2 days, NS).

**Intragraft leukocyte infiltration in CCR7-deficient mice**

Heart allografts with a full MHC disparity (recipients: CCR7<sup>−/−</sup> recipients and control littermates, donor: C3H) were examined by immunohistology at days 4 and 6 after transplantation. At day 4, infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was considerably reduced in CCR7-deficient recipients compared to WT controls. However, analysing grafts 6 days after transplantation revealed similar levels of leukocyte infiltration in both groups. These results confirm the functional observations of delayed, but eventually effective skin and heart allograft rejection in CCR7-deficient mice.

**Lack of T cell expansion in draining lymph nodes of CCR7<sup>−/−</sup> compared to WT recipients after fully mismatched unilateral skin transplantation**

To examine the intensity of the allogeneic immune response in CCR7<sup>−/−</sup> mice, C3H skin was transplanted to the recipient’s left flank. Recipients were sacrificed at different time points (days 4 and 7). Lymph nodes and spleen were harvested and analysed by flow cytometry. Lymph node cell counts were reduced to a tenth in CCR7<sup>−/−</sup> mice compared to those in WT mice. Following fully MHC- mismatched skin transplantation cell numbers in the draining lymph nodes of CCR7-deficient recipients were five to ten times lower compared to those in WT mice. Whereas cell numbers in draining lymph nodes in WT recipient mice continued to increase at day 7, lymph node cells in CCR7<sup>−/−</sup> mice showed no further expansion. Both on day 4 and day 7 after syngeneic grafting, increased cell numbers were observed in the draining lymph nodes. These were considerably lower than in allografted mice, but were significantly higher compared to those in CCR7-deficient mice. In summary, CCR7<sup>−/−</sup> recipients exhibited a significant defect in the homing of lymphocytes to draining lymph nodes and showed severely reduced alloantigen-induced T cell expansion in these organs as compared to WT recipients.
Additional prolongation of allograft survival in splenectomised CCR7-deficient mice

In accordance with previously reported results in aly/aly mice lacking lymph nodes, which after splenectomy were unable to reject allogeneic heart grafts, we attempted to extend allograft survival in CCR7-/- mice by splenectomy (to this end, WT and CCR7-/- mice were splenectomised and grafted with C3H skin). Compared to non-splenectomised recipients, splenectomy significantly prolonged the survival of WT skin grafts by more than four days (8.9 +/- 0.9 days vs 13.6 +/- 1.3 days, p < 0.01). In CCR7-/- mice, splenectomy extended skin graft survival by 5 days (12.3 +/- 0.9 days vs 17.3 +/- 2.2 days, p < 0.01). Heart graft survival could also slightly be enhanced by splenectomy in CCR7-/- recipients (10.7 +/- 2.8 days vs 13.3 +/- 1.0 days, p < 0.05), whereas WT recipients showed no significant increase in graft survival (7.3 +/- 0.5 days vs 8.3 +/- 1.2 days, NS). These findings demonstrate that splenectomy further prolonged skin and heart allograft survival in CCR7-/- as well as in WT mice, but was unable to induce indefinite graft survival.

Subtherapeutic doses of Cyclosporin A abrogate the CCR7-/- effect

Synergistic effects between cyclosporine A (CsA) and the deficiency of different chemokine receptors (e.g. CXCR3, CCR5, CX3CR1, CCR1) have previously been reported. To elucidate whether CsA might augment allograft survival in CCR7-deficient mice, we treated C3H (H-2k) heart–grafted and skin-grafted WT and CCR7-/- mice (H-2b) with subtherapeutic daily doses of CsA.

In WT mice, CsA did not significantly prolong heart allograft survival (7.8 +/- 1.8 days vs 7.3 +/- 0.5 days without CsA, NS). Interestingly, CsA abrogated the previously observed prolongation of heart-graft survival in CCR7-/- mice (8.0 +/- 2.0 days vs 10.7 +/- 2.8 days without CsA, NS).

In contrast, in the presence of CsA, skin graft survival was extended in both WT (15.5 +/- 2.4 days versus 8.9 +/- 0.9 days without CsA, p < 0.01) and CCR7-/- mice (15.7 +/- 1.5 days vs 12.3 +/- 0.9 days without CsA, p < 0.05). As for the heart-grafted mice, there was no significant difference in allograft survival between CsA-treated WT and CCR7-/- mice (15.5 +/- 2.4 days in WT vs 15.7 +/- 1.5 days in CCR7-/- mice, NS). Therefore, in both models the previously observed prolongation effect of CCR7 deficiency was neutralised by CsA therapy.

In summary, we could show a prolongation of skin allograft and heart allograft rejection in CCR7-deficient mice. Future studies aim to address the role of memory vs. naive T cells in this model, the effect of CCR7 deficiency on tolerance induction, the mechanism of CCR7-mediated prolongation of allograft survival in TCR-transgenic mice, and strategies to develop a therapeutic protocol for allograft protection targeting chemokines and their receptors.
Identification of Novel Proteins Involved in Rejection of Transplanted Organs

Background and Aims of the Proposal
Reducing the rejection rate of transplanted organs represents a major challenge to modern medicine. While it is readily appreciated that cyclosporine A (CsA) or FK506 can effectively suppress T cell activation via the Signal-I pathway, they do not necessarily inhibit the expansion of activated T cells that rely on Signal III. Moreover, it remains less clear whether effector proteins within the later pathway belonging to the interleukin 2 (IL-2) receptor, hold greater potential. Based on our new findings, we believe that IL-2 activation of two key T cell transcription factors, signal transducer and activator of transcription Stat5a and Stat5b which exhibit 96% homology, represent novel molecular targets for pharmacological inactivation to control T cell expansion and activity in this late cell signaling cascade. Our principal hypothesis was that active Stat5a/b transcription factors are critical for T-cell-mediated immunity and allograft rejection. We base this hypothesis on our earlier findings that IL-2 cannot activate Stat5a/b in lymphocytes isolated from immune-suppressed tumor-bearing mice and HIV-infected patients. To identify T cell regulators that block Stat5a/b activation, we mapped activating tyrosine and serine phosphorylation sites within these transcription factors and searched for drugs that would selectively inhibit them. A variety of traditional pharmacological drugs, as well as antisense strategies, were employed to test our hypothesis. Those agents that effectively and selectively blocked this T cell signaling pathway were subsequently validated using allograft rejection models.

Results

Selective inhibition of Janus tyrosine kinase (Jak)3 blocks Stat5a/b activation and T lymphocyte function.
We identified two selective agents that were able to inhibit Stat5a/b activation in primed T cells by employing a tyrphostin family member AG-490, or the prodigiosin analogue PNU156804, to target their upstream regulator Jak3. We reported that AG-490 or PNU156804, were competent to selectively uncouple Stat5a/b activation of human primary and tumor-derived T cells based on phosphorylation, DNA-binding experiments, and cell-proliferation assays. Indeed, these drugs ablated Stat5a/b tyrosine and serine phosphoryla-
tion by cytokines competent to activate them, including the T cell growth factors IL-2, IL-7, IL-9 and IL-15 without affecting the expression of their respective receptors. Interestingly, T cell proliferation regulated by these cytokines was also completely uncoupled. We also demonstrated that PNU156804 preferentially inhibited Jak3 catalytic activity as compared to its closest related family member, the ubiquitously expressed enzyme Jak2, in blocking the engagement of the Stat5 signaling pathway. Both agents disrupted the ability of Stat5a/b to translocate to the nucleus, bind DNA and regulate T-cell-mediated gene transcription. This effect was selective since neither agent inactivated other signaling pathways including the ubiquitously expressed and recruited transcription factors NFκB, or T-cell-receptor-regulated effectors controlled by Zap70 and p56Lck. Lastly, neither AG-490 nor PNU156804 had a significant effect on unprimed T cells that fail to express Jak32-4.

**Inhibition of the Jak3-Stat5a/b signaling pathway is immunomodulatory.**

To determine whether uncoupling the Jak3/Stat5a/b signaling pathway holds immunosuppressive potential, we employed major histocompatibility complex (MHC) mismatch rat heart allograft transplant models. To test this notion, a seven-day course of intravenous therapy with 5-20 mg/kg AG-490 was used to inhibit rejection of heterotopically transplanted Lewis (RT1\(^\text{a}\)) heart allografts in ACI (RT1\(^\text{a}\)) recipients. We reported that AG-490 significantly prolonged allograft survival in a dose-dependent manner. Moreover, this agent acted synergistically when used in combination with the Signal-I inhibitor CsA by displaying combination index (C.I.) values less than 1.0 at a variety of drug ratios\(^3\). However, this synergy was not observed when AG-490 was used in combination with another Signal-III inhibitor, rapamycin (RAPA), which typically resulted in C.I. values greater than 1. AG-490 treatment reduced graft infiltration of mononuclear cells (GICs). Of importance, Stat5a/b DNA binding of *ex vivo* stimulated IL-2-stimulated GICs was blocked, confirming our *in vitro* findings. Lastly, this treatment failed to affect IL2R\(^\alpha\) mRNA expression, as judged by ribonuclease-protection assays, a marker of T cell priming\(^3\).

Similar results were obtained with PNU156804, however, this agent was water soluble and could be readily delivered orally in a heterotopic heart transplant model combining WF (donor: RT1\(^\text{a}\)) to BUF (recipient: RT1\(^\text{a}\)). For transplanted rats that received an alternate-day 14-day oral gavage of 40, 80, or 120 mg/kg PNU156804 alone, survival was extended to day 55 +/- 21.0 (\(p < 0.001\)); in combination with CsA to ratios of 1:1 to 1:64 to 85.8 +/- 23.8 (\(p < 0.001\)); or in combination with RAPA ratios of 40:1 to 160:1 to 45.5 +/- 14.5 (\(p < 0.001\)) were obtained. From both sets of findings we conclude that agents that disrupt early T cell signaling pathways (e.g. CsA), as opposed to late T cell signaling pathway antagonists such as RAPA, will act synergistically with Stat5a/b inhibitors\(^6\). Uncoupling the Stat5 signaling pathway may provide an alternative model for the design of immunosuppressive agents and tolerogenic strategies\(^5\).
A role for Stat5a/b as an effector pathway critical for T cell survival.

One earlier observation that was made from the above studies was that prolonged inhibition of Stat5a/b yielded cell death in primary human T cell lines. This suggested that perhaps one mechanism by which the aforementioned drugs may prolong allograft survival might occur via deletion of immunoreactive T cells. To specifically address this question and specifically delineate the role of Stat5a/b in T cell function, we sought an antisense approach to accomplish this task. Since no previous work had been published on the use or design of antisense oligonucleotides for ablating Stat5 expression, an extensive effort was made to identify an antisense oligonucleotide that could simultaneously disrupt both mRNAs thereby accounting for any compensatory action of these highly conserved transcription factors. Additionally, novel delivery conditions were developed to allow for highly efficient incorporation of oligonucleotides into lymphoid cells. We identified a unique phosphorothioate-2’-O-methoxyethyl antisense oligodeoxynucleotides (asODN) that was shared by Stat5 and Sta5b, but not other Stats. Efficient delivery was confirmed by the presence of fluorescent TAMRA-labeled ODN in > 95% of IL-2-responsive cell lines and 55% in primary T cells. Acute asODN administration reduced levels of Stat5a (90%) in 6 hours while Stat5b required nearly 48 hours to attain the same inhibition, suggesting that the apparent turnover rate for Stat5a was eight-fold higher than that for Stat5b. However, expression of the closely related Stat3 protein was unchanged at 72 hours following asODN treatment. Molecular ablation of Stat5a/b promoted apoptotic cell death in a significant population of primary PHA-activated T cells (74%) within 48 hours, as assessed by visualization of karyolytic nuclear degeneration and other generalized cytoarchitectural alterations. Additionally apoptotic T cells were quantified using enzymatic detection of TdT-positive DNA degradation, and automated cytometric detection of annexin-V translocation. However, little cell death was observed in non-primed primary human T cells.

These findings suggest that antigen-activated T cells may reach a “point of no return” threshold that is critically dependent on Stat5 propagating signals. As such, Stat5 activating cytokines such as IL-2, IL-7 and IL-9 may be required to maintain T cell survival via Stat5a/b-dependent genes. Additionally, while the mechanism by which cell death occurs is not immediately known, we propose that the cytotoxicity associated with Stat5a/b ablation may also derive, in part, from activation of caspase-8 and -9, effectors critical for apoptotic cell commitment. On the whole, we conclude from these studies that Stat5a and Stat5b may act preferentially in lymphoid cells as anti-apoptotic survival factors. In contrast to earlier models for Stat5a/b as a cell-cycle regulator, our results suggest that Stat5a/b may participate only transiently in this capacity. Future studies are planned to test the efficacy of these antisense oligonucleotides in vivo to disrupt T cell activity and determine their possible therapeutic role in ablating allograft rejection using the aforementioned heterotopic rat heart transplant models. Lastly, we seek to identify Stat5a/b-responsive target genes that are critical for regulating this biology.
Conclusions
We conclude from these studies that the Jak3/Stat5 signaling pathway is critical for maintaining T cell survival via regulation of anti-apoptotic signaling pathways. Depletion of Stat5a/b may directly promote cell death via caspase-8 activation as well as affect downstream genes yet to be identified. Antigen activated, but not unprimed, T cells appear to be most susceptible to cell death following inactivation of the Jak3/Stat5a/b signaling pathway. Within the transplant setting, inhibition of this effector pathway can prolong allograft survival. It is believed that one mechanism by which this occurs is via deletion of T cells. This model is inferred from both in vitro studies and loss of PMN infiltration into the site of the graft based on H&E staining. Additionally, inactivation of the Jak3/Stat5a/b pathway, when performed in combination with immunosuppressive agents that target the Signal-I pathway, provide a synergistic response. In contrast, inhibition of Jak3/Stat5a/b with a Signal-III inhibitor may, at best, provide an additive response. In summary, it is believed this work has provided direct molecular rationale and preclinical evidence that immunosuppressive strategies targeting the Jak3/Stat5a/b signaling cascade hold therapeutic promise for minimizing rejection of transplanted organs and when used in combination with early T cell signaling modifiers may be given at lower doses thus reducing the toxic side effects of these agents.

Publications
Donor Stem Cell Infusion to Induce Allospecific Graft Tolerance

Aims
The goal of the project was to explore, in a mouse model with heart allograft, the possibility that infusion of immunologically immature allogeneic totipotent stem cells into a graft recipient could induce tolerance to a solid organ graft from the same donor.

Specific aims were:
1. To investigate the immunogenicity and differentiation capacity of allogeneic totipotent stem cells
2. To assess the effectiveness of intravenous infusion of donor totipotent stem cells to induce long-term allogeneic heart graft survival
3. To evaluate the role of the thymus in the tolerogenic process induced by donor totipotent stem cell infusion
4. To investigate the mechanism(s) involved

To evaluate whether infusion of recipient stem cells, transfected with donor major histocompatibility complex (MHC) class I and/or class II molecules induces long-term graft acceptance.
This was designed to be a three-year project, of which one year was funded by ROTRF. In the first year, the aim was to investigate the engraftment capability and the tolerogenicity properties of mouse totipotent stem cells derived by in vitro fertilisation as reported in the results below.
Preliminary results of experiments aimed at obtaining mouse totipotent stem cells, by nuclear transfer of an adult somatic cell into an enucleated oocyte, are also presented.

Results
1. Isolation and characterisation of murine embryonic stem cells.
C57BL6/J x C3H/He mouse embryonic stem (ES) cells were isolated from the inner cell mass of the blastocyst-stage embryo obtained by in vitro fertilisation. We isolated several inner-cell masses to establish different ES cell cultures. For the in vivo experiments, we used cells derived from the inner-cell mass named 26 (ICM26) which presents all the characteristics of ES cells in undifferentiated states as will be described below.
The analysis of the trophoectoderm allowed us to demonstrate the male sex of the embryo-
derived stem cells by amplifying one Y-linked gene (Sry) and one X-linked gene (Dxn) using
PCR (Fig. 1). The H2b x H2k genotype was verified by PCR with two sets of primers: p1 and p2,
specific for mouse H2-I/A αb, amplified a DNA fragment of 789 bp; p3 and p4, specific for
mouse H2-I/Aαk, amplified a DNA fragment of 584 bp. Results confirm H2b x H2k genotype
(Fig. 2).

ES cells were grown in vitro under appropriate culture conditions taking into account the
presence in the culture medium of 1) leukaemia inhibitory factor (LIF) and 2) a mitotically
inactivated feeder layer of murine fibroblasts (STO SNL2) in order to prevent cell differentiation.
ES-cell colonies were enzymatically dissociated when they reached 200-300 μm diameter to
avoid differentiation. The typical morphological characteristic of ES-cell colonies that we
obtained is shown by phase-contrast microscopy in (Fig. 3, panel A). The undifferentiated
state of ES cells was confirmed by their positivity for alkaline phosphatase activity using
specific histochemical Fast Blue staining (Fig 3, panel B). Moreover, ES-cell colonies expressed
the Stage-Specific Embryonic Antigen 1 (SSEA-1) epitope, as shown by immunofluorescence
experiments (Fig. 3, panel C) and by FACS analysis (SSEA-1 +: range 38% to 70%; Table 1).
The murine fibroblasts STO SNL2 were negative for alkaline phosphatase as well as for the SSEA-1 antigen. Finally, we showed that ES colonies also highly expressed the POU-Domain transcription factor Octamer-4 (Oct-4) regarded as a classical marker of pluripotent stem cells. The results of the real-time PCR analysis for Oct-4 are reported in Table 1.

FACS analysis showed that ES cells were negative for MHC I and MHC II antigens (Fig. 4). We have also extended our work to obtain C57BL/6 x 129/SVJ embryonic stem cells. These cells will be available for future in vivo transplant experiments in fully mismatched combinations.

**Table 1.** The percentage of SSEA-1 positive cells was determined by FACS analysis (FACSort, Becton Dickinson) using a mAb specific for mouse SSEA-1 (from Chemicon). Oct-4 mRNA expression was evaluated by quantitative real-time PCR (TaqMan ABI Prism 5700, Applied Biosystem) using specific primers. GAPDH mRNA expression was analysed as a housekeeping to assess the overall cDNA content. The DDCt equation was used to compare the Oct-4 expression in each sample with the expression in mouse fibroblasts (STO SNL2) taken as reference unit = 1 (calibrator).
2. Engraftment of ES stem cells after infusion into a syngeneic recipient

In all the experiments reported below C57BL/6 x C3H/He (H2b x H2k) male ES cells (derived from ICM 26) were infused into the portal vein of female mice. Injection into the portal vein was performed on the basis of previous studies showing that the liver is a privileged organ for donor allogeneic stem cell engraftment and development of tolerance.

In the first series of experiments, 1x10^6 ES cells were injected into female syngeneic C57BL/6 x C3H/He mice. The animals were sacrificed at 24 hours, 4-, 7-, and 21- days after cell infusion (n = 2 each). From all animals blood, spleen, bone marrow, liver, lymph nodes, lung, thymus, heart, kidney and small bowel were collected. A fragment of each tissue was frozen at -80°C for subsequent DNA extraction and determination of donor chimerism. Another fragment was fixed in 10% buffered formaldehyde and embedded in paraffin for histologic examination.

Donor chimerism was evaluated by real-time quantitative PCR using primers and a fluorescent probe specific for Sry on the Y chromosome, as previously described. This method allows the specific determination of male DNA into female DNA up to a dilution of 1:5,000 (0.02%; a representative standard curve is shown in Fig. 5).

Up to now we have completed the determination of chimerism in all tissues from animals sacrificed at 21 days and in livers and thymi from the other animals. Results show that stem cells engrafted the recipient liver and the amount of donor DNA progressively increased with time to such a degree that, at 21 days, more than 70% of DNA was of donor origin (Table 2).
By macroscopic examination, the liver appeared abnormally enlarged with multiple ectopic lobes to give a cauliflower appearance. An image of the liver from one mice sacrificed at 21 days is shown in Figure 6. Histologic examination of liver tissue showed the presence of a teratoma that included gut epithelial (endoderm), adipose, smooth muscle, bone, cartilage (mesoderm) and neural epithelial (ectoderm) components (Fig. 7). Rare small teratoma-like structures were also found in the liver of animals sacrificed 7 days after infusion.

No detectable microchimerism was found in the thymus at any time point examined. As for the other tissues examined at day-21 post infusion, we found low but specific microchimerism in blood, lung, lymph nodes, kidney and heart (Table 2).
3. Engraftment of ES cells after infusion into an allogeneic recipient

Once documented that ES cells engraft and expand in a syngeneic environment, we then explored the ability of these cells to engraft in MHC-incompatible recipient mice. Thus, different numbers (1x10⁶, 0.5x10⁶ or 0.1x10⁶) of ES cells were injected into the portal vein of female C57BL/6 (H₂ b) mice (n = 2 each) which were sacrificed 7 days later. An additional group of 2 mice received 1x10⁶ stem cells and were sacrificed 21 days after cell infusion. From all animals blood, spleen, bone marrow, liver, lymph nodes, lung, thymus, heart, kidney and small bowel were collected for microchimerism determination and histologic examination as described above. Up to now, we have completed the determination of chimerism in all tissues from animals sacrificed at 21 days and in livers and thymi from the other animals. A low but specific microchimerism was detected in the livers of all animals sacrificed 7 days after stem cell infusion, as documented by the presence of donor male DNA (Table 3).

<table>
<thead>
<tr>
<th>Tissues/organs</th>
<th>Time after infusion (7 day)</th>
<th>Time after infusion (21 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1x10⁶ ES</td>
<td>0.5x10⁶ ES</td>
</tr>
<tr>
<td>Liver</td>
<td>0.72</td>
<td>0.08</td>
</tr>
<tr>
<td>Thymus</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Blood</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
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<tr>
<td>Bone Marrow</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Small Bowel</td>
<td>0.02</td>
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</tbody>
</table>

Table 3. Donor chimerism allogeneic combination (% donor DNA)

Figure 7. Histological sections (3 µm) are stained with hematoxylin and eosin. Panels A, B and C show livers from syngeneic animals infused with ES cells: 7 days after infusion areas of undifferentiated cells (A) and rare small teratoma-like structures (B) are present. Teratoma are visible 21 days after ES-cell infusion (C). Panels D, E and F show livers from allogeneic animals: small areas of undifferentiated cells are present (arrows) 7 and 21 days after cell infusion. Control liver was shown Panel G. Original magnification 10x.
Of note, the amount of donor DNA found in recipient liver was proportional to the number of donor stem cells infused (Table 3). At 21 days, no detectable microchimerism was found in the liver. Thus, the capability of stem cells to engraft into an allogeneic recipient was very low as compared with their engraftment in a syngeneic recipient. By macroscopic examination, the livers of animals sacrificed either at 7 days or at 21 days after cell infusion appeared normal but for the presence of several white spots on the Glisson's capsule that, on histological examination, appeared as to be composed of adipose tissue with areas of inflammation and necrosis. Histological examination also showed the presence of rare areas containing undifferentiated cells (marked with an arrow in Fig. 7) within liver parenchyma. It is possible that those areas represent engrafted donor male stem cells that were not detected by real-time PCR due to the random sampling or to the limit of detection of the assay. Experiments of real-time PCR on serial sections containing the above areas and in situ hybridisation studies are in progress to establish whether they actually derive from donor stem cells. No donor DNA was found in the thymus or in the other tissues examined by real-time PCR. Further analysis on serial sections and by in situ hybridisation will be performed to look for the presence of rare engrafted donor cells.

4. Effect of donor stem cell infusion on T cell alloreactivity

To determine whether infusion of donor allogeneic stem cells allowed the development of a state of T cell hyporesponsiveness, the proliferative response of splenocytes isolated from C57BL/6 (H2b) mice sacrificed 7 days after 1x10^6, 0.5x10^6 or 0.1x10^6 ES cell infusion, towards donor or third-party Balb (H2d) antigens was assessed. Splenocytes from C57BL/6 mice infused with 1 x 10^6 ES cells had around 35% reduction of the proliferative response toward donor antigens, as compared with splenocytes from naive mice (Fig. 8). Similar results were obtained with splenocytes taken from mice receiving 0.5 x 10^6 or 0.1 x 10^6 ES cells (Fig. 8). T cell hyporesponsiveness was not donor specific, since the proliferative response towards

Figure 8. Splenocytes were cultured at 37°C in 5% CO2 for 4 days in quadruplicate containing 4x10^6 stimulators from syngeneic (C57BL/6), donor (C57BL/6 x C3H/He) and third party (Balb/c) mice (irradiated with 40 Gy) in RPMI 1640 medium supplemented with 10% FCS. Then cells were pulsed with ^3H-thymidine and collected 18 h later. Results are expressed as mean counts per minute (cpm).
third-party Balb antigens was reduced too. To evaluate whether the state of immune hypo-
responsiveness in animals treated with ES cells was long lasting, the same experiments were
repeated in mice sacrificed 45 days after donor stem-cell infusion. Splenocytes from animals
who had received either 1 x 10^6 or 0.5 x 10^6 ES cells were hyporesponsive to donor antigens
in vitro, as documented by a lower than normal proliferative response in MLR, while the pro-
liferative response toward third-party antigens was normal (Fig. 8). On the other hand, the
proliferative response of splenocyte from mice infused with 0.1 x 10^6 ES was comparable with
that of splenocytes from naïve mice (Fig. 8).

5. Effectiveness of donor stem-cell infusion to induce long-term allograft survival
Either 1x10^6 or 0.5 x10^6 ES cells were infused into the portal vein of allogeneic C57BL/6 (H2b)
mice. Seven (n = 3) or 45 days (n = 4) after donor cell infusion animals underwent heterotopic
heart transplantation from donor C57BL/6 x C3H/He mice. No immunosuppressive drug was
given to the animals. An additional group of naïve C57BL/6 (H2b) mice (n = 5) received a
C57BL/6 x C3H/He heart as controls. In all animals, graft survival was monitored thereafter by
daily palpation of the transplanted heart. Control untreated mice rejected the heart graft within
13 days (9, 10, 13, 12, 13 days, median: 12 days, Fig. 7). The animals receiving an allogeneic
heart graft 7 days after stem-cell infusion had a slight prolongation of heart graft survival
(13, 22, >21). On the same line, 3 out of 4 mice receiving an allogeneic heart 45 days after ES-
cell infusion had a prolonged graft survival (13, 21, >100, >100) (p < 0.05 vs naïve mice) (Fig. 9).

6. Nuclear transfer: preliminary results
Three different experiments have been performed so far for the isolation of ES cells derived
from cloned blastocysts (cumulus cells were employed as nuclear donor). Table 4 shows the
rates of development of cloned blastocysts in each experiment. The amount was calculated
as the number of blastocysts over the total number of reconstructed embryos (zygotes). Each
blastocyst was placed into one well of a 4-well plate seeded with STO fibroblasts feeders and
cultured for four days in KO-DMEM (20% ES/FCS, 1% MEM/ non essential aminoacid, LIF
1000 U/ml, pen-strep 1%) to allow the attachment of trophoblasts to the feeder layer.
Table 4. Rates of development of cloned blastocysts in three experiments. The percentage was calculated as the number of blastocysts over the total number of reconstructed embryos (zygotes).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5/50</td>
<td>4/54</td>
<td>4/58</td>
<td>13/162</td>
</tr>
<tr>
<td>%</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>8</td>
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</table>

In these preliminary experiments no ES-cell lines were obtained from cloned embryos. Failure may be due to a low rate of embryo development or to a reduced capacity of trophoblasts to adhere to fibroblast feeder layer. Next experiments will be focused on finding the correct culture conditions to improve both the developmental rate of cloned embryos to the blastocyst stage and the capacity of cloned embryos to give stable ES-cell lines.

7. Conclusion and perspectives
The results obtained so far document that:
1. ES cells infused into the portal vein of a syngeneic mouse colonise the liver and form teratoma. Further studies are in progress to establish whether any of the engrafted stem cells differentiate into mature functional hepatocytes and to find out the experimental conditions to possibly prevent/limit the formation of teratoma.
2. Engraftment of ES cells into an allogeneic recipient is rather poor even though these cells are immunologically immature, since they do not express MHC I nor MHC II antigens. Further studies are in progress to establish whether ES cells, after infusion into the allogeneic host, acquired a phenotype of mature cells expressing MHC antigens and are recognised by the recipient immune system.
3. Animals receiving allogeneic stem cells had T cell hyporesponsiveness that, 45 days after cell infusion was donor specific. Consistently, the majority of these animals showed a prolonged survival of a heart graft from the same stem-cell donor strain. Altogether these results suggest that allogeneic stem cells were capable of modifying the host immune response to induce a state of hyporesponsiveness and favour graft acceptance. Evaluation of the mechanisms involved in ES-induced T cell hyporesponsiveness are undergoing. Further studies will be done to ameliorate stem cell engraftment and optimise tolerance induction, by testing different doses of ES cells and different infusion timings. The effect of combining ES-cell infusion with anti-CD3 antibodies or costimulatory blockade on the survival of a subsequent allogeneic heart graft will also be evaluated. Finally, we will test the tolerogenic properties of infusion of adult allogeneic bone marrow-derived mesenchymal stem cells.
4. Next efforts will focus on obtaining stable ES-cell lines by nuclear transfer.
Publications

Publications derived from this grant
1. Casiraghi F, Cravedi P, Remuzzi G. E se il trapianto di cellule staminali fosse utile anche per migliorare i risultati del trapianto d’organo; in “Le risorse cellulari della vita”. Eds Bugio GR, Locatelli F. *In press*
Derivation of Insulin-Producing $\beta$ Cells from Embryonic Stem Cells

A better understanding of islet ontogeny and the phenotype of putative islet stem cells would further our ability to generate a cell-replacement therapy for treating diabetes. However, it has proven difficult to isolate islet stem/progenitor cells because of their uncertain phenotype and transient existence during development. As pluripotent embryonic stem (ES) cells are derived from the blastocyst inner-cell mass, the embryonic ancestors of all adult tissues, they are a potential source for generating tissue progenitor cells \textit{ex vivo}. Differentiating in culture, ES cells follow many critical developmental stages found in normal embryos, including initial specification of lineage-restricted progenitor cells, ultimately resulting in the formation of highly specialized post-mitotic cell types. Based on these features, ES cells are recognized as a valuable tool for studying commitment and differentiation of mammalian embryonic tissues under \textit{in vitro} conditions.

The overall goal of this project is to develop an \textit{in vitro} ES-cell-based differentiation system to study pancreatic islet development. In addition, an ES-cell-based strategy could permit the generation of an unlimited supply of insulin-producing $\beta$ cells from an abundant, renewable, and readily accessible source for transplantation.

Previously, we have found that, under simple \textit{in vitro} culture conditions without the addition of exogenous growth factors, mouse ES (mES) cells are able to differentiate into cells expressing each of the 4 major islet hormones, insulin (Ins), glucagon (Glu), somatostatin (Som), and pancreatic polypeptide (PP). Islet precursor cell markers, peptide YY (YY) and islet amyloid polypeptide (IAPP) were expressed abundantly in focal clusters and many, but not all, Ins-expressing cells and Glu-expressing cells were found to co-express both early endocrine markers, indicating the presence of islet precursor cells among mature $\alpha$-cell and $\beta$-cell types. Islet-restricted transcription factor genes, including $PDX1$, \textit{neurogenin 3}, $PAX4$, $Nkx6.1$, and $Nkx2.2$, were also found to be transcribed by mES-cell progeny. More recently, we found that PDX1 protein, a transcription factor critical to pancreas development and expressed in pancreatic foregut progenitors, was produced by discrete foci of cells within differentiating ES-cell clusters. Many other features of islet development, such as 1) the acute transition from Ins/Glu double-positive early endocrine cells to single hormone-positive cells, and 2) the differentiation of ES cells into phenotypically mature cells capable of producing C-peptide I
and PDX1 nuclear protein and exhibiting electron dense secretory granules by electron microscopy, were also reproduced in this murine ES-cell culture differentiation system1.

Summary of recent results

1. Mouse ES cells differentiate into PDX1+ pancreatic precursor cells.
When we immunostained early post-embryoid body (EB) cultures of mES cells with anti-PDX1, many PDX1+ cells were seen (Fig. 1A, B, C), some of which were also PYY+. These PDX1+ cells appeared in distinct foci suggesting a local inductive microenvironment or clonal growth from a single precursor might be responsible for these clusters. The fact that we were unable to see PDX1 and Ki-67 co-staining in immunofluorescence double-staining studies suggests that these cells are not proliferating under these conditions and that a local inductive microenvironment may be present. The PDX1+ cells first appeared approximately 4 days post-plating of embryoid bodies (EBs) and their numbers increased over time throughout the culture period up to 21 days.

In mouse ES-cell cultures, PDX1 was occasionally co-expressed with Ins (probably representing more mature islet β cells; Fig. 1C, upper right hand corner) and some Som+ cells as would be expected of adult-like delta cells. However, many PDX1+ cells did not co-express Ins or Som suggesting that these might represent uncommitted pancreatic precursors (Fig. 1B, C).

Figure 1.
PDX1 expression in mES-cell differentiated cultures.
A. Time course of the number of mES cell-derived PDX1+ cells.
B. 7-day post-plating cultures showing focus of PDX1+ cells (red) surrounded by YY+ (blue) and glucagon+ (green) cells.
C. Another focus on day 14 post-plating showing a central area of PDX1+ cells and cells stained with a cocktail of antibodies to Ins, Glu, and Som (green) and antibody to PP (blue).
D. Focus of cells expressing IAPP (red, top panel) and YY (green, middle panel). Lower panel shows merged image. Many cells co-express YY and IAPP in cytoplasmic granules.
E. Ins+ cells (red panel) co-express both YY (green panel) and IAPP (blue panel), as seen in merged image (lower right panel).
F. Early cells (before day 21 post-plating) co-express insulin with glucagon in cytoplasmic granules.
2. Both D3 and R1 ES-cell lines as well as human ES-cell lines have the capacity to differentiate into islet lineages.

We differentiated D3 and R1 ES-cell lines in parallel, then immunostained for PDX1 and the islet hormone proteins. We found that both cell lines are capable of generating PDX1, Ins, Glu, and YY expressing cells (data not shown).

3. Differentiation through an embryoid-body (EB) phase is required for generation of PDX1+ cells.

To test whether the EB phase is necessary for islet lineage differentiation, we differentiated D3 ES-cells for 21 days either directly on adhesive plastic (no EB cultures) or in suspension culture for 7 days to allow formation of complex, cystic EBs followed by plating for an additional 14 days to adhesive plastic (EB cultures; baseline cultures which allowed the differentiation into PDX1+ cells previously). The rationale for this was to test whether the cell-cell interactions that occur in the EB phase which mimic early embryonic tissue structure and germ-layer development are needed for islet lineage specification as defined by differentiation into PDX1+ cells.

We found that the ES cells differentiating without formation of EBs do not generate significant numbers of PDX1+ cells demonstrating that, under these serum-supplemented conditions (without added growth factors), an EB phase is required for generation of PDX1+ pancreatic precursors.

4. Enrichment of PDX1+ cells using selective growth-factor-supplemented culture conditions.

We tested a series of growth factors and serum conditions in a first attempt to enrich cultures for pancreatic precursors since under baseline conditions the frequency of this phenotype is less than 1%.

We found that FGF10 (50 ng/ml) increases the number of PDX1+ cells by a factor of 2-3-fold. In contrast, FGF10 or BMP4 or their combination does not enrich for YY+ cells. Likewise, reducing the serum concentration of the post-plating phase from 10% to 1% had no salutary effect, nor did replacing 10% serum with a 10% knockout serum replacement (KSR) supplement.

Publications:

Cytokine-Activated Signalling in T Cells is Required for Tolerance Induction by Allochimeric Protein

The project has been focused on induction of transplantation tolerance (TT), namely permanent acceptance of allografts without the need for chronic immunosuppression. During a three-year period we expanded this project to reflect our most recent results. Now, we postulate that potent tolerogenic protocols require two sequential phases: 1) clonal deletion of alloreactive T cells; and 2) generation of potent regulatory T cells (Treg) with interleukin (IL)-4/stimulators and activators of transcription (Stat)6-driven Th2-type function.

Hypothesis 1: Effective IL-4/Stat6-driven upregulation of Th2 is required for induction of TT by allochimeric protein.

Three cell membrane signals are necessary for full T cell activation: 1) alloantigen/T cell receptor (TCR); 2) B7/CD28 and CD40/CD40L; and 3) cytokine/cytokine receptor. It is also known that Stat4 is needed for development of IL-2/IFN-γ-producing Th1, and Stat6 for IL-4/IL-10-producing Th2. To examine the role of IL-4/Stat6-driven Th2-type function in TT, we have used Stat4- and Stat6-deficient mice. Normal Balb/c (H-2d) as well as Balb/cStat4–/–, Balb/cStat6–/–, and Balb/cStat4/6–/–-deficient mice were transplanted with C57BL/6 (H-2b) heart allografts. Our results showed that all Balb/c recipients treated with anti-CD40L (MK1) mAb alone (days 0, 2, 4 and 6) accepted C57BL/6 heart allografts for more than 100 days. In contrast, only 50% of Balb/cStat4/6–/–- and none of Balb/cStat6–/–-deficient recipients treated with the same tolerogenic protocol displayed tolerance (>100 days). Furthermore, tolerance was also induced in Balb/c recipients treated with anti-CD40L mAb (day 0) combined with CTLA4-Ig (day 2) or with donor allo-antigens. Thus, tolerance induced by signal 2 blockade (inducing early apoptosis of donor-specific T cells) also requires development of IL-4/Stat6-dependent regulation to maintain tolerance.

We have also tested some allochimeric proteins in mice. The nucleotides for ten polymorphic amino acids (a.a.) in the α1 helical region of H-2Kd were introduced onto the H-2Kb gene to produce α1h62-83d-H2Kb. In a similar fashion, nine polymorphic a.a. located in the α1 helical region of H-2Dd were introduced onto the H-2Db gene to produce α1h52-83d-H2Db. The E. coli system was utilized to produce proteins. The normal C57BL/10 (H-2b*) recipients acutely rejected Balb/c (H-2d*) heart transplants (9.0 ± 1.2 days). Although a single p.v. injection of 50 µg α1h52-83dH2Dd or α1h70-83dH2Kd alone had little effect, the combination of 25 µg
α1h52–83\(^a\)-H2Db and 25 µg \(\alpha_{1h}70-83\(^a\)-H2Db\) prolonged survival to 22.4 ± 5.0 days, but without tolerance induction. Since allochimeric protein alone did not induce tolerance, we plan to use first clonal deletion (days 0–7) by MR-1 mAb, followed by development of regulation by using allochimeric protein (day 8–14).

TT was consistently induced by allochimeric proteins in rats [Wistar Furth (RT1\(^u\)) to ACI (RT-1\(^a\))]. For example, just four polymorphic a.a. in \(\alpha_{1h}62-69\(^u\)-RT1.Aa\) protein alone (produced in a baculovirus system) induced tolerance after single p.v. administration and a short treatment of recipients with cyclosporine (CsA). Oral feeding (p.o.) with other \(\alpha_{1h}70-77\)-RT1.Aa protein (Fig. 1A and B) alone also produced tolerance, blocking both acute and chronic rejection (Fig. 1C). Adoptive transfer experiments demonstrated that Treg mediates donor-specific tolerance, and RT-PCR confirmed that Treg had predominantly Th2-type function (Fig. 2A). Western blots (WB) showed a significant, but lower than rejector, expression of Jak3 (Fig. 2B), and electrophoretic mobility shift assay (EMSA) displayed a unique pattern of signal transduction, with reduced activation of Erk2, NF-κB, AP-1 and Stat5 (Fig. 2B). T cell clone (CD4+/CD25+) showed a potent proliferative response to immunogenic but significantly reduced (70%) response to tolerogenic peptide, and WB showed reduced tyrosine phosphorylation of ZAP70 following challenge with tolerogenic peptide.

**Figure 1.** Induction of tolerance by \(\alpha_{1h}70-77\)-RT1.Aa protein. **A.** Four polymorphic a.a. were introduced in recipient RT1.Aa to obtain tolerogenic proteins produced in a baculovirus expression system. **B.** Oral delivery of allochimeric protein (days 0–6) induced tolerance to heart allografts. **C.** Allochimeric protein prevented induction of chronic rejection when examined 100 days after grafting. CsA-induced tolerance did not prevent chronic rejection but addition of allochimeric protein protected from chronic rejection.

**Figure 2.** Characterization of T cell activation in tolerance. **A.** PCR was used to examine cytokine expression in tolerant heart allografts at 100 days post graft. **B.** Spleens from the same tolerant recipients were examined by WB for expression of Jak3 and phosphorylation of Erk1/2. EMSA was used to examine presence of AP1, NFκB and Stat5 in nuclear fraction (S: syngeneic; A: allograft rejection; and T: tolerant recipient). **C.** EMSA was used to measure Stat5 translocation in recipients treated with tolerogenic protocol without or with CsA, RAPA or AG490.
We also proved that tolerogenic allochimeric proteins work in a different strain combination [Brown Norway (BN; RT1\(^n\)) to PVG (RT1\(^c\))]. In particular, the \(\alpha_{1h}^{52-90^-}\text{-RT1.A}^c\) protein (Fig. 3A) produced in an \textit{E. coli} bacterial expression system injected by p.v. in combination with p.o. CsA (4 mg/kg; days 0–6) induced tolerance (> 100 days) in 4 out of 6 recipients (Fig. 3B). ELISpot assay showed a marked increase in the number of T cells producing IL-2, IFN-\(\gamma\), IL-4, and IL-10 in untreated recipients (Fig. 3C). CsA alone reduced the number of T cells producing cytokines on days 5 and 7, but not 14 post graft; \(\alpha_{1h}^{52-90^-}\text{-RT1.A}^c\) induced a marked increase IL-4-producing Th2 cells, and a reduction of IL-2-/IFN-\(\gamma\)-producing Th1 on days 5 and 7 post graft; and \(\alpha_{1h}^{n}\text{-RT1.A}^c\) protein/CsA combination consistently reduced Th1 cells, and increased Th2 cells on days 5, 7, 14, and 200 post graft. Thus, allochimeric proteins alter TCR signals, and, when followed by full signals 2 and 3, promote generation of Th2reg cells.

Newly discovered cytokine-driven repressor regulatory elements seem to be involved in differentiation of IL-12/Stat4-controlled Th1 and IL-4/Stat6-controlled Th2 cells. Jaks and Stats are regulated by a series of negative feedback loops by a family of suppressors of cytokine signaling (SOCS)1–7 and CIS1. Human PBLs were challenged with phytohemagglutinin (PHA) for 48 hours and rested without PHA for the next 48 hours. These PBLs displayed tyrosine-phosphorylated Stat5a and Stat5b when re-stimulated for 15 minutes with IL-2, IL-7, IL-9, or IL-15 (Fig. 4A); the same PBLs showed Stat5a/b activation after IL-2 stimulation at 5, 10, 30, 60, and 120 minutes (Fig. 4B). Furthermore, rested PBLs re-challenged with IL-2, IL-4, IL-7, IL-9, IL-12 or IL-15 for 0.5, 1, 2, 4, 24, and 48 hours displayed very diverse patterns and kinetics of SOCS and CIS1 mRNA levels, as shown by RNase Protection Assay (RPA); results were normalized for expression of housekeeping genes (Fig. 4C). Thus, SOCS and CIS1 are expressed differently in response to each cytokine.
We examined whether SOCS are involved in regulation of tolerance. Although tolerance (>100 days) was induced in Balb/c recipients by treatment with anti-CD40L mAb (MR1) alone (days 0, 2, 4 and 6; n=5), the same therapy failed to induce tolerance in Stat6−/− recipients (20.2 ± 7.0 days; n=5; Fig. 5A). The levels of SOCS1, SOCS2 and SOCS3 mRNA were measured by real-time PCR (Fig. 5B). Purified T cell rejectors (day 7; n=3) stimulated for 6 hrs with Th1-promoting IL-12 showed increased levels of SOCS2 (0.06 ± 0.001; SOCS/β-actin ratio × 104; p=0.001) in comparison to tolerant recipients (none; day 200; n=3). In contrast, T cells from tolerant hosts challenged with Th2-promoting IL-4 for 6 hours had 5-fold greater levels of SOCS3 (3.1± 0.02; p=0.02) when compared to T cells from rejectors (1.5 ± 0.002). There was no difference in SOCS1 mRNA levels (NS; n=3) between rejector and tolerant groups. Similar results were obtained in tolerant mice treated with MR1 (day 0) and donor cells (data not shown). We also examined tolerant recipients in which tolerance was induced by combined MR1 (day 0) and CTLA4-Ig (day 2) therapy (Fig. 5C). In this experiment, tolerant (day 100) or rejector (day 7) T cells were stimulated with irradiated (2000 rads) donor splenocytes once a week for 3 weeks in the presence of IL-4 (upper panel) or IL-12 (lower panel); some of these cultures were additionally challenged with IL-4 (or IL-12) for 2 hours; other cultures were additionally kept overnight without cytokines and then re-challenged with IL-4 (or IL-12) for 1, 2 or 24 hours. All these cultures were examined for SOCS3 mRNA by real-time PCR. Without exception, tolerant T cells displayed significantly higher SOCS3 mRNA levels in comparison to rejectors. In contrast, IL-12 did not induce SOCS3 mRNA levels. Thus, in three in vivo models, the tolerant state correlated with elevated SOCS3 expression.
To further confirm these results, we have performed in vitro experiments to examine SOCS3 mRNA expression in primary culture conditions promoting Th1 versus Th2 cells. In particular, Balb/cStat4–/– spleen cells were stimulated with ConA in cultures supplemented with IL-4 and anti-IL-12 MAb to promote generation of Th2 cells. In a similar fashion, Balb/c Stat6–/– splenocytes were cultured with ConA and IL-12 with anti-IL-4 MAb to obtain Th1 cells. Real-time PCR was used to measure SOCS3 mRNA levels (Fig. 5D). Normal T cells showed low levels of SOCS3 mRNA (0.3 ± 0.015), which in Th2-culture conditions displayed elevated SOCS3 levels at day 7 (1.5 ± 0.08), day 14 (1.4 ± 0.08) and day 35 (3.3 ± 0.6). In contrast, Th1-culture conditions showed that SOCS3 levels observed at day 7 (1.1± 0.2) decreased at days 14 (0.3 ± 0.1; \( p<0.01 \)) and especially at day 35 (0.05 ± 0.001; \( p<0.01 \); \( n=3 \)). Regular PCR methods confirmed the commitment of Th1 and Th2 cells by measuring expression of IL-4/IL-10 and IL-2/IFN-\( \gamma \) mRNA, respectively. Thus, Th2 commitment correlates with preferential expression of SOCS3 mRNA.

Figure 5. Elevated expression of SOCS3 mRNA by Th2reg cells in tolerant recipients. A. Tolerance was induced by anti-CD40L (MR1) MAb (days 0, 2, 4, and 6) in Balb/c but not in Stat6–/– recipients. B. Purified T cells from tolerant (>100 days) or rejector (day 7) recipients were stimulated in vitro with IL-4 for 6 hrs and SOCS1, SOCS2, and SOCS3 mRNA was measured by real-time PCR. C. Purified T cells from tolerant (>100 days) or rejector (day 7) recipients were cultured for 21 days, stimulated 3× with irradiated donor cells and IL-4 (upper panel) or IL-12 (lower panel); these cultures were used without any stimulation (None) or with stimulation by indicated cytokine for 2 hrs. Some cultures were additionally kept without cytokines overnight and re-challenged with indicated cytokine for 1, 4, or 24 hrs; SOCS3 mRNA was measured by real-time PCR. D. T cells from Stat4–/- mice were cultured with IL-4 and anti-IL-12 Ab, whereas T cells from Stat6–/- mice were cultured with IL-12 and anti-IL-4 Ab for 0, 7, 14, or 35 days; SOCS3 mRNA was measured by real-time PCR.
Hypothesis 2: Delivery of a full second B7/CD28 signal is needed to induce TT by allochimeric protein.

We examined whether induction of regulatory T cells requires signals 2 and 3. Injections of α1h70-77-RT1.Aa protein alone or with CsA (4 mg/kg; days 0–7) induced tolerance to WF heart allografts, documenting that inhibition of TCR signaling by CsA did not affect tolerance induction. In contrast, addition of signal 2 blockade by CTLA4-Ig (50 mg; days -1, 0, and 1), or signal 3 blockade by RAPA (4 mg/kg; days 0–6), targeting the mammalian target of rapamycin (mTOR) ANGEBEN, or AG490 (40 mg/kg; days 0–6), targeting Jak3, abolished induction of tolerance (Fig. 6). These observations were confirmed by the pattern of Stat5a/b activation: the tolerogenic protocol (protein/CsA) did not block activation of Stat5a/b that was necessary for development of Treg cells (Fig. 2C). Thus, induction of potent regulation requires signals 2 and 3 to drive Th2reg cells.

Hypothesis 3: Apoptosis of alloantigen-specific T cells (AICD) is not necessary for induction of TT by allochimeric protein.

Although the role of Stat5a/b in T cell activation is well established, little is known about the role of Stat5a/b in allograft rejection and induction of tolerance. Therefore, we examined the role of Stat5a/b in allograft rejection and in the function of T cells. C57BL/6Stat5a/b+/-, C57BL/6Stat5a/b–/–, and C57BL/6Stat5a/b+/- mice were transplanted with C3H (H-2k) heart allografts. Although both C57BL/6Stat5a/b+/- and C57BL/6Stat5a/b–/– acutely rejected heart allografts, lack of

Figure 6. Inhibition of signal 2 (CTLA4-Ig) or signal 3 (SRL or AG490) blocked induction of tolerance by allochimeric protein. Recipients were treated with tolerogenic protein alone or in combination with CsA, CTL4-Ig, SRL (RAPA) or AG490.

Figure 7. Survival of heart allografts in Stat5a/b-deficient recipients. Stat5a/b+/-, Stat5a/b+/- and Stat5a/b–/– recipients were transplanted with C3H (H-2k) heart allografts; survival was evaluated daily on a scale of 0–4 and the day without graft heart function was considered to be rejection. Lower part shows individual survival of hearts in Stat5a/b+/- recipients; all hearts lost the majority of function around 15 to 25 days; two displayed marginal function up to 60 days.
Stat5a/b tripled the survival times of grafts (Fig. 7). To understand the mechanism of delayed rejection, purified T and B cells from C57BL/6^{Stat5a/b+/+} and C57BL/6^{Stat5a/b−/−} mice were stimulated for 48 hours with ConA and LPS, respectively, showing production of different cytokines (Fig. 8). However, although activated B cells had very little apoptosis (Fig. 9A), the majority of activated T cells entered apoptosis (Fig. 9B), as shown via TUNEL assay. These observations were also supported by other experiments in which human PBLs were stimulated with PHA for 48 hours, rested overnight in medium alone and exposed to 7.5µM Stat5a/b-specific antisense oligonucleotides in the presence of IL-2. TUNEL results confirmed that Stat5a/b inhibition resulted in apoptosis within 48 hours in more than 30% of T cells (Fig. 9C). Gene array showed that pro-apoptotic genes were expressed predominantly in Stat5a/b-deficient T cells but not in normal T cells after ConA activation (Fig. 10A). In addition, Western blot documented that anti-apoptotic Bcl-2 protein expression increased in normal T cells but not in Stat5a/b-deficient T cells (Fig. 10B), and IL-4 or IL-12 increased Bcl-2 protein expression (Fig. 10C). Based on these results we suggest that Stat5a/b is absolutely necessary for generation of effector and regulatory T cells.

Based on our studies we postulate that: 1) induction of apoptosis of donor-specific T cells shortly after transplantation is beneficial for graft survival; 2) development of Th2-type regulatory cells requires both signal 2 and signal 3; 3) Stat5a/b transcription factor is absolutely required for activation of T cells; 4) lack of inhibition of Stat5a/b induces apoptosis of activated T cells; 5) IL-4/Stat6 signaling is required for development of regulatory Th2-type cells; 6) and SOCS3 regulates IL-4/Stat6-driven Th2-type regulatory cells.
Figure 10. A. Apoptosis of Stat5a/b−/− T cells correlates with increased expression of pro-apoptotic genes and reduced expression of anti-apoptotic genes. Stat5a/b+/+ or Stat5a/b−/− T cells were activated for 36 hrs and examined by microarray for mRNA of 100 apoptosis-related genes. B. Stat5a/b+/+ or Stat5a/b−/− T cells were activated for 48 hrs with ConA and examined by Western blot for expression of Bcl-2 protein. C. Stat5a/b−/− T cells were activated for 48 hrs with ConA without cytokines or with addition of IL-2, IL-4 or IL-12 and examined by Western blot for expression of Bcl-2 protein.
Publications


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Improving the Preservation of Heart Transplants: the Role of Mitochondrial Ion Channels

Abstract
The mitochondrial ATP-regulated potassium channel (mitoKATP channel) is present in the inner membrane of heart mitochondria. Like the plasma membrane KATP channel, the mitochondrial channel is inhibited by antidiabetic sulfonylureas and activated by potassium channel openers, such as diazoxide. In the present project, we have analysed the cytoprotective properties of diazoxide on the H9c2 cardiac myoblast cell line and neonatal rat ventricular cardiomyocytes. We have observed that diazoxide protects neonatal rat ventricular cardiomyocytes, but not H9c2 myoblasts, against injury induced by hydrogen peroxide or ischemia. Moreover, diazoxide prevented hydrogen-peroxide-induced mitochondrial potential depolarization in neonatal rat ventricular cardiomyocytes. Diazoxide, at the same time, did not affect the anti-apoptotic protein bcl-2 expression level in these cells. The protective effects of diazoxide were suppressed by 5-hydroxydecanoic acid, a potassium channel blocker. These observations suggest that activation of the mitochondrial ATP-regulated potassium channel plays an important role in protection of neonatal cardiomyocytes against injury. Additionally, single channel properties of the mitoKATP channel were studied in a planar lipid bilayer. It was shown that this channel is activated by diazoxide and BMS-191095 and inhibited by Mg2+. Single-channel studies also indicated that the mitoKATP channel possess multiple nucleotide-binding sites.

Introduction
A mitochondrial ATP-regulated potassium channel (mitoKATP channel) was identified by a patch-clamp technique in the inner membrane of liver mitochondria1. Later, a similar channel was described in heart2, brain3, 4 and skeletal muscle mitochondria5. Like the plasma membrane ATP-regulated potassium channel (KATP channel), the mitochondrial channel is inhibited by antidiabetic sulfonylureas such as glibenclamide and activated by potassium channel openers (KCOs)6. Diazoxide seems to be an especially potent activator of the mitoKATP channel7. The primary function of this channel is to allow K+ transport into the mitochondrial matrix. This phenomenon could be involved in maintaining mitochondrial volume homeostasis. The molecular identity of the mitoKATP channel is unknown. Probably, it is composed of two types of subunits; a pore-forming subunit (Kir family) and a sulfonylurea
receptor. Several observations based on antibodies suggest that the mitoK$\text{ATP}$ channel belongs to the inward rectifier K$^+$ channel family–Kir6.x$^9$. Similarly, the molecular properties of the mitochondrial sulfonylureas receptor are not clear. The use of [125I]-glibenclamide lead to the labeling of a 28 kDa protein in heart mitochondria$^{10,11}$. Recently, with the use of the fluorescent probe BODIPY-glibenclamide, a 64-kDa protein was labeled in brain mitochondria$^4$.

The cardiac mitoK$\text{ATP}$ channel plays an important role in protecting cardiomyocytes during ischemia/reperfusion$^{12-14}$. Activation of the channel by diazoxide seems to mimic ischemic preconditioning in heart muscle$^{15,16}$. The cardioprotective action of mitochondrial KCOs probably involves multiple mechanisms, including an effect on ischemia-induced cellular apoptosis. In the present work, we have analyzed the effects of KCO diazoxide on H9c2 cardiac myoblast cell line and neonatal rat cardiomyocytes. We have shown that diazoxide caused protection of cardiac ventricular myocytes (but not H9c2 cells) against ischemia/reperfusion injury. These effects have been antagonized by the mitoK$\text{ATP}$ channel inhibitor 5-hydroxydecanoic acid (5HD). Further, we have investigated the effect of oxidative stress induced by hydrogen peroxide on the mitochondrial membrane potential in cardiac ventricular myocytes. We have observed that diazoxide protects cardiac mitochondria in ventricular myocytes against depolarization induced by oxidative stress. Diazoxide-induced protection is not accompanied by changes in the expression level of anti-apoptotic protein bcl-2 in ventricular myocytes. Moreover, studies on single channel properties of the mitoK$\text{ATP}$ channel were performed.

**Results**

In the search for a cellular model to study the consequences of mitoK$\text{ATP}$ channel activation, we studied cardiac myoblast cell line H9c2 and neonatal rat ventricular cardiomyocytes. Recently, we have shown that, in skeletal muscle cell line L6, potassium channel openers such as diazoxide and nicorandil caused a stimulation of cellular oxygen consumption, implying mitochondrial effects of these drugs$^3$. Hence, in our studies we applied cardiac myoblast cell line H9c2 to establish whether the potassium channel opener diazoxide is able to protect these cells against ischemia/reperfusion injury. Incubation of H9c2 cells under an atmosphere of 95% Argon and 5% CO$2$ for 10 hours followed by 2 hours of reoxygenation increased cell injury (Fig. 1A), measured as lactate dehydrogenase (LDH) release, from 14 ±1% in untreated cells to 51 ±4% after ischemia/reperfusion. Application of 100 mmol/l diazoxide did not influence cell injury in either control cells (14 ±1%), or in cells that underwent the ischemia/reperfusion protocol (48 ± 6%). Values obtained for cells additionally treated with 100 mmol/l 5-HD were also not significantly different from untreated controls, (12±1% and 51 ±2%, for control and ischemia/reperfusion cells, respectively; Fig. 1A).
We also show that ischemia, ischemia + 100 mmol/l diazoxide, ischemia + 100 mmol/l 5-HD treatment does not influence the mRNA expression level of apoptosis related proteins in H9c2 cells (Fig. 1B). Cells were subjected to 3 hours ischemia followed by 2 hours reperfusion and the total RNA was isolated. Using multiplex RT-PCR, we studied the mRNA levels of bax, bcl-xL, caspase-3 and bcl-2. A fragment of constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was additionally amplified and used as an internal control (Fig. 1B). Diazoxide had no effect on protein kinase C isoform distribution in H9c2 cells (data not shown). A similar ischemia/reperfusion protocol was applied to neonatal rat ventricular cardiomyocytes.

We also show that ischemia, ischemia + 100 mmol/l diazoxide, ischemia + 100 mmol/l 5-HD treatment does not influence the mRNA expression level of apoptosis related proteins in H9c2 cells (Fig. 1B). Cells were subjected to 3 hours ischemia followed by 2 hours reperfusion and the total RNA was isolated. Using multiplex RT-PCR, we studied the mRNA levels of bax, bcl-xL, caspase-3 and bcl-2. A fragment of constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was additionally amplified and used as an internal control. Diazoxide had no effect on protein kinase C isoform distribution in H9c2 cells (data not shown). A similar ischemia/reperfusion protocol was applied to neonatal rat ventricular cardiomyocytes.

**Figure 1.** Effect of ischemia/reperfusion on LDH release and mRNA level in H9c2 cells.

**A.** Cell injury was estimated using LDH measurements. LDH activity was measured in the medium and cell lysates. Results are expressed as a percentage of total releasable LDH. Results are shown for: control – untreated cells, diaz – cells treated with 100 mmol/l diazoxide, diaz/5-HD – cells treated with 100 mmol/l diazoxide and 100 mmol/l 5-HD, –O2 – indicates cell subjected to 10 hours ischemia and 2 hours reperfusion. Experiments were performed in triplicate and the results are shown as means ±SEM.

**B.** mRNA expression of apoptosis-related proteins. Levels of bax, bcl-xL, caspase 3 and bcl-2 mRNA were assessed. GAPDH gene fragment was used as an internal control. Fragments of the studied genes, amplified by RT-PCR, were resolved in 2% agarose gels and visualised by ethidium bromide staining. Control – untreated cells, IS – cells subjected to 3 hours ischemia and 2 hours reperfusion, IS/diaz – cells treated with 100 mmol/l diazoxide prior ischemia, IS/diaz, 5-HD – cells treated with 100 mmol/l diazoxide and 100 mmol/l 5-HD prior to ischemia.

**Figure 2.** Effect of ischemia on rat neonatal cardiomyocytes.

Cell injury was estimated using LDH measurements. LDH activity was measured in the medium and cell lysates. Results are expressed as a percentage of total releasable LDH. IS - the effect of 120 min simulated ischemia and 24 hours reperfusion on cell injury, IS/diaz – cells were incubated with 100 mM diazoxide for 30 min prior to ischemia, IS/diaz, 5-HD – cells incubated with 100 mM diazoxide and 500 mM 5-HD for 30 min prior to ischemia. *** p < 0.0001 vs. control, †† p < 0.01 vs. IS, control ñ untreated cells, diaz ñ cells treated with 100 mmol/l diazoxide, diaz/HD – cells treated with 100 mmol/l diazoxide and 500 mmol/l 5-HD. Experiments were performed in triplicate and the results are shown as means ±SEM.
Figure 2 shows the cell injury after 2 hours simulated ischemia followed by 24 hours reoxygenation. These experiments were also performed in the presence of 100 mmol/l diazoxide and 500 mmol/l 5-HD. Cell injury was measured by estimating the level of LDH released from the cells. We show that ischemia increases the amount of damaged cells from 33 ±2% in control to 56 ±2% in cells incubated with ischemic buffer. The injury of cells exposed to 100 mmol/l diazoxide for 15 min before, then during ischemia significantly decreases the level to 43 ±2%. When 500 mmol/l 5-HD is applied together with diazoxide this partly abolishes diazoxide protection with cell injury at 50 ±3% (Fig. 2). We have also studied neonatal rat cardiomyocyte injury after exposure to oxidative stress induced by 16 hours incubation with 100 mmol/l hydrogen peroxide, known to be a strong oxidant and agent leading to hydroxyl-radical production. We show that diazoxide protects cardiomyocytes against oxidative stress in a dose-dependent manner (Fig. 3A). Cell injury in control cells, as measured by LDH release, was 33 ±3%; 100 mmol/l H$_2$O$_2$ increased this value to 70.7±0.3%. In the presence of 100 mmol/l diazoxide, LDH is released from 56 ±1% cells. Incubation with 50 mmol/l and 10 mmol/l diazoxide gave the values of 62 ±1% and 65 ±1%, respectively, which were still significantly different from the value obtained for cells treated with only 100 mmol/l H$_2$O$_2$.

Figure 3A. Cell injury measurements. Cardiac myocytes were incubated with 100 mM H$_2$O$_2$ and diazoxide (as indicated) for 16 hours. LDH activity was measured in the medium and cell lysates. Results are expressed as a percentage of total releasable LDH. †††p < 0.0001 vs. control, ***p < 0.0001 vs. H$_2$O$_2$. Experiments were performed in triplicate and the results are shown as means ±SEM.

Figure 3B shows the results of mitochondrial membrane potential measurements in neonatal rat cardiomyocytes after exposure to oxidative stress. Cells were incubated with 200 mmol/l H$_2$O$_2$ for 2 hours, loaded with the fluorescent mitochondrial potential indicator JC-1 and analyzed using flow cytometry. We show that the amount of cells with a high mitochondrial
potential (upper left corners of panels, marked with *) in control cells is 14.3%. In cells treated with H$_2$O$_2$, this value is reduced to 7.1%; 100 mmol/l diazoxide reverses the effect of oxidative stress and again 14.7% of cells have high mitochondrial membrane potential. As expected, only 2.6% of cells incubated for 10 min with uncoupler, 1 mmol/l CCCP, had a high mitochondrial membrane potential. The results shown are representative for three independent experiments (Fig. 3). We have shown that diazoxide protects neonatal rat cardiomyocytes against injury caused by simulated ischemia and oxidative stress. From previous reports, it is known that this protective action might be connected with the inhibition of apoptosis, so we decided to study the influence of diazoxide on bcl-2 protein expression. Using Western blotting, we show (Fig. 4A) that incubation of neonatal rat cardiomyocytes with 100 mmol/l diazoxide for 2, 3, 6, 8, 10, 16, 24 and 40 hours does not significantly change the level of bcl-2 protein present in the cells with respect to controls. Densitometric analysis of the result, normalized to the β-actin signal, is shown in Figure 4B.

![Figure 4. Diazoxide effect on bcl-2 protein expression in neonatal rat cardiomyocytes.](image)

**A.** Cardiomyocytes were incubated with 100 mmol/l diazoxide for indicated times. Cell lysates were collected and analyzed by Western blot using antibodies against bcl-2 protein and β-actin (to ensure equal loading). The result shown in the panel is representative of three independent experiments.

**B.** Bcl-2 protein signal changes after cardiomyocyte treatment with 100 mmol/l diazoxide. Densitometric analysis of bcl-2 protein signal (normalised to β-actin signal) from Western blots shown in panel A. Untreated cells – white bars, diazoxide treated cells – striped bars.

Additionally, single-channel properties of the mitoK$_{ATP}$ channel were studied in a lipid bilayer. A purified inner-mitochondrial membrane was reconstituted into planar lipid bilayer (Fig. 5). It was shown that this channel is activated by diazoxide and BMS-191095 and inhibited by Mg$^{2+}$. Single-channel studies also indicated that the mitoK$_{ATP}$ channel possess multiple nucleotide-binding sites (data not shown).
It is well established that activation of the mitoK\textsubscript{ATP} channel in cardiac myocytes plays an important role in protection against cell injury\textsuperscript{14,16}. It is also believed that the mitoK\textsubscript{ATP} channel is a key element in ischemic preconditioning\textsuperscript{13}. Since discovery of the role of the mitochondrial channel in cardioprotection\textsuperscript{15,17}, its pharmacological properties have been clarified. Many questions, however, remain about the biochemical mechanisms leading from channel activation to cytoprotection. One of the possibilities is the mitoK\textsubscript{ATP} involvement in regulating ischemia-induced cellular apoptosis. Recently, it has been reported that apoptosis may significantly contribute to irreversible myocyte injury during ischemia and reperfusion\textsuperscript{18,19}. The appearance of apoptotic cells in the peri-necrotic zone during reperfusion suggests that apoptosis may be important in the process of extending infraction over time, after the onset of reperfusion\textsuperscript{20}.

In this study, we have used cardiac myoblast cell line H9c2 and primary culture of rat ventricular cardiomyocytes. Recently, we have shown that the activation of potassium transport upon K\textsubscript{COs} application, in rat skeletal muscle myoblast cell line L6, caused a stimulation of cellular oxygen consumption, implying a mitochondrial target for K\textsubscript{COs}\textsuperscript{5}.

Moreover, the effect induced by diazoxide and nicorandil was blocked by a K\textsubscript{ATP} channel blocker-glibenclamide\textsuperscript{5}. Hence, we have explored the protective properties of diazoxide in myoblast cell line H9c2 upon ischemia/reperfusion. Myogenic cell line H9c2 was previously applied to study cell-death mechanisms upon oxidative stress\textsuperscript{20}, hypoxia\textsuperscript{22} and overexpression

**Discussion**

Figure 5. Effect of Mg\textsuperscript{2+} on activity of the mitoK\textsubscript{ATP} channel.  
**A.** Single-channel recording in gradient 50/150 mM KCl (cis/trans) solution at -30 mV under control condition and after addition 10 mM, 1 mM and 10 mM MgCl\textsubscript{2} - indicates the closed state. Recordings were low pass filtered at 500 Hz.  
**B.** The dose-response curve for Mg\textsuperscript{2+} inhibition of K\textsuperscript{+} current amplitude after addition 1 mM, 3 mM, 10 mM, 30 mM, 100 mM, 300 mM, 1 mM, 3 mM, 10 mM MgCl\textsubscript{2}. GAPDH bcl-xL caspase 3 bax bcl-2 control IS IS/diaz IS/diaz, 5-HD B 10 30 50, control diaz diaz/ 5-HD Cell injury (% of LDH release) control diaz diaz/ 5-HD-O2 A.
of calreticulin. In our studies, no protective effect of diazoxide on cardiac myoblast cells H9c2 was observed. Moreover, diazoxide was not able to modulate the mRNA level of bax, bcl-xL, caspase-3 and bcl-2 proteins. The above observations suggest that the mitoKATP channel is not present in the mitochondria of H9c2 cells. However, we have shown that the mitochondrial potassium channel opener diazoxide was able to protect neonatal rat ventricular cardiomyocytes against ischemic injury and, moreover, protects cardiac neonatal cells against oxidative injury, as observed previously. At the same time, the protective action of diazoxide cannot be explained by its putative antioxidant properties; its action is not mimicked by other antioxidants. Recently, similar results were obtained with another potassium channel opener-nicorandil. Protection after ischemic insult was suppressed by the potassium channel blocker 5-HD, confirming that the activation of mitoKATP is a key event upon diazoxide application. The only partial effect of 5-HD is probably due to low penetration through cell membranes, which diminishes the effective concentration of this drug in the cytosol. Reperfusion of ischemic myocardium results in apoptotic cell death, which can be blocked by adapting the heart to ischemic stress induced by cyclic episodes of brief ischemia and reperfusion-ischemic preconditioning. It has been shown that ischemic preconditioning reduces apoptosis in isolated rat heart by up-regulating bcl-2 protein and mRNA. Studies using in vivo viral gene transfer of Bcl-2 and transgenic mice also show that this protein is efficient in blocking apoptosis and is able to preserve ventricular geometry and function after ischemia/reperfusion. The use of antisense bcl-2 oligodeoxynucleotides reversed the protective effect of ischemic preconditioning by eliminating the anti-death signal from bcl-2. Our experiments with neonatal cardiac myocytes, however, exclude that changes of bcl-2 protein expression are involved in diazoxide-induced cytoprotection.

In summary, the findings presented in this study provide functional data showing that diazoxide (and probably BMS-191095) is able to protect neonatal cardiac myocytes against ischemic and oxidative injury and suggest that activation of the mitochondrial ATP-regulated potassium channel plays an important role in protection of these cells against injury. Single-channel studies indicate that magnesium ions are potent blockers of the mitoKATP channel and probably are also able to lower the protective effects of channel activation. Moreover, the mitoKATP channel possesses multiple nucleotide-binding sites.
Publications


**Publications derived from this grant**


AIM 1: To induce mixed chimerism and tolerance through the transplantation of allogeneic mobilized peripheral blood stem cells (mPBSC) with costimulation blockade.

Mixed chimerism and tolerance can be induced without cytoreductive conditioning through transplantation of approximately 200x10^6 bone marrow cells (BMC) under costimulation blockade. Since such high numbers of BMC are clinically not feasible, we evaluated the use of mPBSC, which is the source providing the highest numbers of hematopoietic stem cells (HSC) from a living donor. Since little is known about the properties of mPBSC in non-myeloablative protocols, we first investigated the engraftment potential of murine mPBSC in a CD45-congenic model. The transplantation of moderate doses of mPBSC (20x10^6/mouse) after non-myeloablative total body irradiation (TBI) induced high levels of chimerism. Even though chimerism levels were lower than after the transplantation of the same number of BMC, the persistence of stable multi-lineage chimerism (>20 weeks) suggests that sufficient numbers of HSC are contained in 20x10^6 mPBSC to allow lasting reconstitution.

We next transplanted increasing numbers of allogeneic mPBSC under standard costimulation blockade (1 mg anti-CD154 (CD40L) mAb plus 0.5 mg CTLA4Ig). Unexpectedly, long-term chimerism was not observed in any group (Table 1).

<table>
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<th>HSC transplantation (d 0)</th>
<th>TBI in Gy (d -1)</th>
<th>Rate of chimeras</th>
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<tr>
<td>20x10^6 BMC + c.b.</td>
<td>2 or 3</td>
<td>16/16</td>
</tr>
<tr>
<td>20x10^6 mPBSC + c.b.</td>
<td>2 or 3</td>
<td>0/12</td>
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<tr>
<td>75x10^6 mPBSC + c.b.</td>
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<tr>
<td>200x10^6 mPBSC + c.b.</td>
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<td>0/3</td>
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<td>3</td>
<td>0/9</td>
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Table 1. Transplantation of various numbers of BMC or mPBSC under standard doses of costimulation blockade (c.b.: 1 mg anti-CD154 mAb, 0.5 mg CTLA4Ig) after the indicated doses of TBI. The rate of successful long-term chimeras achieved with each protocol is shown.
Not even the transplantation of $200 \times 10^6$ Balb/c mPBSC into B6 recipients irradiated with 3 Gy TBI yielded lasting chimerism under standard costimulation blockade. As early as 1 week post-transplant, chimerism was undetectable in most of the mPBSC recipients. Preserved donor reactivity was observed in mPBSC recipients in mixed lymphocyte reaction assays, while control chimeras induced with BMC typically demonstrated hyporesponsiveness towards donor-type stimulators. Notably, following the transplantation of $20 \times 10^6$ BMC together with $60 \times 10^6$ mPBSC, no chimerism was detected even 1-week post-transplant, while all mice transplanted with $20 \times 10^6$ BMC alone became chimeric (Fig. 1). Thus, allogeneic mPBSC are rejected under standard doses of costimulation blockers.

We next used an intensified conditioning regimen designed to overcome the increased alloresistance posed by mPBSC. B6 recipients received a donor-specific transfusion, increased doses of costimulation blockers, transient immunosuppression, 3 Gy TBI and $200 \times 10^6$ Balb/c mPBSC. With this protocol high-levels of lasting mixed chimerism were observed (Fig. 2). Donor skin was accepted by most recipients while third-party grafts were promptly rejected. The dose $200 \times 10^6$ mPBSC is considerably above a clinically relevant amount of mPBSC (the equivalent of roughly $75 \times 10^6$/mouse). Non-myeloablative mPBSC protocols with feasible cell numbers thus still need to be developed.

**Figure 1.** B cell and myeloid chimerism in representative mice 1 week after transplantation of BMC alone (right panels) and BMC plus mPBSC (left panels).

**Figure 2.** Multilineage chimerism after transplantation of $200 \times 10^6$ Balb/c mPBSC into B6 recipients under a conditioning regimen employing donor-specific transfusion (d-6, $60 \times 10^6$ Balb/c splenocytes plus anti-CD154 d-6), 3 Gy TBI (d-1), transient immunosuppression (rapamycin, mycophenolate mofetil, methylprednisolon; d0 to d27) and increased doses of anti-CD154 and CTLA4Ig post-transplant. Rates of chimeras are shown at 6 and 12 weeks post-transplant.
Doses of costimulation blockers and TBI allowing the establishment of chimerism and tolerance with allogeneic BMC are not sufficient to prevent the rejection of mPBSC. mPBSC appear to contain a deleterious APC as they trigger rejection of BMC when transplanted in combination. The lower tolerogenic potential of mPBSC in recipients of costimulation blockade warrants consideration in the development of (pre-)clinical tolerance protocols employing mPBSC.

**AIM 2: To increase the reliability of HCT with costimulation blockade through the transient use of immunosuppressive drugs.**

Using a model employing a standard dose of BMC, costimulation blockade and non-myeloablative TBI, we found that transient treatment post-BMT with cyclosporine or tacrolimus inhibited the induction of long-term chimerism, while the administration of rapamycin, mycophenolate mofetil, steroids or FTY720 did not impair chimerism. Cyclosporine and tacrolimus also significantly inhibited tolerance induction, while rapamycin promoted it (compared to the standard protocol without immunosuppressive drugs). We also evaluated the addition of the combination of rapamycin, mycophenolate and steroids to the BMT model, with the rationale that this immunosuppressive regimen has been used successfully after renal transplantation. Treatment with this immunosuppressive drug cocktail allowed a reduction in the dose of TBI to 1 Gy, which otherwise does not reliably permit allogeneic-mixed chimerism. It is thus not only possible to use a clinically relevant immunosuppressive regimen in combination with a tolerance protocol using BMT with costimulation blockade, but certain immunosuppressive drugs can even help to reduce the required host conditioning.

Previous studies revealed that mixed chimerism and tolerance can be induced in the majority of recipients (approximately 60%) without irradiation if very high numbers of allogeneic BMC (approximately 200x10⁶ BMC/mouse) are transplanted under costimulation blockade. We now evaluated whether short-course immunosuppression improves the reliability of this strategy and whether it allows chimerism and tolerance to be achieved with lower numbers of BMC. B6 mice received 200, 100, or 50x10⁶ BMC from Balb/c donors plus an anti-CD154 mAb and CTLA4Ig with or without a four-week course of immunosuppression (IS; rapamycin, methylprednisolone and mycophenolate mofetil). From the groups treated with IS, 9 of 10 mice receiving 200x10⁶, all mice (n = 10) receiving 100x10⁶ and 4 of 5 mice treated with only 50x10⁶ BMC developed long-term chimerism (24 weeks). From the groups without IS, in contrast, 1 of 4 (p < 0.05) mice which received 200x10⁶, 6 of 9 (p < 0.05) transplanted with 100x10⁶ and 1 of 5 receiving 50x10⁶ BMC developed chimerism (Fig. 3). Donor skin graft survival correlated with chimerism as 9 of 10 mice transplanted with 200x10⁶ and all mice transplanted with 100x10⁶ BMC treated with IS accepted donor grafts (>170 days), while all mice (n = 5, p < 0.05) from the 200x10⁶ BMC groups and 3 of 9 (p < 0.05) from the 100x10⁶ BMC groups without IS rejected donor skin. Furthermore, 3 of 5 mice treated with 50x10⁶ BMC plus IS, but only 1 of 5 mice from the untreated group accepted donor grafts (>170 days).
These data suggest that a one-month course of rapamycin, mycophenolate and steroids improves the reliability of high-dose BMT protocols. Besides, transient immunosuppression allows a reduction in the cell dose required for the induction of mixed chimerism without cytoreduction. Models inducing durable mixed chimerism without cytoreduction have used BMC doses that cannot be obtained clinically. Modifications of such protocols allowing success with lower numbers of BMC are thus a step towards the clinical applicability of this approach.

While the dose of donor BMC necessary to induce lasting mixed chimerism under non-cytoreductive conditions can be lowered by immunosuppressive treatment, we did not observe mixed chimerism with a clinically more feasible dose of 20 million BMC, even when they were transplanted under an intensified conditioning regimen including increased doses of costimulation blockade, immunosuppression and donor-specific transfusion. Therefore, models achieving chimerism and tolerance without cytoreduction using practicable BMC numbers still need to be developed.

AIM 3: To increase the reliability of HCT with costimulation blockade by improving peripheral deletion of donor-reactive cells through the additional infusion of selected donor populations. First we aimed to identify the donor population mediating the peripheral deletion of donor-reactive T cells after BMT with costimulation blockade. Towards this goal, we followed the amount of relevant superantigen-reactive CD4⁺ PBL (Vβ5⁺ or Vβ11⁺) after treatment with anti-CD154 mAb and transplantation of separated allogeneic cell populations (Balb/c to B6). In the experiments performed, we did not observe deletion after the transplantation of either selected B cells or dendritic cells. Since this approach has not identified the sought population, additional studies are planned in which the candidate population is eliminated from the transplanted inoculum to investigate whether deletion is inhibited in this way.
Publications
1. Koporc, Sykes, Wekerle et al. (in preparation)
3. Blaha, Sykes, Wekerle et al. (in preparation)
Use of a Death Decoy Protein DcR3/TR6 to Treat Organ Graft Rejection

With the one-year financial support from ROTRF during the period from Nov. 2002 to Nov. 2003, our laboratory has investigated several aspects of immune responses related to graft rejection, and has published 5 peer-reviewed articles during this period in high impact journals with Dr. Jiangping Wu as the senior author. ROTRF has been duly acknowledged in these articles. The results from our laboratory are summarized as follows.

We have discovered that a death decoy receptor DcR3/TR6, a tumor-necrosis factor (TNF) receptor family member, can interfere with T cell aggregation during the T cell activation process, and this function coincides with increased secretion of DcR3/TR6 by activated T cells. The possible physiological role of TR6 in this regard might be to disperse T cells to their destiny once the necessary cellular interaction for activation is over.

We have also found that DcR3/TR6 can inhibit in vitro and in vivo T cell chemotaxis toward chemokines such as SDF-1α and 6-Ckine. In consistence with this finding, tumors secreting DcR3/TR6 have reduced lymphocyte infiltration. This implies that administration of DcR3/TR6 locally in grafts can prevent T cells infiltration to the grafts, hence reducing the rejection force. Using a diabetic mouse model, we have reported that the administration of DcR3/TR6 in vivo can protect transplanted islets from primary non-function, which is defined as loss of islet function due to non-immune factors. Mechanistically, such protection is due to reduced Fas-mediated apoptosis of islets after their isolation.

With ROTRF support and co-funding from other agencies, we have found that ephrinB2 and ephrinB3, both of which are ligands to members of the largest family receptor kinases, Eph kinases, can costimulate T cells. The function of Eph kinases in the immune system was previously unknown. This novel finding has opened a new field in T cell biology, and implies that interfering with such costimulation might lead to aborted T cells to respond to antigens; in relation to organ transplantation, this could be a new approach to control rejection and to induce tolerance.

With the intention to express soluble DcR3/TR6 in grafts to ameliorate graft rejection, transgenic mice were generated with CMV-promoter-driven DcR3/TR6 expression. The mice
indeed had high serum DcR3/TR6 levels, but unfortunately, their DcR3/6 expression extinguished around age of 6-8 weeks. As an alternative, transgenic mice were then generated with actin-promoter-driven DcR3/TR6 expression. These mice were fertile, had no gross anomaly, and had persistent high serum DcR3/TR6 levels. We are currently in the process of testing whether local DcR3/TR6 expression in grafts from these transgenic mice can indeed ameliorate acute and chronic rejection responses.

Publications
6. How to apply for an ROTRF grant

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- Originality
- Scientific excellence

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