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

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

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

7. Board of Trustees (BT)

8. Scientific Advisory Committee (SAC)
We are pleased to announce that the second five years of activity of the Roche Organ Transplantation Research Foundation (ROTRF) have started with the beginning of funding Cycle XI (submission deadline for the Letters of Intent was 1 October 2003).

The funding provided by the ROTRF 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.

In light of the success obtained until now, the ROTRF has decided to further promote clinical research in transplantation and is proud to announce the launch of a new initiative to fund more clinical research projects. This particular initiative aims at supporting research projects proposing work with human clinical material, organ transplant patients, human organ preservation, human transplant pathology and other human transplant problems, and which promise to have a significant impact in the clinical setting in the near future. Therefore, as of 1 October 2004, the ROTRF will award two types of grants, conventional research grants and clinical research grants.

In September 2004, the ROTRF will organise a series of State-of-the-Art Lectures during the International Congress of the Transplantation Society in Vienna. Five ROTRF grantees will be given the opportunity to present the results achieved during their ROTRF-funded research. The lectures will be chaired by Prof. Megan Sykes and Prof. Jean-Paul Souillou, two members of the ROTRF Board of Trustees.

The ROTRF activities have benefited from the generous support of numerous people. We would like to thank F. Hoffmann-La Roche Ltd for its support to the Foundation. We would also like to thank all scientists and clinicians involved: the Board of Trustees; the Scientific Advisory Committee; and, most of all, the investigators.

On the behalf of the Board of Trustees

Philip F. Halloran, MD, PhD
2. Facts and Figures

Funding Cycle XI – Letter of Intent Submission in October 2003

The Roche Organ Transplantation Research Foundation (ROTRF) is pleased to announce that in the first cycle of the second five-year period, 2.0 million Swiss Francs (CHF) have been distributed to twelve applicants who were awarded an 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 XI) the ROTRF received 75 Letters of Intent from scientists around the world. More than half of the applications (53%) came from European scientists, in particular from the UK (20%), France (12%), Germany (5%) and the Netherlands (4%). 36% of the Letters of Intent were submitted by scientists in the USA, 5% by Canadian, and the remainder by Australian and South American 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 twelve full or partial grants of up to 300’000 CHF to five applicants from the USA, two from the UK and one each from Australia, France, Germany, Italy, and the Netherlands.

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.
Statistics on Applications to the ROTRF

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

Distribution of the ROTRF applications worldwide

North America: <47% of applications

South America: <1% of applications

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

Africa: >1% of applications
Objective
Our goal is to generate alloreactive immunosuppressive CD4+ T cells by transducing the Foxp3 gene into conventional CD4+CD25- T cells (Tsup). We will then test the capacity of Tsup to control allograft rejection and graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell transplantation (HSCT).

Rational
In the field of organ transplantation, it has been demonstrated that CD4+CD25+ regulatory T cells (Treg) can be used to induce specific tolerance to solid organ transplants. However, this immunosuppressive effect was obtained using Treg purified from mice that had previously received an in vivo treatment for tolerance induction, a strategy hardly applicable to humans due to its toxicity. In the field of allogeneic HSCT, we have demonstrated that high numbers of Treg can inhibit the life-threatening GVHD in mice. Although effective in terms of immune reconstitution and antileukemic effect, this approach requires several weeks of culture to generate sufficient numbers of Treg. Such a procedure would therefore be difficult to transpose into a clinical setting because of the high cost of long-term culture and because of the risk that expanded T cells would show reduced survival after injection. Thus, cell therapy using Treg is a very promising strategy for transplantation but still requires further research in order to fulfill clinical criteria.
**Hypothesis**
The ectopic expression of Foxp3 is sufficient to confer suppressor function on peripheral transduced CD4⁺CD25⁻ T cells. Upon stimulation of conventional CD4⁺CD25⁻ T cells with allo- geneic antigen presenting cells (APC), only the CD4⁺ T cells that are capable of recognizing alloantigens will proliferate. After several days of culture and a phase of *in vitro* selection for alloantigen-specific activated CD4⁺ T cells, Foxp3-containing lentiviral vectors added to the culture will specifically transduce alloreactive CD4⁺ T cells. These transduced cells will consequently acquire immunosuppressive properties and may have the potential to induce specific tolerance to alloantigens.

**Specific Aims**
1. To produce lentiviral particles expressing Foxp3 and Thy-1, a non-immunogeneic membrane marker useful for sorting transduced cells
2. To develop a culture procedure allowing efficient lentiviral transduction of conventional CD4⁺ T cells and, consequently, generate Tsup that are specific for particular alloantigens
3. To test these Tsup for their ability to specifically control alloreactive responses in different clinical contexts such as skin and islet transplantations as well as GVHD

**Expected Results**
We expect that CD4⁺ cells collected after transduction of the Foxp3 gene will acquire immunosuppressive properties that are specifically directed toward selected particular alloantigens. Since the role of Foxp3 in Treg has been described in mice and in humans, we expect that our pre-clinical work will define technological procedures that will be transferable to humans.
Molecular Virology of Post-Transplant Malignancy: A Novel Large Animal Model

Objective
To determine the molecular virologic basis of post-transplant lymphoproliferative disease (PTLD) as a basis for new therapies.

Background
PTLD is a common complication of solid organ transplantation. Risk of PTLD is related to intensity of immune suppression, particularly T cell depletion, and Epstein-Barr Virus (EBV) infection. Increased EBV viral loads and expression of latent cycle antigens precedes PTLD. This risk is increased by cytomegalovirus (CMV) co-infection. Optimal therapies for PTLD remain undefined.

Preliminary Data
PTLD is a common complication in our miniature swine undergoing non-myeloablative conditioning (thymic irradiation, in vivo T cell depletion, cyclosporine) for allogeneic hematopoietic cell transplantation. In this model, there is a rapid rise in viral load of the novel porcine lymphotropic herpesvirus 1 (PLHV-1), preceding B cell expansion and PTLD. PLHV-1 has not yet been grown in vitro but has been partially sequenced (73 kb of 150 kb). This sequence contains genes homologous to EBV including a variety of homologues of mammalian oncogenes, chemokines and receptors thought to be associated with lymphoma. These homologues of EBV and Kaposi’s sarcoma herpesvirus (KSHV) in PLHV-1 are transcribed in PTLD-affected pigs but not in healthy swine undergoing the same immune suppression. We have developed quantitative molecular assays to measure porcine viruses (porcine CMV, PLHV, porcine endogenous retrovirus).

Hypothesis
The molecular mechanisms underlying development of virally mediated post-transplant lymphoproliferative disorders can be characterized in the miniature swine model of allogeneic stem cell transplantation. Differential gene expression by viruses and host tissues will be studied prior to and during development of tumors. Novel genes will be compared with homologous genes from human viruses and tissues to provide a basis for new approaches to the diagnosis and therapy of PTLD.
**Specific Aims**

The proposed studies are designed to elucidate fundamental mechanisms in the pathogenesis of post-transplant lymphoma. We will use the PTLD model in porcine allogeneic hematopoietic stem cell transplantation to:

1. Examine the molecular pathogenesis of PTLD due to PLHV-1 using genetic microarrays to identify genes activated in the development of PTLD. PLHV-1 gene expression will be studied in serial samples (PBL, lymph node or thymus) from control animals without immune suppression, and in PLHV-infected animals with and without PTLD.

2. Correlate genes activated from PLHV-1 with homologous genes or proteins active in EBV- or KSHV-mediated human disease. Such gene products are potential diagnostic markers for PTLD as well as potential targets for immunotherapy in human transplant recipients.

3. Study the role of other viruses (porcine cytomegalovirus and porcine endogenous retrovirus) in predisposition to PTLD.
Modification of Dendritic Cells and DNA Vaccination for the Induction of Tolerance

T cell activation is dependent on co-stimulation; in particular through CD28 interacting with CD80/86 on antigen presenting cells (APC). Engagement of the T cell in the absence of co-stimulation can lead to anergy and the induction of regulatory T cells. We have developed an approach to prevent co-stimulation, by expressing in APC a fusion protein between CTLA4 and KDEL, an endoplasmic reticulum retention signal. CTLA4-KDEL binds to CD80/86 in the endoplasmic reticulum, and prevents it reaching the cell surface. Expression of CTLA4-KDEL in APC leads to hyporesponsiveness and anergy in antigen-specific T cells both in vivo and in vitro.

We will transfect murine dendritic cells (DCs) with CTLA4-KDEL using non-viral gene delivery. We will confirm they no longer express CD80/86 and the expression of other surface markers is normal. We will then test their ability to stimulate alloreactive T cells in vitro, expecting that alloreactive T cells will not respond but will become anergic. These cells will be administered in vivo to determine if they can induce tolerance to alloantigens. Using a strain combination (CBK→CBA) in which rejection is due to the indirect pathway of allorecognition, we will determine if we can prolong graft survival for corneal and cardiac allografts. We will go on to look at combinations that test the direct pathway.

There is at present considerable interest in the use of DCs that have been ‘frozen’ by drug or cytokine treatment in an immature phenotype to induce tolerance. We expect that our CTLA4-KDEL-modified DCs will have advantages for this because they will be incapable of expressing CD80/86 even under extreme activation conditions. In addition, they can be administered as activated DCs (albeit without CD80/86 expression) which express high levels of MHC class II and home well to lymph nodes. They will therefore be better than immature DCs at presenting antigen to alloreactive T cells, and so anergising them. We will therefore compare the efficacy and other properties of CTLA4-KDEL-transfected DCs and dexamethasone-treated immature DCs.

Finally, we will develop a novel method of tolerance induction by administering a DNA vaccine encoding both an alloantigen and CTLA4-KDEL. This will result in both molecules being expressed by DCs and so lead to tolerance to the antigen. This approach offers a new strategy for tolerance induction that might have wide applicability.
Impact of Sanglifehrin A, a Novel Immunosuppressant on Dendritic Cell Function in Solid Organ Transplantation

Objective
To investigate the novel cyclophilin-binding immunosuppressant Sanglifehrin A (SFA) in experimental solid organ transplantation with emphasis on dendritic cells (DCs) and the underlying mechanisms of its unique suppressive effects on DCs.

Background
DCs are uniquely well-equipped antigen-presenting cells that initiate and regulate immune responses and represent ideal targets for pharmacological manipulation of immune responses. Sanglifehrins, are novel immunophilin-binding immunosuppressants that are produced by the actinomycetes strain Streptomyces A92-308110. Although SFA, like Cyclosporin A (CsA), binds with high affinity to cyclophilin, unlike the latter, SFA does not inhibit calcineurin phosphatase activity or any other known target of immunosuppressive drugs. We found that SFA rapidly blocked IL-12p70 production by human DCs. Emerging data from the recent literature indicates that the decisive signal that transfers tolerogenic DCs into immunogenic DCs is the release of proinflammatory IL-12.

Specific Aims
1. Dissection of the signaling events that are targeted by SFA in DCs to understand the molecular mechanism of action
2. Characterization of the effects of SFA on DC function in different in vivo models
3. Treatment of organ (heart, skin) recipients with SFA-exposed DCs and parenteral application of SFA with subsequent analysis of donor-specific immunity and graft survival

Preliminary Results
This proposal takes advantage of data of our group showing that SFA abrogates bioactive IL-12p70 production by human dendritic cells. In direct comparison to the related immunosuppressants CsA and rapamycin, SFA acts uniquely within 1 hour to inhibit 80–95% of IL-12p70 production by differentiated DCs. We have developed a parenteral SFA formulation that can be applied for animal experiments. Our preliminary, as yet unpublished data show that a 10-day course of SFA blocked 94% of LPS/IL-4-induced IL-12p70 and 97% of CPG-induced murine IL-12p70 production in vivo.
Proposed Methods
1. Utilization of phospho-specific Alexa647-conjugated mAbs specific for ERK1/2, p38 MAPK, Stat1p/6p to study phosphorylation events specifically in DCs by flow cytometry
2. Employment of the calcium sensitive dye Fluo-3 and non-radioactive cAMP assays to study calcium influx and cAMP formation in SFA-exposed DCs
3. Established murine model ± the DC growth factor Flt3-ligand to analyze SFA’s effects on DCs in vivo under steady-state and dynamic conditions
4. Murine skin and vascularized murine and rat heart transplant models with defined minor and MHC disparate strain combinations

Expected Results
We expect to gain novel insights into the mechanism of action of SFA and into its potential clinical use as a novel immunosuppressive agent in solid organ transplantation.
Imperial College London, London, UK

Promoting Transplantation Tolerance: Adoptive T Cell Therapy Using Customised Regulatory Cells

One of the central challenges in the field of transplantation tolerance is to translate the insights accumulated from experimental models into clinically applicable protocols. Based on a large body of *in vitro* and *in vivo* data it appears that two key events in the induction of transplantation tolerance are the deletion of anti-donor T cells with direct allospecificity, and the induction of regulatory T (Treg) cells that can control indirect, and residual direct, pathway T cells. Many questions remain unanswered concerning the nature of the regulatory cells that maintain tolerance *in vivo* and their specificity.

In this application we describe a strategy for the generation, expansion and characterisation of donor-specific Treg cells with direct and indirect allospecificity. Two types of Treg will be studied, those derived from naturally occurring CD4+CD25+ T cells, and Tr1 cells induced by antigen exposure in the presence of IL-10. CD4+ T cell lines will be generated from C57BL/6 mice against the class I alloantigen Kd. The cell lines will be fully characterised in terms of phenotype, expression of key genes, specificity, relative efficiency and mechanism of action (*contact dependence versus cytokine secretion*) *in vitro*. The efficacy of the Treg lines will be investigated *in vivo* using two approaches: first they will be injected with carboxy-fluorescein diacetate, succinimidyl ester (CFSE)-labelled TCR-transgenic T cells with direct or indirect allospecificity for Kd. Proliferation and cytokine secretion by the CFSE-labelled cells will be measured. Second, the ability of the cell lines to prevent rejection of heart grafts in Rag-/- C57BL/6 mice reconstituted with naïve CD4+ or CD8+ T cells.

To combine regulation with deletion of direct pathway cells, adoptive T cell therapy using the cell lines will be combined with short term rapamycin treatment. Deletion will be visualised by injecting CFSE-labelled TCR-transgenic T cells, and similarly labelled Tregs in Rag-/- C57BL/6 mice. These experiments will be followed by the sequential administration of rapamycin and regulatory cells to heart graft recipients of fully immunocompetent C57BL/6 mice.
The final aim of this study is to extend this approach into a human system. Similar regulatory cell lines, with indirect allospecificity for HLA-A2 will be generated against autologous HLA-DRB1*0101 DCs with an HLA-A2 peptide. To select antigen-specific cells, we will use a DR1:A2 peptide tetramer for cell sorting.

Preliminary data is presented to demonstrate that we have established the conditions for generating and selecting Treg cells. These data will provide an invaluable platform for pilot clinical studies in transplant patients.
Modulation of TRAF-Dependent Signaling in EBV+ B Cell Lymphomas

Epstein-Barr Virus (EBV) B cell lymphomas are the most serious form of post-transplant lymphoproliferative disease. We propose to examine how EBV alters signaling through tumor necrosis factor receptor (TNFR) family members that control growth and survival in B cells. Latent membrane protein 1 (LMP1) is an EBV-encoded membrane protein that constitutively signals in a manner similar to CD40 to provide NFκB-mediated survival signals and to inhibit apoptosis. Activated B cells express the TNFR family members CD27, CD30, CD40, TNFR1, and TNFR2. The TNFR family is critical in mediating survival, differentiation and cell death signals. Importantly, both LMP1 and TNFR family members interact with multiple members of the six known TNFR-associating factor (TRAF) family adaptor proteins in different combinations to transduce their signals.

We hypothesize that LMP1, by usurping the cellular TRAF signaling pathway, modulates the function of cellular membrane proteins that require TRAF adaptor proteins to propagate their signal. This is important because impaired function of molecules involved in B:T interactions such as the TNFR family could contribute to the outgrowth of EBV-infected B cells and the pathogenesis of post-transplant lymphoproliferative disease (PTLD).

Specific Aims
1. To determine the role of EBV in modulating the signaling pathways used by TNFR family members to activate responses in B cells. We will determine the expression of the TNFR family members by flow cytometry and of TRAF1-6 by Western blot. Cell lines will be treated with agonist antibodies- or ligand-transfected CHO cells to trigger TNFR proteins. The effects on proliferation, cell cycle progression, and apoptosis will be assessed. Immunoprecipitation and Western blot experiments will assess TRAF association with TNFR and LMP1 to determine if EBV infection alters the interaction of specific TRAF molecules with specific receptors.
2. To determine the role of LMP1 in modulation of TNFR function by two complementary approaches. To test if LMP1 is necessary for EBV-mediated alteration of TRAF signaling we will use RNA interference (RNAi) to inhibit LMP1 expression in EBV⁺ B cells. To determine if LMP1 is sufficient we will introduce LMP1 into EBV⁻ B cells. We will define the cytoplasmic domain/s of LMP1 involved in signaling interactions between LMP1 and TNFR molecules by using vectors encoding wild-type LMP1 and LMP1-deletion mutants for C-terminus activation region (CTAR) domains. Immunoprecipitation and Western blot experiments will assess specific interactions between TRAF and TNFR in LMP⁺- and LMP cell lines.

Understanding the effects of EBV and LMP1 on the B cell molecules that control growth and survival will enhance the development of therapies for PTLD.
BK-Virus Load Measurements in Kidneys: New Strategies for Assessing the Risk of BK-Virus Nephropathy

Background
BK-virus (BKV) causes BK-virus nephropathy (BKN) in up to 5% of renal allograft recipients. It has a major detrimental impact on long-term graft function. Re-activation of latent BKV (transplanted with the donor kidney) is a potential pathway resulting in BKN. However, surprisingly little is known about the possible risk for BKN arising from latent BKV infections in native/donor kidneys and ureters: What is the prevalence of latent BKV, where are the specific sites of latency, and what are the viral load levels? In addition, the definition of viral load levels can also help with establishing quantitative PCR as an adjunctive clinical tool to make an early “pre-histological/pre-clinical” diagnosis of BKN in graft biopsies. Traditionally, the diagnosis of BKN has required the identification of viral inclusions by light microscopy (LM). LM, however, provides “late” evidence of BKN since inclusions are only formed in the presence of high viral copy numbers. Quantitative PCR could help to detect pre-histological stages of BKN, which are currently undefined. Such an early diagnosis of BKN would likely improve patient management and long-term graft survival.

Aim
Our quantitative PCR analysis aims to establish BKV load levels in graft biopsies from patients with and without BKN and native kidneys. The definition of BKV load levels will help with better understanding BKN and improving patient management.

Study Design
Renal tissue from different patient populations will be analyzed by PCR. Group 1: 26 renal transplant recipients with BKN, retrospective analysis of consecutive graft biopsies (n=66) from different time points (including implantation biopsies; on average 3 biopsies/patient). BKV load curves will be established. Group 2 (control): 66 control biopsies (matched to group 1) from renal transplant recipients without BKN, analysis identical to group 1. Group 3 (control group, 80 native kidneys from 40 deceased patients, “mirror image of potential organ donor pool”): per kidney 4 samples will be analyzed. Data from group 3 serves as baseline control readings for groups 1 and 2.
Summary
We aim at defining BKV load levels by PCR in native and transplanted kidneys (i.e. “reference values”) in order to establish whether a) high latent BKV loads in donor organs (i.e. “pre-loaded organs”) represent a risk for the development of BKN post-transplantation, and b) whether BKV load measurements in graft biopsies can be used to diagnose BKN at an early pre-histological stage.
Donor-Engineered Dendritic Cells to Generate Tolerogenic Regulatory T Cells for Renal Transplantation

The ability of dendritic cells (DCs) to induce immunity or tolerance is related to their state of functional maturation. Immature DCs, lacking enough costimulatory activity can induce T cell hyporesponsiveness \textit{in vitro} and prolong allograft survival \textit{in vivo}, suggesting their involvement in the induction of graft tolerance. Thus, manipulation of DCs by controlling their functional and maturation status has been offering potential for therapy of allograft rejection. In preliminary studies we found that rat immature DCs, obtained by cell transfection with adenoviral vector encoding for dominant negative kinase IKK2 (dnIKK2), are capable of generating hyporesponsive T cells with potent regulatory activity on naive T cells. However, the phenotype of DC-induced T regulatory cells, the mechanism by which regulatory cells are generated, as well as the mediators involved in their regulatory function, remain ill-defined. Increasing evidence is available that T regulatory cells may provide the most favorable route to operational tolerance in organ transplantation.

Here we propose to characterize \textit{in vitro} phenotypically and functionally regulatory T cells generated by allogeneic immature dnIKK2 DCs, and to explore \textit{in vivo} their capacity to control the effector arm of the immune response and induce tolerance to a solid organ graft from the same DC donor strain. To pursue these goals, the following specific aims will be addressed \textit{in vitro} and \textit{in vivo} experiments in rats:

1. To characterize the phenotype of the T regulatory cells generated by allogeneic immature dnIKK2 DCs
2. To assess the mechanisms by which stably immature DCs promote the emergence of T cells with regulatory activity
3. To define the pathways and the mediators involved in the regulatory function of these T cells
4. To evaluate the effectiveness of intravenous infusion of donor-specific dnIKK2 DC-induced T regulatory cells as a tool to promote tolerance in a rat model of kidney allotransplantation.

We expect that donor-derived immature engineered DCs allow the development of regulatory T cells capable of inhibiting naive T cell response \textit{in vitro} and inducing long-term kidney allograft survival \textit{in vivo}. 
**Correction of Diabetes Using Primary Liver Cells**

**Background**
Type I diabetes mellitus is caused by the autoimmune destruction of pancreatic β cells. The problems of chronic complications and lack of organs for transplantation could be overcome by genetically engineering an "artificial β cell". The target cells in this proposal are liver cells which express glucokinase and the glucose transporter GLUT-2, as do pancreatic β cells. Studies by our group have shown that the introduction of the insulin gene into two liver cell lines (HEP G2ins/g and Huh7ins) has resulted in synthesis, storage and regulated secretion to glucose and other metabolic substances, and correction of diabetes in a mouse model. This ability to store insulin, is linked to the expression of the β cell transcription factor NeuroD. Using an efficient lentiviral transduction system (HIV/MCSV) we have established that delivery of furin-cleavable insulin (INS-FUR; which allows for the production of fully processed insulin from liver cells) and Neuro D directly into the liver of streptozotocin-diabetic rats, resulted in storage of insulin, normal glucose response curves and reversal of diabetes for eight months.

**Research Plan**
Using the HIV/MSCV system we aim:
1. To determine if the expression of INS-FUR alone and together with Neuro D induces storage of insulin and permanent reversal of diabetes in non-obese diabetic (NOD) mice
2. To determine if we can achieve better physiological control of INS-FUR production from the livers of NOD mice, if we transfer INS-FUR under the control of a glucose- and insulin-sensitive promoter
3. To determine if the insulin-secreting cells in NOD mice are subject to autoimmune attack
We will examine the storage and secretion of insulin to metabolic stimuli in NOD mice by performing oral and IV glucose tolerance tests, measuring insulin and human c-peptide by radioimmunoassay. Insulin storage will be examined by extraction, immunohistochemistry and immunoelectron microscopy. The tissue distribution of the vectors will be examined by RT-PCR and Western blot analysis. General pancreatic transdifferentiation will be examined by RT-PCR, Western blot and immunohistochemistry. Normal hepatocyte function will also be examined. We will perform histological studies of lymphocyte homing and cell death and look at lymphocyte cytotoxicity, proliferation and cytokine secretion to determine if insulin-secreting liver cells in NOD mice are subject to autoimmune attack.

**Outcomes**

The results from this research proposal should result in the development of a new and exciting protocol for the somatic gene therapy of type I diabetes.
Recently, indoleamine 2,3-dioxygenase (IDO) production by specific murine dendritic cell (DC) subsets has been shown to block alloAg-specific T cell responses in vivo. In addition, cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA4Ig), that blocks the B7-CD28 costimulatory pathway, induces high levels of IDO in splenic plasmacytoid (p)DC. This enzyme promotes T cell apoptosis by converting tryptophan into pro-apoptotic kynurenines. Its inhibition prevents the induction of spontaneous liver transplant tolerance in mice, suggesting a role for IDO in the inherent tolerogenicity of the liver.

Our preliminary data show that freshly isolated liver pDC but not other DC subsets, strongly express IDO. pDC have been shown to induce T cell anergy and to promote the differentiation of regulatory T cells, implying an important role for pDC in the induction of tolerance. Indeed, our preliminary data show that highly purified pDC of donor origin are very effective in prolonging organ allograft survival and the most effective regulatory DC subset that we have examined.

We hypothesize that IDO production by hepatic pDC and its influence on T cell responses plays a key role in the induction of liver transplant tolerance.

**Specific Aims**

1. **We will determine IDO production by hepatic pDC in murine models of spontaneous liver transplant acceptance and rejection.** In vitro experiments will determine the potential of freshly isolated, CTLA4Ig-stimulated or interferon (IFN) γ-treated hepatic pDC to secrete functional IDO and its role in inhibiting allogeneic T cell responses and inducing T cell apoptosis. In vivo experiments will determine whether IDO is differentially expressed by hepatic pDC in liver transplant tolerance and rejection models.

2. **We will assess the impact of CTLA4Ig on IDO production by pDC in a hepatic allograft rejection model.** These experiments will ascertain whether further induction of IDO in pDC can reverse acute rejection resulting in prolonged liver allograft survival, by promoting apoptosis of donor-reactive T cells.
3. We will determine the potential of upregulated pDC IDO production for therapy of liver allograft rejection by adoptive transfer of pDC. We will determine the impact of the IDO-producing DC on liver allograft rejection and apoptosis of host alloreactive T cells.

This investigation will provide new insights into the role of hepatic pDC and IDO in the regulation of alloreactive T cell responses in relation to organ transplant tolerance. Our findings may provide the basis of a novel cell-based therapeutic strategy to promote tolerance induction in organ transplantation.
Dr. Cees van Kooten, Principal Investigator

Leiden University Medical Center, Leiden, The Netherlands

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

Dendritic cells (DCs) serve as an essential link between innate and adaptive immune responses. They can induce both primary and secondary immune responses and play a key role in immuno-stimulatory as well as immuno-suppressive responses. This dual function has made them potential targets in vaccine development for the prevention and treatment of infections and cancer as well as autoimmune diseases and allograft rejection.

Recently, we have demonstrated that different DC subsets display a marked heterogeneity in sensitivity for immune modulating agents. Importantly, we showed that the immunosuppressive drug rapamycin specifically induces apoptosis in human monocyte-derived DCs and CD34-derived DCs, but not in monocytes and macrophages. We found that expression of the cyclin-dependent kinase inhibitor p27kip1 plays a key role in the regulation of DC survival. Adenoviral transduction of the human p27kip1 gene in DCs directly causes apoptosis, associated with downregulation of the anti-apoptotic protein mcl-1.

The longevity of DCs has been proposed to be a critical factor influencing the outcome of immune responses. However, relatively little is known about the regulation of apoptosis and survival in DCs, or how DC apoptosis/survival affects cellular immunity and allograft survival.

The specific aims of the present proposal are:
1. To identify the molecular mechanisms of survival in human DCs
2. To prolong graft survival through pretreatment with long-lived immature DCs

Using monocyte-derived DC, we will study in detail the mechanism of mTOR-regulated p27kip1 expression and the regulation of bcl-2 family members at mRNA and protein level. Additional target genes will be identified using reverse transcriptase-multiplex ligation-dependent probe amplification (RT-MLPA). Specific targets will be inactivated at the mRNA level using antisense oligo’s and small interfering RNA’s, or introduced by adenoviral transduction. We will specifically focus on the role of FOXO transcription factors, which in recent years have been identified as a critical switch in the regulation of cell survival. We will use DC derived from Mcl-1-Tg mice to investigate the impact of immature DC longevity on allograft survival in an experimental transplant model.
These studies will lead to a thorough understanding of the survival mechanisms of DC. The identification of molecules that regulate the survival of DC will provide a platform for the design and implementation of protocols aimed at inhibiting alloimmune reactivity or increase donor-specific tolerance in organ transplantation.
Activation of Macrophages and Endothelium by Non-Complement Fixing Antibodies in Allograft Rejection

Alloantibodies (AlloAbs) are clinically significant components of the immune response to organ transplants. We documented a critical role of AlloAbs in acute allograft rejection of B10.A (H-2^a) hearts transplanted to C57BL/6 (H-2^b) recipients. Wild-type (WT) recipients reject B10.A hearts within 7–14 days (n=18). In contrast, over 85% of B10.A cardiac allografts survive more than 14 days in Ig knock out (IgKO) C57BL-Igh-6 mice (n=39).

We found that some subclasses of monoclonal antibodies against donor MHC class I antigens can reconstitute allograft rejection in IgKO recipients. Passive transfer of a single 100 μg dose of complement-activating IgG2b (15-1-5P) 3 days after transplantation does not cause IgKO recipients to reject their hearts rapidly (n=8). In contrast, injection of a single 50–200 μg dose of IgG2b at 10 days after transplantation reconstitutes acute rejection of cardiac allografts to IgKO recipients (n=11). Although passive transfer of a subthreshold dose (25 μg of IgG2b (n=5) or a single 100–200 μg dose of non-complement-activating IgG1 (n=5) does not restore acute cardiac rejection in IgKO recipients, a combination of these alloreactive monoclonals does cause acute graft rejection (n=12). Flow cytometry and ELISA for C3b and C3d documented that IgG1 does not activate complement and does not augment complement activation by IgG2b. Preliminary in vitro experiments with microarrays and ELISA indicate that IgG1 stimulated isolated mouse endothelial cells (SVEC4-10) that express donor MHC class I antigens to produce MCP-1, KC and RANTES, and the addition of macrophages to the culture results in high expression of IL-1, IL-6 and MIP-2.

These findings led us to the hypothesis that non-complement-activating IgG1 can augment injury and cardiac allograft rejection through activation of chemokines that attract macrophages. We will test the mechanisms by which antibody and complement activate graft endothelial cells and macrophages.
The following specific aims are designed to test the role of FcRs and complement receptors (CR) on macrophage activation:

1. Macrophage activation via engagement of FcγRI and FcγRIII by AlloAbs bound to endothelial cells \textit{in vitro}
2. Macrophage activation via engagement of CR1/2 and CR3 by C3 and C4 split products will be tested \textit{in vitro} with macrophages from CR3 and CR2 KO mice
3. Based on the findings from the preceding two \textit{in vitro} specific aims, IgKO will be crossed with the relevant FcγR or CR KO mice for passive transfers of IgG1 and IgG2b AlloAbs.
4. Progress Reports of ROTRF Grantees

M.D David Briscoe, Principal Investigator
Dr. Stuart Robertson, Co-Investigator

Children’s Hospital, Boston, USA

Vascular Endothelial Growth Factor in Acute and Chronic Rejection

Specific Aims

The overall objective of this proposal is to define the function of vascular endothelial growth factor (VEGF) in allograft rejection. We hypothesize that VEGF is a proinflammatory cytokine via its ability to regulate intragraft mechanisms of rejection. To test this hypothesis, we have a research plan to investigate the mechanism of function of VEGF in ischemia reperfusion injury, acute and chronic rejection.

Progress on Specific Aims includes:

1. Recent studies suggest that chemokine and chemokine-receptor interactions: interferon-inducible protein-10 (IP-10)/CXCR3 or monocyte chemoattractant protein-1 (MCP-1)/CCR2 play key roles in allograft rejection. VEGF, a known regulator of MCP-1 and IP-10 is expressed and is functional for leukocyte recruitment into allografts. In this study, we evaluated the interactions between VEGF-mediated trafficking of leukocytes and rejection, and MCP-1 or CXCR3 function in vivo. Fully MHC-mismatched hearts from BALB/c mice were transplanted into C57BL/6 wild-type (WT), MCP-1, or CXCR3 gene knockout (-/-) recipients; and were treated with either neutralizing VEGF anti-serum or normal rabbit serum i.p. on days -1, 0, 2, 4, 6, 9, 12, 15. Our findings were that anti-VEGF inhibited rejection and prolonged survival from 9 days (n=5) to as much as 14 days (n=5, p<0.005) in WT recipients. Anti-VEGF also prolonged survival in MCP-1-/- recipients (median survival time MST 15 days, n=2, -> 21 days, n=4) and in CXCR3-/- recipients (MST 15 days, n=3, -> 19 days, n=6). Histological examination of the grafts confirmed massive infiltration and vasculitis in rejecting WT grafts but less infiltrates and less vasculitis in all anti-VEGF-treated chemokine-deficient recipients. To further address the role of intragraft chemokines for VEGF function, we also transplanted C57BL/6 WT, MCP-1-/-, or IP-10-/- grafts into BALB/c recipients that were untreated or treated with anti-VEGF. Surprisingly, while IP-10-/- grafts had a prolonged survival, MCP-1-/- grafts were rejected at a similar rate as WT grafts (MST 9 days, n=6). Moreover, whereas anti-VEGF further prolonged survival of IP-10-/- grafts (MST 19 days, n=6, -> 34 days, n=7), in initial studies, anti-VEGF failed to prolong survival of MCP-1-/- grafts (MST 10 days, n=5). Taken together, these data suggest that VEGF function in vivo is independent of recipient MCP-1 and CXCR3 interactions. Moreover, it appears that donor MCP-1 alone is not sufficient or necessary for the
development of acute rejection of cardiac allografts; and that intragraft MCP-1 may be important for VEGF function in vivo. Our findings are consistent with the possibility that VEGF is a potent pro-inflammatory cytokine in rejection and that it is a potential therapeutic target. Importantly, these findings define VEGF as functional for leukocyte trafficking in vivo in both a chemokine-dependent and a chemokine-independent manner.

2. MCP-1 and the CXC chemokine receptor 3 (CXCR3) are two of a family of chemokines and chemokine-receptors that are proposed to play a critical role in leukocyte trafficking into allografts and in effector-cell activation. CXCR3 is the receptor for the chemokines IP-10, I-TAC and Mig that are produced mainly by activated Th1 and NK cells. The MCP-1 chemokine is produced mainly by monocytes and endothelial cells, and binds to its hematopoietic cell receptor CC chemokine receptor 2. Both CXCR3 and MCP-1 are expressed in association with rejection and promote the migration of T cells into inflamed tissues. We used fully MHC-mismatched BALB/c – C57BL/6 tail to trunk skin or cardiac transplant models to determine the relative role(s) of donor or recipient MCP-1 and CXCR3. Compared with WT control mice (MST= 9 days, n=8) CXCR3−/− or MCP-1−/− recipients did not show delayed rejection of fully mismatched skin allografts. To our surprise, when CXCR3−/− and MCP-1−/− mice were used as graft donors, all skin allografts survived significantly longer than their WT controls (MST=15 days, 12 days vs. 9 days; n=10, 10 and 8 respectively, p< 0.0001). This finding is in contrast to results following cardiac transplantation in which CXCR3−/− cardiac allografts do not survive significantly longer, and our preliminary observation that the lack of MCP-1 expression in cardiac allografts does not affect leukocyte trafficking or acute rejection (MST=8 days vs. 9 days, n=6 and 5 resp). Also, we found that WT cardiac allografts in CXCR3−/− or MCP-1−/− recipients had prolonged survival (p< 0.005). We hypothesized that the reason for the different role of CXCR3 and MCP-1 in skin and cardiac allograft rejection is due to vascularization. To address this possibility, we developed a model in which fully mismatched skin grafts were allowed to heal in SCID recipients. Four weeks later SCIDs received recipient WT spleen cells, and subsequently rejected the vascularized skin grafts by day 16 (n=8). Adoptive transfer of CXCR3−/− or MCP-1−/− spleen cells in this vascularized skin model did not result in a significant prolongation of survival. We conclude that donor expression of CXCR3 and MCP-1 in the heart and skin have different functions in rejection. Our data imply that there are organ specific differences in the usage of chemokine pathways that mediate selective leukocyte trafficking into allografts.
3. We have started to evaluate the expression and function of VEGF in an established model of renal ischemia reperfusion (I/R) injury. In brief, for these initial studies, C57/BL6 mice are anesthetized and unilateral acute tubular necrosis (ATN) is produced by clamping of the renal pedicle to produce ischemia for 30–60 minutes. Clamps are removed and reperfusion injury occurs. Blood samples were taken daily for assessment of creatinine and tissues were harvested at 2 hours, 6 hours, 12 hours, 24 hours and then at day 2 and day 6 to assess recovery. RNase-protection assays using the RiboQuant multiprobe template (Pharmingen) demonstrated that MCP-1 and IP-10 are induced early (2–24 hours); and that VEGF is induced simultaneously. We treated animals (n = 2–4 per group) with control Ig, anti-VEGF on days -1, 0, 1, 2, 4 and 6 or PTK 787 (a VEGF-receptor antagonist) and found that chemokine expression is decreased at early times but not at later times following I/R. We are currently assessing the effect of the immunosuppressants rapamycin and mycophenolate mofetil (MMF) alone or in combination with anti-VEGF treatment on the expression of proinflammatory chemokines in this model.

4. VEGF, a potent angiogenesis factor induced by activated T cells, stimulates angiogenesis in the process of inflammatory response. Our previous studies have shown that CD4+ T lymphocytes play an important role in Ag-independent proinflammatory response in I/R injury and in the expression of VEGF. We have reported that CD40L interacting with CD40 is a mechanism by which the immune system can induce VEGF expression and function in vitro and in vivo. This study explores the role of VEGF in rat liver inflammatory injury triggered by I/R and modulated by gene transfer of CD40lg. Sprague-Dawley (SD) rats were infused with Ad-CD40lg or Ad-βgal-reporter gene (2.5x10^9 pfu i.v.). One day later, livers were harvested, preserved for 24 hours at 4°C in UW solution, and then transplanted orthotopically into syngeneic SD recipients. Animals were followed by survival; separate groups of OLTs were harvested at day 1, 3, 7 and 14, and analyzed histologically. VEGF and cytokine gene pattern was screened by RT-PCR. Intragraft expression of anti-oxidant HO-1 and anti-apoptotic Bcl-2/Bcl-xl genes was assessed by Western blots. We found that 100% of Ad-CD40lg pretreated OLTs survived >14 days (vs. 50% in Ad-βgal controls and untreated group; n = 6 rats/gr). Unlike Ad-βgal controls, which showed significant edema, moderate sinusoidal congestion, and moderate to severe necrosis (>60%), the Ad-CD40lg gene therapy group revealed minimal sinusoidal congestion/necrosis. These correlated with decreased sGOT levels in the Ad-CD40lg group. Ad-CD40lg-gene-transfer significantly reduced apoptosis in OLTs treated with Ad-CD40lg (p < 0.005). Intragraft expression of mRNA coding for VEGF in Ad-βgal and untreated controls consistently increased, as compared with sham controls. However, Ad-CD40lg-gene-transfer significantly decreased VEGF expression. Unlike in controls, TNF-α, IL-2/IFN-γ remained depressed, whereas that of IL-4/IL-10 reciprocally increased selectively in the Ad-CD40lg group. The expression of HO-1 and Bcl-2/Bcl-xl increased throughout in Ad-CD40lg-transduced OLTs, as compared with Ad-βgal group.
**Conclusion**

This is the first report, which documents that VEGF is associated with the process of hepatic inflammatory injury induced by I/R. Ad-based CD40lg-gene-therapy down-regulated VEGF expression and protected rat OLTs against otherwise severe cold I/R injury. Ad-CD40lg-gene-transfer prevented hepatic apoptosis, facilitated Th1 to Th2 shift, and triggered the expression of anti-oxidant/anti-apoptotic genes with cytoprotective functions.

**Publications:**

The original trial was designed to enroll 100 renal transplant recipients into a steroid/cyclosporin A (CsA) withdrawal protocol resulting in mycophenolate mofetil (MMF) monotherapy. The project was modified during the human subjects review process. The current trial will enroll 75 patients who are over the age of 55, more than one year post-renal transplant with good renal function and without leukopenia (the delayed-type hypersensitivity DTH assay requires 40 million cells). The patients are randomized into two groups, 50 will be withdrawn from steroids and 25 will be maintained on steroids. The steroid withdrawal trial itself is fully funded from other sources and is being conducted independently of the DTH study. The ROTRF grant covers the basic science/DTH studies of patients enrolled in the MMF monotherapy trial.

Two DTH assays will be performed on each patient, one at enrollment in the study and a second after steroid withdrawal or at an equivalent time point for those in the control arm. It was also the recommendation of the human subjects committee that the DTH-testing and steroid withdrawal be blinded (i.e., we are performing the DTH assay, but do not know which patients are being withdrawn from steroids and which are in the control arm; likewise the clinicians who are following the patients do not know their DTH status). The final change to the protocol was that the reduction to monotherapy be performed in two stages. The first consists of a slow withdrawal of steroids over a 12-week period. This will allow the clinicians to determine if the steroid withdrawal is putting the patients at risk for rejection. If not, the second stage of the trial will be a withdrawal of CsA for patients on whom steroid withdrawal was successful. The endpoint for our research remains the same, i.e., does a patient’s DTH status predict their tolerance for reduced immunosuppression?

Of the 75 patients, 21 have been enrolled to date and we have performed the first DTH test on all enrolled patients. In addition, 9 patients have reached the 6-month time-point and have been tested twice. Because of the blinded nature of the trial, we do not know which of these 9 patients have had their steroids withdrawn. The second stage of the trial (CsA withdrawal) has not been initiated with any patients. Although we are a year into the trial and have only enrolled 21 patients, we are confident that the goal of 75 will be met within the next year. The protocol was modified approximately 4 months into the trial to increase the pool of eligible subjects.
Results to Date
The DTH assay is performed by injecting 7 million patient peripheral blood mononuclear cells (PBMC) into the footpad of a SCID mouse with either phosphate buffered saline (PBS, control), donor antigen (sonicated cells from donor spleen or PBMC to assess sensitization to the donor), recall antigen (Epstein-Barr Virus, EBV, or tetanus toxoid, depending on the patients exposure), and donor antigen with recall antigen (to assess linked suppression-the ability of the donor antigen to trigger a suppression of the recall response). Prior to injection and 24 hours after injection, the footpad thickness is measured with a spring-loaded caliper. In all experiments, the change in thickness measured when PBMC and PBS are injected is considered "background" and the value is subtracted from all other measured values. A net swelling of more than 25 x 10^-4 inches is considered a positive response. Based on the net swelling measured with the 3 test injections (donor antigen, recall, donor + recall) a phenotype is assigned as follows:

<table>
<thead>
<tr>
<th>Donor Antigen</th>
<th>Recall Antigen</th>
<th>Donor + Recall</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive*</td>
<td>Positive</td>
<td>≥ 50% of recall</td>
<td>Sensitized</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>≥ 50% of recall</td>
<td>Non Regulator</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>≤ 50% of recall</td>
<td>Regulator</td>
</tr>
</tbody>
</table>

Table 1. Description of DTH-phenotypes. *Positive, ≥ 25 net swelling; Negative, < 25 net swelling

Patients who respond to donor antigen are sensitized, while those that suppress the recall response (more that 50% reduction in net swelling compared to recall antigen alone) in the presence of donor antigen are regulators. Non-regulators respond to recall antigen, but the response is not modified by the addition of donor antigen.

<table>
<thead>
<tr>
<th>Tx type</th>
<th>N</th>
<th>Regulator</th>
<th>Non-Regulator</th>
<th>Sensitized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Haplo-LRDα</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>LURDβ</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CADγ</td>
<td>10d</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>9 (43%)</td>
<td>8 (38%)</td>
<td>3 (14%)</td>
</tr>
</tbody>
</table>

Table 2. DTH phenotype before steroid withdrawal. *LRD = living related donor, βLURD = living unrelated donor, γCAD = cadaver donor, *we recovered too few cells from one patient to perform the background, PBS, injection.
Of the patients tested, 43% are regulators while 38% are non-regulators and only 14% are sensitized (Table 2). This is very similar to a recent study (partially funded by Roche Pharmaceuticals) of 62 renal transplant recipients from our center, where 53% were regulators, 39% were non-regulators and 8% were sensitized\(^1\). The number of regulators was higher in the larger study as there were 6 recipients of human leukocyte antigen (HLA)-identical living related transplants and all of those patients were regulators. As in the larger study, none of the haplo-identical recipients of a living related transplant were sensitized. Another striking similarity between the larger study and the first analysis of the patients in this trial is that the patients who received a cadaver donor, but are matched for HLA-DR are significantly more likely to be regulators. In this study, all four of the HLA-2 DR matched CAD recipients were regulators.

![Figure 1](image)

**Figure 1.**

A. % inhibition of net swelling in response to recall antigen in the presence of donor antigen. An inhibition of more than a 50% (solid line) is considered regulation. Each line represents DTH from a single patient tested before steroid withdrawal (1\(^{st}\) reading) and then 6 months later (2\(^{nd}\) reading). B. Change in response to donor antigen alone (net foot pad swelling). A net swelling of >25 (solid line) is positive. Similar symbols on both graphs represent data from the same individual.

As noted above, 9 enrolled patients have been tested twice with the DTH assay. Because of the blinded nature of the study, we do not know which have been withdrawn from steroids or how well those that have been withdrawn have fared. However, most patients do not change their phenotype (Fig. 1A). That is, regulators continue to regulate 6 months later, although the percentage (%) inhibition may change. To date, no patients have become regulators at the second testing who were not already regulators. However, if we look at the level of response to donor antigen alone, we see that two sensitized patients lost their sensitization, while two previously non-sensitized patients have become sensitized. None of the regulators developed a positive response to donor antigen.
**Future Directions**

We will continue to enroll patients to reach our goal of 75 and continue to perform 2 DTH tests on each patient. In addition, we will work with the clinicians to determine if there is enough success from the early stages of this trial to begin phase 2, i.e. CsA withdrawal. If phase two is initiated, we will perform a third DTH test on each patient at the time of MMF monotherapy as planned under the ROTRF research proposal design.

**Publications:**

Mechanisms of Renal Allograft Rejection

Hypotheses

1. The rejection response is compartmental, with individual responses in different compartments: lymphoid, systemic, graft interstitial, and graft epithelial/endothelial. Homeostasis is altered when T cells enter the epithelial compartment.
2. Tubulitis is mediated primarily by CD103 CD8 T cells engaging E-cadherin on the basolateral membranes of epithelium. Tubulitis is independent of immunoglobulin, perforin, granzyme A and B, and Fas ligand (FasL).
3. Engagement of epithelial cells profoundly alters epithelial cells, which lose polarity, develop senescence changes, and express mesenchymal and fibroblast-like features, with varying degrees of apoptosis. Epithelial-mesenchymal transformation contributes to atrophy and fibrosis.
4. CD103KO mice will develop arteritis but will be protected from tubulitis.
5. Microarray patterns can be identified that correlate with tubulitis.

Specific Objectives to Define

1. Differential T cell homeostasis in tubulitis lesions.
2. The mechanisms of tubulitis, including changes in cadherin expression and the potential changes in epithelial cell biology, e.g. altered transcription.
3. The evolution of tubulitis in CD103 deficient hosts.
4. Compare arteritis lesions to tubulitis lesions.
5. Correlate the evolution of tubulitis with microarray patterns.

Since the funding of the grant we have concentrated on Aims 2, 3, and 5.

1. Specific Aim 2: Mechanisms of Tubulitis and Epithelial Mesenchymal Transdifferentiation (EMT)

Changes in CD103 expression during tubulitis in mice. Intra-organ accumulation of CD103⁺ T lymphocytes is thought to trigger tubulitis, a pathologic manifestation of kidney rejection. We thus monitored the evolution of tubulitis and expression of CD103 during the course of rejection. Tubulitis was absent or minimal at day 5 and 7, and severe at day 21. CD103 mRNA was detectable at day 5, peaked at day 7 and slightly decreased at day 21.
post-transplant. Early increase in CD103 expression could be partially attributed to the systemic effect of IFN-\(\gamma\). Laser capture microdissection revealed that at day 21, CD103 transcripts were present mainly in the tubules. Rejecting kidneys showed typical manifestations of graft reprogramming already between day 5 and 7, as judged by the upregulation of smooth muscle actin and downregulation of E-cadherin. However, tissue injury as exemplified by the distortion of tubules, aberrant intracellular localization of E-cadherin and loss of LDH was observed at day 21. We conclude that the early accumulation of CD103\(^+\) lymphocytes in the interstitium correlates with the onset of graft reprogramming, while tubulitis and the tissue injury are caused at the later time, when a subset of CD103\(^+\) lymphocytes relocates to tubules. Thus epithelial-mesenchymal transformation comes ahead of tubulitis and tissue disintegration. As predicted, tubulitis was unaffected by perforin knockout and by double knockout of granzyme A and B.

**Evidence for EMT in human kidney transplants.** The hallmark of renal transplant deterioration is tubular atrophy and interstitial fibrosis, usually without specific glomerular lesions. The linkage between these elements may be mechanistic: tubular epithelial cells (TECs) could contribute to fibrogenesis via epithelial-mesenchymal transdifferentiation (EMT) in kidney transplants developing tubular atrophy/interstitial fibrosis (TA/IF). We evaluated the relationship between epithelial and mesenchymal markers in human kidneys with various degrees of fibrosis and atrophy, compared to their one hour post-transplant biopsy. Biopsies from ten kidneys with TA/IF, as defined by Banff CT CI CV scores and impaired function, were studied by immunohistochemistry and compared to their implantation biopsies, and to ten protocol biopsies from transplants with stable function. Epithelial markers (E-cadherin, cytokeratin) were reduced and E-cadherin expression was redistributed from the basolateral membrane to the cytoplasm and apical membrane. New expression of mesenchymal markers [vimentin, S100A4 (human homologue of fibroblast-specific protein-1), alpha-smooth muscle actin] and collagen synthesis marker (heat-shock protein-47, HSP-47) was demonstrable in cytoplasm of TECs, both in deteriorating and atrophic tubules. Double immunostaining showed coexpression of cytokeratin and vimentin, S100A4, and HSP-47 in some TECs, suggesting an intermediate stage of EMT. Moreover, EMT features correlated with serum creatinine, the degree of TA/IF, history of T cell-mediated rejection (TCMR), calcineurin inhibitor effect (hyalinosis), and proteinuria. The tight relationship between loss of epithelial markers and appearance of mesenchymal markers in renal transplants with emerging TA/IF support the concept that TECs contribute to fibrogenesis in kidney transplants, via EMT. EMT changes correlate with graft function and extent of fibrosis. The association of EMT with TCMR as well as post-transplant stresses (i.e. CNI effect, proteinuria) suggests that damage to TECs either by immunologic or non-immunologic processes leads to loss of epithelial features and induction of fibroblast features.
2. Specific Aim 3: The Evolution of Tubulitis in CD103-Deficient Hosts
We obtained from Dr. Gregg Hadley in Baltimore some mice which had disruption of gene \( Itgae \), the \( \alpha \)-integrin-E chain. This is the chain which is responsible for CD103 expression in the integrin \( \alpha_{E}\beta_{7} \). These mice lack expression of CD103. We then performed CBA/J kidney transplants into these mice. To our surprise, the first mice in this group have florid tubulitis at day 21. Thus we have already established an answer to the question we posed, but we must fully describe this. The answer is that CD103 expression is not necessary for the development of tubulitis lesions. However, CD103 could still have unique roles within tubulitis lesions, by mediating direct epithelial engagement by T cells. We are now proceeding with electron microscopy of these lesions. There still could be unique effects of CD103 in tubulitis lesions.

3. Specific Aim 5: Correlate the Evolution of Tubulitis with Microarray Patterns
We have now made extraordinary progress under the ROTRF grant in establishing the Affymetrix microarray description of the evolution of kidney transplantation in mice. We have now performed 40 microarrays on batches of kidneys from the combination CBA/J kidneys into C57BL/6J hosts, at days 5, 7, and 21. We have also performed CBA kidneys into C57BL lacking B cells (the so called \( Igh-6 \) and \( Igh-J \) mice from Jackson). These mice have no ability to produce antibody and are deficient in mature B cells. We have previously described the fact that the biology of rejection differs in mice lacking B cells\(^2\). Finally, we have performed arrays on mixed lymphocyte culture day 4 (i.e. allogeneically simulated effector T cells) for comparison with the rejecting kidneys.

In addition, we have performed kidney transplants and microarrays on those transplants in hosts lacking IFN-\( \gamma \), and in hosts that have been treated with recombinant IFN-\( \gamma \). In these hosts we are examining the effect of IFN-\( \gamma \) in graft rejection and identifying the IFN-\( \gamma \) inducible genes.

4. Summary
Our hope is that these studies of the molecular biology of mouse kidney transplants will establish a new basis for our understanding of the effect of T cells and antibody on kidney and other epithelial organs. We also hope to establish a website in which people can browse the Affymetrix microarray data, gene by gene, on all of the above mouse combinations, once all of the data has been subjected to peer review. In this website the ROTRF will be acknowledged as the funding sponsor, and the website will be under the auspices of the newly formed Alberta Transplant Institute.

Publications:
Reduction of Gal\(\alpha(1,3)\)Gal for Xenotransplantation: Studies of HAR/DXR

**Aim 1: iGb3, a second glycosyltransferase-producing Gal\(\alpha(1,3)\)Gal.**

We have now isolated a full-length cDNA for the mouse iGb3 synthase, and fully characterised the genomic organisation of this gene in both mouse and rat. Mouse genomic clones have been isolated and are being characterised to identify which would be useful for a gene knockout construct. As an alternate means to reduce the effect of this enzyme siRNA experiments will begin shortly.

Several pig tissues have been examined for iGb3 expression by Northern blot analysis and a cDNA library is being constructed from salivary gland mRNA (the tissue with the highest expression).

**Aim 2: Removing Gal\(\alpha(1,3)\)Gal by \(\alpha\)-galactosidase and decreasing Gal\(\alpha(1,3)\)Gal using modified fucosyltransferases.**

The \(\alpha\)-galactosidase ENDO-Gal C was to be redirected and targeted to the Trans-Golgi-Network (TGN) to improve the efficacy of cleaving Gal\(\alpha(1,3)\)Gal on proteins before export to the cell surface. To achieve this, chimeras of ENDO-Gal C containing the transmembrane domain and cytoplasmic tail of human Furin (Fur, an enzyme located in the TGN and known to cycle to the cell surface) have been constructed and are being examined for efficacy.

We have shown that the H-type fucosyltransferase (FT1) localises in the Golgi, whilst the secretor fucosyltransferase (FT2) is found in vesicles throughout the cell. When the cytoplasmic tail or the cytoplasmic tail and transmembrane was replaced with that of FT1, the chimeric enzymes were relocalised to the Golgi. When FT1 or FT2 were coexpressed with GT the amount of Gal\(\alpha1,3\)Gal expressed on the cell surface was reduced. However, the relocalised FT2 reduced the level of Gal\(\alpha1,3\)Gal on the cell surface by a factor of 2 as compared to wildtype FT1 and FT2. More importantly these chimera also reduced Gal\(\alpha1,3\)Gal produced by iGb3 synthase.
Vascularization of Engineered Human Skin Equivalent

The performance of skin equivalents as well as other engineered tissues is limited by inadequate perfusion in the post-transplantation period. The goal of this project is to develop a strategy for improving the function of living skin equivalents by engineering an optimal human vascular bed. To complete this task we have three specific aims. Significant progress has already been made in two of these three aims within the first year of funding.

The first specific aim is to optimize conditions for effective revascularization of skin grafts using cultured human endothelial cells (EC) by a) improving the *in vitro* formation and persistence of neo-vessels in skin equivalents by selective addition of supporting cells and pro-angiogenic media supplements, b) testing the potential for Bcl-2 overexpression to improve *in vitro* stability and subsequent engraftment and function of synthetic vascular beds, and c) comparing the capacity of EC derived from human umbilical vein (HUVEC), skin, and blood to effectively perfuse skin grafts transplanted into immunodeficient mice.

We have developed the methodology for optimally seeding supportive fibroblasts, keratinocytes and EC onto acellular dermis-based skin grafts. First, neonatal-foreskin-derived fibroblasts are seeded on the cut underside of the dermal graft, followed 5 days later by keratinocytes, and after another 5 days by EC. The fibroblasts distributed throughout the dermis and improved the survival of EC in the grafts from 24 hours to 48-72 hours. This extension in survival should greatly improve the “shelf life” of grafts long enough to allow delivery to a healthcare provider for clinical usage. We are currently expanding blood-derived fibrocytes and bone-marrow stromal cells to determine whether cells autologous to the recipient could be utilized for this purpose, and if these cells provide additional support by differentiating into smooth muscle cells. In a related collaborative study performed in Dr. Rakesh Jain’s laboratory we have demonstrated that the addition of mesenchymal cells to engineered tissues improves the long-term stability of human synthetic vascular beds is a proof-of-concept. Here we showed that mesenchymal cells incorporated into tissue structures readily associate with human endothelial cells *in vivo*, and extend survival of HUVEC-lined vessels for at least a year after implantation into immunodeficient mice. Conversely, dysregulation of molecules in the angiopoeitin signaling pathway, which is essential for controlling the association of smooth-muscle cells with blood vessels, results in the *in vivo* generation of venous malformations.
Significant progress has also been made in evaluating the \textit{in vivo} function of HUVEC incorporated into synthetic human vascular beds, as well as the benefit of overexpressing the anti-apoptotic gene Bcl-2 in these cells\(^3\). We have shown that HUVEC can readily perfuse human skin equivalents \textit{in vivo} and the performance of the EC is significantly improved by overexpression of Bcl-2 (Fig. 1).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Orthotopic transplantation of vascularized human skin equivalents. Hematoxylin and eosin staining of epithelialized and vascularized acellular dermis-based grafts seeded with Bcl-2 HUVEC at 2 (A), 4 (B), and 6 (C) weeks after implantation on to mice (arrows highlight some of the perfused blood vessels). Staining with anti-human- (D) and anti-mouse- (E) CD31 antibodies show that many human EC lined vessels are present at 2 weeks (D) at which time mouse vessels are rare (E) and limited to the edge of the graft\(^3\).}
\end{figure}

We established stringent criteria for determining what constitutes successful transplantation of a vascularized graft, namely the presence of perfused vessels lined by human EC extending 2/3 of the thickness of the dermis, with at least 3 of these vessels per high power field, and the presence of a continuous differentiated epidermis composed of human keratinocytes. By these criteria, approximately 75\% of the grafts seeded with Bcl-2 transduced HUVEC were
successfully transplanted at 2, 4, and 6 weeks after engraftment, compared with 25% of EGFP-transduced controls. Furthermore, Bcl-2 overexpression enhanced vascular maturation resulting in a greater degree of investiture with supportive smooth-muscle cells, a hallmark of vascular stability. Another significant finding was that inclusion of human EC greatly expedited the perfusion of grafts. By two weeks after implantation the grafts were almost exclusively perfused by human endothelial lined vessels with murine vessels limited to the edge of the grafts, indicating that these grafts were almost exclusively perfused by the human vessels at this time point (Figs. 1, 2). Together, these results highly suggest that utilization of Bcl-2-transduced HUVEC in human skin equivalents can expedite perfusion in the critical post-transplantation period, which is likely to translate to better overall graft performance.

Progress has also been made in the incorporation of potential autologous sources of EC into the skin equivalents. Dermal microvascular EC derived from adult skin have been successfully cultured, and when seeded on to epithelialized grafts formed perfused vessels in vivo. Utilization of this cell type revealed an important limitation, namely that the yield of EC from adult tissue is relatively low, ultimately limiting the capacity for expansion in culture. Our experience was that the cells usually lost the capacity to vascularize grafts if they were expanded into more than 3-T75 flasks. The net result is that the area of graft that could be generated was actually less than the size of the split thickness skin graft from which the cells were harvested, therefore use of dermal microvascular EC was not pursued further. On the other hand, we have successfully vascularized skin equivalents with EC derived from both
umbilical cord and adult blood-derived progenitor cells. After use of our selection protocol the acquisition of pure populations of differentiated EC was confirmed by flow cytometric detection of endothelial markers CD31, Tie-2, and TNF-induced expression of E-selectin.

Epithelialized skin equivalents were seeded with both endothelial progenitor cell (EPC) derived EC types, and grafted onto SCID-beige mice. By 2 weeks after transplantation perfusion through human EC lined vessels, reactive with UEA-1 lectin and anti-human-CD31 antibodies, could only be seen in the grafts seeded with human EC, with murine CD 31-reactive vessels limited to the periphery of the grafts. Similarly, grafts not seeded with human EC were essentially avascular after 2 weeks. The resultant human vessels showed evidence of maturation with deposition of human type IV collagen and investiture with smooth-muscle cells/pericytes (as determined by immunostaining directed at smooth muscle α-actin; Fig. 2). Perfusion of the human vessels was confirmed by rapid binding of intravenously injected UEA-1 lectin (Fig. 2), and casting of the vasculature with silicone microfilm. These data demonstrate the feasibility of vascularizing human skin equivalents with a source of autologous EC that can be obtained relatively non-invasively through phlebotomy. In further studies the in vivo function of these vessels will be compared to that of Bcl-2-transduced HUVEC, and further beneficial effects of Bcl-2 transduction in EPC derived EC will be evaluated.

The second specific aim is to examine whether adverse graft-host interaction can occur that may limit the safety and clinical utility of vascularized grafts. The specific focus of this aim is to evaluate whether a) transgenes such as Bcl-2 are tumorigenic, and b) whether the inclusion of EC may increase the likelihood of allograft rejection. We have begun making progress in both parts of this aim. In the initial proposal there was significant gross and histologic evidence that overexpression of Bcl-2 in a SV40-immortalized murine endothelial-tumor model did not increase the risk of angiosarcomas, formation or metastases. We have now confirmed, using highly sensitive reverse-transcriptase PCR with SV40-large-T-antigen-specific primers in lung, liver and spleen, that Bcl-2 does not confer the capacity for tumor micro-metastases. In subsequent experiments a similar analysis will be performed with Bcl-2-transduced human EPC-derived EC. In preliminary experiments currently in progress, SCID-beige mice were reconstituted with human peripheral blood mononuclear cells (PBMC) allogenic to the EC within the grafts, to evaluate allograft interactions.

In summary, in the first year of funding, the majority of experiments proposed in Specific Aim 1 have been completed and experiments addressing the proposed second Specific Aim are well under way.
Publications:
5. Final Reports of ROTRF Grantees

Dr. Simi Ali, Principal Investigator
Prof. John A Kirby, Co-Investigator

The Medical School, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, UK

Anti-Rejection Therapy: Modifying Intragraft Immunity by Specific Blockade of Th1 Cell Recruitment

Specific aims outlined in the grant:
1. Identify the specific amino acid residues required for interaction with heparan sulphate (HS); these residues will then be mutated individually or in combination to generate a non-glycosaminoglycan (GAG) binding RANTES variant.
2. Compare the specific receptor binding capacity of these mutants with wild-type RANTES.
3. Determine whether GAG-binding capacity is a pre-requisite for biological activity using in vitro Ca²⁺-flux and chemotaxis assays.
4. Determine the potential of mutant RANTES for therapeutic blockade of the migration of Th1 lymphocytes.

The chemokines are a family of small chemoattractant proteins that have a range of functions including the activation and promotion of vectorial migration of leukocytes. RANTES/CCL5, a member of the CC-chemokine sub-family, has been implicated in a variety of immune responses. In addition to the interaction of CC-chemokines with their cognate cell-surface receptors, it is known that they also bind to GAGs, including HS. This potential for binding to GAG components of proteoglycans on the cell surface, or within the extracellular matrix, might allow formation of the stable chemokine concentration gradients necessary for leukocyte chemotaxis. In this study, we created a panel of mutant RANTES molecules containing neutral amino acid substitutions within putative, basic GAG binding domains. Despite showing reduced binding to GAGs, it was found that each mutant containing a single amino acid substitution induced a similar leukocyte chemotactic response within a concentration gradient generated by free solute diffusion. However, it was found that the mutant K45A had a significantly reduced potential to stimulate chemotaxis across a monolayer of microvascular endothelial cells. Significantly, this mutant bound to the CCR5 receptor and showed a potential to mobilise Ca²⁺ with an affinity similar to the wild-type protein. These results show that the interaction between RANTES and GAGs is not necessary for specific receptor engagement, signal transduction or leukocyte migration. However, this interaction is required for the induction of efficient chemotaxis through the extracellular matrix between confluent endothelial cells1.
Furthermore, using confocal microscopy we examined the relative expression of GAG species including HS, chondroitin-4-sulphate (C4S) and chondroitin-6-sulphate (C6S) by a cultured human microvascular endothelial cell line. It was demonstrated that HS is expressed more abundantly than either C4S or C6S respectively. Significantly, the potential of HS on these cells to bind RANTES/CCL5 was increased following stimulation with IFN$_{\gamma}$ and TNF$_{\alpha}$. This correlated with the transient increase in the level of N-deacetylase/Nsulphotransferase (NDST-1) enzyme which catalyzes the reaction that initiates sulphation and modification of HS. A series of renal allograft biopsies was examined to determine the function of GAG expression during rejection. In the normal kidney it was found that HS was largely restricted to the tubular basement membranes whilst C4S and C6S were expressed at lower levels within the interstitial tissues. The expression of all three GAGs was increased during acute rejection, but HS remained predominant. Dual colour immunofluorescence demonstrated that CCL5 was associated with HS-rich tubular basement membrane during rejection. This suggests a mechanism for formation of the static chemokine concentration gradients required for vectorial leukocyte migration during the development of rejection-associated tubulitis$^2$.

We generated a non-GAG binding variant of CCL7 (MCP-3) which antagonizes chemokine-mediated inflammation *in vivo*.

In conclusion, this project has allowed us to examine chemokine/GAG interactions as targets for manipulation of immune response through inhibition of the recruitment of specific leukocytes following inflammation. It has allowed us to:

- Demonstrate that interaction between RANTES/CCL5 and cell-surface GAGs is essential for efficient chemotaxis across the endothelial cell layers.
- Demonstrate that there is upregulation for GAGs during renal allograft rejection and that RANTES/CCL5 associates with HS-rich regions suggesting a mechanism for formation of the static concentration gradients.
- Generate a non-GAG binding variant of CCL7 which antagonizes chemokine mediated inflammation *in vivo*.

Therefore we have made progress beyond our original objectives. We can now block recruitment of many cell types rather than specifically block Th1 lymphocytes.

**Publications:**
Additional publications and abstract arisen from this project:


Understanding how antibodies (Abs) can induce injury or enhance resistance to injury is profoundly important in the context of basic immunology, and in the understanding of the effector functions of Abs following solid organ transplantation. The approach we have taken is a classical reductionist one. While it is clear that humans, non-human primates and Gal−/− mice can mount anti-Gal and non-Gal Ab responses after sensitization with porcine organs or tissues, it is impossible to test whether isotypes, specificities and affinities elicit distinct spectra of tissue damage or accommodation in these models. To circumvent these problems, we have developed two tightly regulated models to determine how anti-Gal mAbs induce graft rejection. Thus our focus on both pathogenicity and protective activities of antibodies should be relevant to our understanding of how antibodies affect xenograft function.

Two specific aims were proposed:

1. The first specific aim of this proposal was to confirm and extend our studies by defining the mechanisms of NK cell-mediated hyperacute rejection, and by characterizing the role of NK inhibitory receptors and Class I molecules in regulating this novel form of IgG1-mediated hyperacute rejection.

2. The second specific aim of this proposal was to test whether different states of graft accommodation are elicited by different sub-classes of anti-Gal IgG and to define the molecular mechanisms of anti-Gal IgG1-induced accommodation.

Studies and Results

In the past year (first year of the ROTRF award), we have confirmed and extended our preliminary observations that different IgG-subclasses of anti-Gal antibodies elicit distinct mechanisms of rejection. Using a series of reagents to inhibit complement activation, FcγR-mediated interactions and NK cells, we have confirmed the ability of anti-Gal IgG3 antibodies to mediate complement-dependent hyperacute rejection. In addition, we have confirmed that an anti-Gal-α1,3Gal IgG1 mAb, with a modest ability to activate complement in vitro, has the ability to induce the rapid rejection of Gal−/+ rat heart xenografts in vivo. Investigations into the mechanism revealed that the rejection was dependent on the activation of complement, on FcγR-mediated interactions, and on the presence of NK cells in the recipient Gal−/− mice.
Inhibition of any one of these events resulted in the abrogation of IgG1-mediated rejection. These observations support a model of IgG1-mediated rejection that is uniquely dependent on FcγR and NK cells.

In the past year, we have also characterized the in vitro and in vivo activities of anti-Gal IgG2a and IgG2b. Only anti-Gal IgG3 and IgG1 hybridomas were generated from spleen cells of Gal⁻/- mice bearing rejected Gal⁺/⁺ Lewis rat hearts. Using sib-selection of natural switch mutants, we generated anti-Gal IgG2a and IgG2b hybridomas.

We are currently breeding mice that are deficient in C3, C5 or C6 with Gal⁻/⁻ mice (Specific Aim 1). We have F1 mice for all the three crosses. We anticipate having double-deficient homozygous mice for C3, C5 or C6 and Gal by summer 2004, and functional transplant studies will be initiated when sufficient mice are available. In addition, we have imported FcγRI and FcγRIII-deficient mice from Dr. J.S. Verbeek (Leiden, The Netherlands). We are currently breeding the mice with the Gal⁻/⁻ mice. We anticipate having double homozygous mice for FcγRI or FcγRIII and Gal by early summer 2004, and functional transplant studies will be initiated when sufficient mice are available. We anticipate that these studies will be completed by the end of 2004, and we hope to have a manuscript for submission by early 2005.

Publications:
1. Yin D-P, Zeng H, Ma L, Shen J, Byrne GW, Xu H, Chong AS. Natural killer (NK) cells mediate IgG1-dependent hyperacute rejection of xenografts. (Submitted)
Interaction of OX2 with its Receptor Controls Organ Rejection

Several years ago, my laboratory described and began characterization of the novel molecule, OX2 (now referred to as CD200), and its receptor, CD200R, which we hypothesized was important in immunoregulation in transplantation, autoimmunity, infection and tumor immunity. The research proposal funded by ROTRF concerned experiments designed to investigate this immunoregulation further, and in particular to provide further “proof-of-principle” experiments documenting the role of CD200 in immunoregulation by developing several new reagents, including anti-CD200R(s), and transgenic mice expressing CD200.

1. Characterization of mAbs to CD200R and their use, with CD200RFc, in manipulating transplant rejection.

Rationale: While our first studies suggested that CD200 was important in immunoregulation in vivo and in vitro, its molecular structure implied it acted primarily following engagement of the receptor CD200R on target cells. In support of this hypothesis, we showed that both whole and Fab anti-CD200R blocked graft prolongation following increased CD200 expression, while soluble CD200Fc was a potent immunosuppressant. CD200R+ cells were identified in an LPS-stimulated macrophage population, and in ConA-activated T cells, and we were able to show that F4/80+ CD200R+ splenic macrophages were immunosuppressive in concert with CD200Fc. These observations prompted us to prepare and characterize the properties of mAbs to CD200R expressed on different tissues/cells.

Results: In association with Dr. P. Mardsen we were able to define, by molecular cloning, heterogeneity in both the mouse and human CD200R gene family. At least 4 major murine CD200Rs were identified (CD200R1-4), with additional subfamily heterogeneity (CD200R31-3). All members of the murine family have been sequenced (at the DNA and amino acid level), and mAbs and cDNA vectors prepared for each. The chromosome map for the murine CD200R family is shown (Fig. 1).
Analysis of tissue expression of the products of these various gene members was performed using real-time PCR, and the results clearly showed discrete patterns of tissue-restricted expression for the different CD200Rs, including unequivocal evidence for CD200R1 expression on activated T cells. Despite a conflicting report in the literature\(^5\) our data showed that all CD200Rs used CD200 as a ligand (Fig. 2). We have suggested that the discrepancy between our data and that of others represents a failure on the part of other groups to identify the full-length clones for other CD200R isoforms\(^6\).

**Figure 1:** Genomic organization of CD200/CD200R gene cluster on mouse chromosome 16 located between markers D16MIT41 and D16MIT138. CD200 is located downstream from its receptors (CD200R’s) and has an opposite direction. RPCI-22 BAC clones 209M03, 402D20, 195O24, and 196K10 spanning this region are indicated.
2. Analysis of transplantation of organs from CD200-transgenic mice (CD200\textsuperscript{tg/tg}) vs littermate non-tg controls into normal or CD200 KO recipients, with/without use of anti-CD200 (or anti-CD200R) mAbs.

**Rationale:** As we had predicted, the mouse CD200 KO has been reported to show an increased susceptibility to spontaneous autoimmune disease\(^7\). Accordingly, we hypothesized that if we constructed a mouse CD200-transgenic line, we would find that organs from CD200-transgenic mice (CD200\textsuperscript{tg/tg}) would be less easily rejected than organs from (+/+) mice, and that CD200-expressing cells would be inefficient in induction of alloreactivity.

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**Figure 2.** FITC-CD200Fc binding to CD200R-transfected COS cells. FACS analysis of binding of FITC-CD200Fc to transfected COS7 cells expressing different CD200R isoforms. FITC-CD200Fc was prepared in standard fashion. \(5 \times 10^4\) COS cells were incubated with 5 µg/ml FITC-CD200Fc for 45 minutes at 4°C, with (solid blue line) or without (solid grey line) a 20-fold excess of non-FITC-labeled CD200Fc, washed three times (4 minutes at 800 rpm), and resuspended in 0.5 ml for FACS analysis. The solid black line shows staining with FITC-rat IgG. Control cells were COS cells transfected with “empty” pBK vector (panel a). Data are representative for 1 of 2 studies.
Results with CD200\textsuperscript{tg/tg} mice: Transgenic mice have been constructed expressing full-length mouse CD200 under control of a Tet-on promoter, on a C57BL/6 background (N10 back-cross). F1 hybrids were made by crossing with an rtTA-tg line (again on a C57BL/6 background) showing inducible expression of CD200 in multiple tissues in the presence of doxycycline. Data (using mice tested at the N5 generation of backcrossing) with splenocytes of doubly-transgenic F1 mice, stimulated with allogeneic cells \textit{in vitro} in the presence of DOX, are shown (Fig. 3). It is apparent that transgenic mice from 3 different founder lines, over-expressing CD200 (in presence of DOX) produce predominantly type-2 cytokines, not type-1 cytokines, \textit{in vitro}, unlike non-tg mice (rtTA).

\begin{figure}
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\caption{Altered cytokines production following exposure of cells of N5 CD200\textsuperscript{tg} mice to doxycycline. Altered induction of cytokine production (increased IL-4, decreased IFN\textsubscript{γ}) measured by ELISA in 40 hours cultures following allostimulation of spleen cells from doubly transgenic (rtTA\textsuperscript{tg} and TRE-CD200-GFP\textsuperscript{tg}) F1 mice in the presence of doxycycline. MLR cultures were initiated using BALB/c spleen stimulator cells, and individual responder spleen cells from three different founder lines, after typing PBL from those spleen donors for expression of TRE-CD200-GFP in the simultaneous presence/absence of the rtTA\textsuperscript{tg}. The number of donors used from each founder line is shown in parentheses. Data show arithmetic mean (+SD) cytokine concentration (pg/ml) for the different groups. Qualitatively equivalent patterns (to IFN\textsubscript{γ}, IL-4) were seen for IL-2 and IL-10 respectively (data not shown for clarity).}
\end{figure}

Finally, we have asked in a skin allograft model, whether forced expression of CD200 in grafts does indeed lead to increased graft survival, as we had predicted. Data confirm that grafts from CD200\textsuperscript{tg/tg} mice survive longer that grafts from non-tg littermate controls, or third-party grafts (Fig. 4).
3. Transplantation of organs from CD200tg/tg vs non-tg controls into normal or CD200RKO recipients, in the presence/absence of anti-CD200 (or anti-CD200R) mAbs and CD200Fc.

Rationale: CD200KO mice show an increased susceptibility to spontaneous autoimmune disease⁷. Since immunoregulatory signaling depends upon engagement of cell surface CD200R, we predicted:

i) that immunoregulatory defects in CD200 KO mice would be “rescued” by infusion of CD200Fc, and

ii) the phenotype of CD200R KO mice would be similar to (CD200KO), but no “rescue” would occur with CD200Fc.

Organs from CD200-transgenic mice (CD200tg/tg) and (+/+) recipients should show equal tendency for rejection in CD200RKO mice (-/-). Graft rejection in CD200R KO mice would be unaffected by anti-CD200R or CD200RFc, though this would improve survival in (+/+). Infusion of CD200Fc into (+/+) or CD200RKO mice would lead to immunomodulation of graft rejection only in (+/+) mice, and this would be modified by a decoy receptor, CD200Fc.

**Figure 4.** Forced expression of CD200 in BL/6 skin grafts enhances survival in C3H. Groups of 8 C3H recipient mice received C57BL/6 skin grafts from normal mice, non-transgenic F1 mice (rtTA) and double transgenic (CD200-rtTA) mice. All recipients received DOX in their drinking water. No other immunosuppression was used. Graft survival was followed daily.
**Results to Date**

Our original strategy to construct a CD200KO mouse was based on our earlier (erroneous) supposition that only one gene for CD200R existed. New data (Fig. 1) shows this is a complex gene family, with redundancy in the expression/function of family members. We have now re-designed our approach, and have constructed 2 vectors which will lead (independently) to deletion of either CD200R1 or CD200R2-4 (in each case under control of temporally and tissue restricted expression of lox/p sites). The constructs have been delivered to Xenogen, and we anticipate delivery of our first chimeric KO mice in ~ 3-4 months. Following construction of the KO we propose that using our cDNA constructs for individual CD200R gene members for transgenic insertion/rescue, we will be able to investigate the functional potential of individual CD200R gene products of different members of this family.

**Publications:**

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

The basis of this ROTRF Grant has been to exploit the inherent specificity of the T cell receptor (TCR) for the major histocompatibility complex (MHC) in order to mediate specific suppression of alloreactive T cells. Exploiting this specific interaction has been made possible by previous discoveries that the MHC can be made soluble in dimeric form. We have demonstrated that this dimeric form is sufficient to interact and trigger the TCR (whereas the monomeric form is not) and have undertaken to study whether interactions between the dimeric MHC and the TCR of alloreactive T cells \textit{in vitro} and \textit{in vivo} would suppress the T cells in a specific manner resulting in graft prolongation. Furthermore, we are studying if an increased efficacy of suppression can be achieved by linking the dimer to a molecule that will deliver a negative signal. Finally, we have interrogated the mechanism of the efficacy of the dimer in its interaction with T cells.

The ROTRF award has enabled us to demonstrate that, \textit{in vivo}, MHC dimers inhibit, with peptide specificity, graft-reactive T cells\(^1\). The suppression of the graft-reactive T cells resulted in the prolongation of the graft. Mechanistically, it was shown that MHC dimers activate the alloreactive T cells as determined by phosphorylation patterns of key molecules. \textit{In vitro}, dimer-mediated activation was shown to stimulate proliferation and even to result in cytolytic effector function. It was necessary to reconcile the results of prolonged graft survival and the activation of graft-specific T cells. It has been shown numerous times that overactivation of T cells with antigen or superantigen results in the collapse of the population of responding T cells. In the case of the \textit{in vivo} dimer experiments summarized here, the dosing regimen of the dimer (every other day, total of 4) resulted in the ultimate collapse of the population of the graft-reactive T cells explaining the observed result of graft prolongation. In contrast, we showed that two administrations of dimer, one week apart, actually increased the population of antigen-specific T cells accelerating graft destruction. In these experiments, cells from animals dosed in this manner were shown to possess antigen-specific cytolytic effector function \textit{ex vivo}.
These observations compelled the interrogation of the mechanism of dimer-mediated T cell activation. This interrogation was based on two separate principles. One, given that the dimer construct possesses an Ig scaffold, does it play a role in augmenting the response potentially through its interaction with Fc receptors of antigen presenting cells (APC)? Two, what role, if any, might the costimulatory molecules B71 and B72 of APC play in augmenting the response? To investigate the first question we utilized mice lacking Fc receptors (Fc−/−). Using irradiated splenocytes from normal and Fc−/− mice enabled a comparison of the potential role of Fc receptors in “facilitating” the presentation of MHC dimers to T cells. Utilizing T cell proliferation as a readout, it was observed that MHC dimer in the presence of APC possessing Fc receptors stimulated a 3-fold increase in proliferation of antigen-specific T cells over MHC dimer in the presence of APC lacking Fc receptors. This result indicted that the Ig moiety of the dimer served as more than a mere scaffold. This observation also suggested that genetic manipulation of the Ig moiety to increase its affinity for the Fc receptor may increase its efficacy in presentation to T cells.

The role of costimulatory molecules, B71 and B72 was examined in a similar fashion. In this case, stimulation of antigen-specific T cells by MHC dimer was examined in the presence of irradiated APC with and without B71,2 molecules. As in the case above with Fc receptors, B71,2 molecules were shown to augment dimer-mediated activation of T cells. The presence of B71,2 on APC resulted in a 3-fold greater stimulation of T cells.

The observation that the presence of costimulatory molecules augmented the effect of MHC dimer brought about the postulation that the covalent inclusion of costimulatory molecules on the MHC-dimer construct may increase the strength of the activation signal to T cells. As a proof-of-principle, we have made a construct comprising MHC-dimer and, for costimulation, anti-CD28. The heterodimeric MHC/co-stimulatory construct stimulated a 5-8-fold increase in proliferation over the homodimeric MHC-Ig of splenocytes from a wild-type mouse. Interestingly, provision of the two molecules in soluble form unlinked, stimulated 8-fold less proliferation. These results indicate that a more rapid collapse of a graft-specific T cell population may be achieved with a heterodimeric construct.

In summary, the ROTRF award has enabled the finding that MHC-dimeric constructs can effectively activate graft antigen-specific T cells. This activation by repeated administration at close intervals results in the collapse of the antigen-specific population. Such collapse results in graft prolongation. These results indicate an effective means to suppress a graft-specific response without the side effect of broad-based immunosuppression. The nature of the MHC-dimer construct, in particular, the Ig moiety, was found to fortuitously facilitate the activity of the dimer presumably through multivalent presentation on APC. This finding indicates that mutating the Ig moiety to obtain stronger Fc receptor binding may further enhance the activity of the dimer. This work has also shown that costimulatory molecules were found to
augment MHC-dimer activity. Heterodimeric constructs comprising Ig, MHC, and costimulatory molecules should provide a means of both specificity and efficiency in collapsing graft-specific T cell populations. In all, this work has demonstrated that **MHC-dimers have the potential to suppress graft-specific T cells in an antigen-specific manner thus allowing a departure from broad-based immunosuppression.** This work has also elucidated mechanistic aspects of this suppression. Furthermore, the work presented here indicates the potency and specificity of a heterodimer that has both MHC and costimulatory moieties.

**Publications:**
2. O’Herrin SM. *In vivo* activation of tumor-specific CD8+ cells that reject tumor. (Abstract).
Polyomavirus BK, JC, and SV40 in the Kidney: Pathogenesis, Early Diagnosis and Improving Graft Outcome

Background
BK virus nephropathy (BKVN) is an emerging infectious complication that develops in up to 8% of renal transplantation recipients. It is characterized by progressive graft dysfunction, which may be confused with acute cellular rejection, and inappropriately treated with steroids. Currently, the clinical management of BKVN is very problematic. It is customary to reduce the immunosuppression and allow the host immune response to mount a successful anti-viral immune response. However, this strategy, even if effective in decreasing the viral load, puts the allograft at risk for acute rejection. When acute rejection develops, clinicians are hesitant to increase the immunosuppression, and risk flare up of viral infection. The resulting therapeutic dilemma results in an unfavorable graft outcome with frequent graft loss. This disheartening situation is unlikely to improve until clinically effective anti-BKV polyomavirus drugs are developed.

Objectives
This grant sought to screen the following compounds previously shown to have anti-viral activity against the polyomavirus family of viruses: (a) Cidofovir, (b) Vitamin A derivatives, (c) Inhibitors of cellular DNA topoisomerase I, (d) Inhibitors of DNA gyrase (topoisomerase II), and (e) phenothiazines.

Experimental Approach
Compounds were screened for their ability to (a) reduce viral replication in culture, as assessed by quantitative PCR for BKV DNA, or (b) suppress viral T-antigen production in a flow cytometric assay. Compared to conventional assays based on plaque reduction, hemagglutination activity, Southern blots, and measurements of DNA synthesis, our proposed assays offer the following advantages: (a) The assays are less laborious and time consuming, (b) The techniques emphasize collection of quantitative data which are less prone to subjective interpretation, and (c) The assays avoid the need to work with bulk quantities of cultured cells and radioactive probes.
Results

1. Cidofovir:
Cidofovir is a nucleotide analog that inhibits DNA-polymerase-mediated incorporation of deoxycytidine (dCTP) in several DNA viruses, including BKV\textsuperscript{11}. This drug has been empirically used in BKVN, and several patients with apparent clinical response to this drug have been reported\textsuperscript{12}. However, no randomized clinical trials have been conducted.

1a. Cidofovir sensitivity studies:
In the quantitative PCR assay, cidofovir showed a SI of 2.45+/−0.7, EC\textsubscript{50} of 39.1+/−27.6, and IC\textsubscript{50} of 76+/−41.6 µg/ml respectively (mean +/- SE, n=5). These \textit{in vitro} experiments confirm that cidofovir possesses anti-BKV properties, although the selectivity index is low, and its known nephrotoxicity continues to make clinicians reluctant to use this drug freely in kidney transplant recipients.

1b. Mechanism of action of cidofovir:
In flow cytometric experiments, WI-38 cells treated with 100 \textmu g/ml and 10 \textmu g/ml cidofovir showed BKV T-antigen expression in 40.4+/−10.1% (mean +/- SD, n=5) and 52.4+/−7.8% cells, respectively, Untreated cells showed T-antigen expression in 59.1+/−7.8 % cells (p=0.011 control vs 100 \textmu g/ml cidofovir, student’s t-test). Thus, cidofovir causes reduction in BKV T-antigen expression when incorporated in the culture medium at high concentration. The action of cidofovir on human viruses is generally regarded to be mediated by its inhibition of DNA polymerase (an enzyme whose coding machinery is lacking in the BKV genome). Our data showing inhibition of T-antigen expression suggests additional potential mechanisms specific for the BKV life cycle. T-antigen is known to regulate the initiation and elongation steps of viral DNA replication through a number of intrinsic biochemical activities\textsuperscript{13}.

1c. Development of cidofovir resistance:
One flask of WI-38 cells infected with BKV several months ago has become resistant to the action of cidofovir. Greater than 90% of these cells now express the VP-1 antigen. DNA extracted from this cell line has been subjected to whole genome sequencing. Although the non-coding control region of the virus shows rearrangements compared to the Gardner strain of BKV originally used to infect these cells, this is probably an artifact of prolonged culture, as has been repeatedly observed in the published literature. The coding sequences in the viral agnoprotein, viral capsid, and T-antigen regions do not show any remarkable changes. It is likely that the cidofovir resistance observed by us has a biochemical rather than genetic basis. However, the actual mechanism remains to be determined.
1d. Mechanism of cidofovir nephrotoxicity:
A significant problem associated with the use of cidofovir is nephrotoxicity, which makes clinicians very hesitant to use this drug in renal transplant patients, whose kidney has already been damaged by viral infection and multiple episodes of acute rejection. In an attempt to understand the mechanism of cidofovir nephrotoxicity, we performed Affymetrix U133 DNA microarray chip analyses to compare the genes expressed in cidofovir treated (100 μg/ml x 7 days, set up in triplicate) with control cells (also set up in triplicate) that received no drug therapy. All chips were scaled to a median intensity of 150, and genes designated as ‘absent’ in all 6 samples were discounted to exclude low quality data. We also discarded any genes absent in half or more of the samples (unless the absence was confined to the drug therapy group). The intensity of the remaining gene signals was log transformed and an F-test (p< 0.05) performed to compare the drug treated and control cells with regards to the genes, which showed the greatest variation across all samples. This allowed compilation of a list of the most changed genes in the cidofovir treated group. Gene annotation was performed with OntoExpress, a web based ontology search engine.

Preliminary analyses indicate that the principal genes upregulated by cidofovir are insulin-like growth factor binding protein 3, chemokine (C-C motif) ligand 2, coated vesicle membrane protein, alcohol dehydrogenase 1B, lumican, lysyl oxidase, protein phosphatase 1, and thrombospondin 1. Important downregulated genes include fibrinogen-like 2, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, leukemia inhibitory factor, core promoter binding protein, BCL2-related myeloid leukemia sequence 1, jun B protooncogene, and inhibitor of DNA binding 1, dominant negative helix-loop helix protein. Thus far, we have not been able to find any genes specifically expressed in the kidney. However, additional analyses of the data will be performed using alternate normalization algorithms in an effort to tentatively identify kidney genes relevant to the mechanism of cidofovir nephrotoxicity.

2. Leflunomide:
Testing for leflunomide [5-methylisoxazole-4-(-trifluoro-methylcarboxanilide)] was not initially included in our initial grant proposal. However, abstracts presented by Dr. James Williams and Michelle Donaldson at the last American Transplant Congress and American Society of Nephrology meetings indicated that empiric use of this drug in BKV nephropathy has led to encouraging clinical results. Hence, we gave high priority to the testing of this agent in our in vitro system. In quantitative PCR, the SI, EC50, and IC50 for leflunomide were 0.97+/−1.05, 44.26 +/- 13.16, and 39.77 +/- 6. 92 μg/ml respectively (data expressed as mean +/- SE, n=3). These initial data indicated that the anti-viral effect of leflunomide is accompanied by significant cytotoxicity and the selectivity index is apparently even lower than cidofovir. Following a suggestion from a virology colleague, we repeated experiments after lowering the multiplicity of infection (MOI) from 200:1 to 20:1. At this lower MOI, an increase in the sensitivity of BKV to leflunomide was observed, and the EC50 was lowered to 8.5 μg/ml. We also tested the
compound A771726, which is the active metabolite of leflunomide, and determined its EC50 to be 17 μg/ml. Thus, it does not appear that using A771726 will have any great advantage over using the parent drug leflunomide. We are now planning experiments seeking to determine if the anti-BKV effect of leflunomide is related to its known ability to inhibit the incorporation of pyrimidine nucleosides in replicating DNA. In addition, we will investigate the effect of leflunomide on the expression of specific viral proteins, namely, T-antigen, viral capsid protein VP-1, and agnoprotein.

While it is encouraging that leflunomide possesses anti-BKV activity, concerns have recently been raised about the occasional occurrence of serious hepatotoxicity during its clinical use. We have, therefore, requested Fujisawa Corporation to supply us with the drug FK778, a derivative of leflunomide for anti-viral screening. Our request has been approved and a Materials Transfer Agreement is currently being processed by the University of Pittsburgh to facilitate transfer of this drug to our laboratory.

3. Retinoic acid:
Vitamin A derivatives inhibit polyomavirus replication in mouse embryo cells, and in transformed fibroblasts, putatively due to decreased transcription of the c-fos proto-oncogene. No clinical trials have been conducted in patients with BKVN. In the quantitative PCR assay, all-trans-retinoic acid showed a SI of 0.7 +/- 0.07 μg/ml, EC50 of 0.22 +/- 0.007 μg/ml, and IC50 of 0.16 +/- 0.01 μg/ml respectively (mean +/- SE, n=2). The low selectivity index indicates that anti-viral activity is not observed until these drugs reach toxic concentrations in the culture medium. In flow cytometric experiments, 15 ng/ml to 1.5 μg/ml of all trans-retinoic acid and 25-500 ng/ml of 13 cis-retinoic acid did not show any inhibition of T-antigen expression.

4. Camptothecin derivatives:
These were tested because of their ability to inhibit cellular DNA topoisomerase I, an enzyme that facilitates nicking and untwisting of viral DNA strands during viral replication. No consistent inhibition of T-antigen was observed with concentrations in the range 0.01 to 0.1 μM. Concentrations exceeding 30 μM caused severe toxicity and cell death. This class of compounds does not look promising as an anti-BKV agent.

5. DNA gyrase inhibitors:
DNA gyrase (DNA topoisomerase II) is an enzyme that facilitates viral replication by relaxing the supercoiled structure of polyomavirus DNA. It was, therefore, hypothesized that inhibitors of this enzyme would inhibit viral replication. In quantitative PCR assays, the SI, EC50, and IC50 for coumermycin were 0.67 +/- 0.19, 12.55 +/- 2.13, and 9.17 +/- 3.11 μg/ml respectively (mean +/- SE, n=4). The disappointing selectivity index is at variance with published data.
that this group of compounds can suppress BK virus replication in Vero cells\textsuperscript{18,19}. However, Vero cells are derived from monkey kidney tissue, whereas we performed our experiments with a human cell line.

6. Phenothiazines:
Chlorpromazine has been reported to inhibit clathrin-dependent endocytosis of polyomavirus JC in a human glial cell line\textsuperscript{20}. The extent to which this mechanism of cellular entry plays a role in the life cycle of BKV has not been determined. Ultrastructural studies performed with the support of this grant show that BKV particles associate with vesicles resembling caveolae rather than clathrin coated pits (data not shown). Confocal microscopy has confirmed the presence of caveolin in virus-associated vesicles in infected WI-38 cells. In these experiments, WI-38 cells were grown in Lab-Tek cell chambers (50,000 cells per chamber) and infected with BKV (1E+07 viral particles per chamber). On day 21, the cells showed marked cytopathic effect, and were double stained using rabbit anti-BK virus VP-1 and mouse anti-human caveolin 1 antibodies. Alexa 488-labeled goat anti-rabbit IgG and Cy3-labeled goat anti-mouse IgG antibodies were used in the second step to obtain antigen specific fluorescence. In figure 1 taken at 40x magnification, VP-1 expression produces a green fluorescence in 3 different microscopic fields (left panel) while caveolin generates a red signal in the same microscopic fields (center panel). Superimposition of these labels generates a yellow signal (right panel) indicative of colocalization of both proteins.
Conclusion

The data collected by us provides conceptual support for the current clinical practice of administering cidofovir and leflunomide to patients with BK virus nephropathy. The challenge now is to determine dosing regimens that are effective while minimizing toxicity. We also need controlled trials to determine the relative contributions of anti-viral treatment and reduced immunosuppression in the apparent observed efficacy of these compounds in the clinical setting. The assays developed during this study will be useful in discovering additional anti-BKV compounds, hopefully with even better selectivity index than cidofovir and leflunomide. Both techniques described here are potentially adaptable to high throughput screening technology in a 96 well format. Such an adaptation would allow anti-BKV screening of several libraries of chemical compounds currently available to the research community.
Publications:


Correction of Diabetes Using Insulin-Secreting Liver Cells

Background
Type I diabetes mellitus is caused by the autoimmune destruction of pancreatic β cells. Current treatment of the disease requires daily insulin injections to control blood glucose levels. Despite rigid maintenance and monitoring of blood glucose levels, the chronic complications of diabetes still develop. Transplantation of pancreatic tissue is restricted by the scarcity of donors and requires patients to be immunosuppressed. This problem could be overcome by genetically engineering an "artificial β cell" to mimic the function of pancreatic cells. The target cells in this proposal are liver cells which express glucokinase and the glucose transporter GLUT 2, as do pancreatic β cells. Studies by our group have shown that the introduction of the insulin gene into two liver cell lines1,2 (HEP G2ins/g and Huh7ins) has resulted in synthesis, storage and regulated secretion to glucose and other metabolic substances, and correction of diabetes in a mouse model (Huh7ins)². Secondly, we have perfected the stable, long-term expression of genes in liver cells using a viral vector delivery system.

Aims
Using our efficient viral transduction system we aimed to determine if:

1. The expression of the human insulin gene together with other factors (insulin cassette) induces storage of insulin in primary liver cells in vitro (0–18 months).
2. The expression of the insulin cassette to primary hepatocytes in vivo can correct diabetes in rats (13–30 months).
3. We can achieve better physiological metabolic control of insulin production, if we transfer insulin under the control of a glucose- and insulin-sensitive promoter into primary liver cells. We will determine if a metabolically sensitive promoter driving the expression of insulin cDNA can regulate human insulin secretion from primary hepatocytes in response to glucose exposure both in vitro and in vivo (13–36 months).
Research Outcomes

It can be seen from the above aims that originally funding was sought from the ROTRF to complete a three-year programme of research. As we were given a one-year proof-of-concept grant, we prioritised the aims and have worked primarily on aim two and to a lesser extent on aim 3 as we believed that if we could show reversal of diabetes in an animal model and some useful function of the inducible promoter system this would prove the concept that our system could be developed into a useful therapeutic regime to tackle Type I diabetes.

1. Reversal of diabetes in an animal model:
Using the vector we have delivered the insulin cassette into the portal circulation of streptozotocin (STZ)-induced diabetic Wistar rats, with the following results:

![Figure 1: Photomicrographs of (A) A section of the liver of a STZ-diabetic Wistar rat transduced with the viral vector alone for a period of 2 months, (B) A section of the liver of a Wistar rat transduced with the viral vector containing the insulin cassette for a period of 2 months. Immunoperoxidase for insulin, positively stained cells are brown.](image)

As can be seen from figure 1B a large number of the cells in the liver have been converted into cells that stain positively for insulin. Unlike other studies that have engineered β cell neogenesis from liver cells\(^4\), the insulin-containing cells are not isolated to the area surrounding the portal circulation, but are scattered throughout the liver tissue (Fig. 1B).
Preliminary electron microscopy indicates the development of typical insulin secretory granules (Fig. 2A, B), without the need for dual expression β cell stimulating hormones as used by other workers\(^4\).

Insulin storage following acid/ethanol extraction was 17,625.8 ± 2.1 ng (\(n=3\)) per liver, which may be compared to 61,000 ng for a non-diabetic rat pancreas. This is 35% of the insulin content, considerably higher than Kojima et al\(^4\) who found mouse livers transduced by adenoviral vectors which expressed NeuorD and betacellulin stored 20% of a non diabetic mouse pancreas and Ber et al\(^3\) who found mouse livers transduced by adenoviral vectors expressing PDX-1 stored 1–3% of the insulin in non-diabetic mouse pancreas. In human islet transplantation, the minimum number of islets required for insulin independence is about 9,000 islet equivalents per kg, or 600,000 islets for a 70 kg person. This corresponds to 30–50% of the number of islets in the pancreas of a non-diabetic individual\(^5\). In islet transplantation all the islets do not survive, so the insulin content of our cells is well within the acceptable limits for insulin independence.

RT-PCR analysis indicated that there was no evidence of insulin expression in other body tissues, indicating that it was specific and isolated to the liver.

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**Figure 2.** Transmission electron micrograph (A) and immunoelectron micrograph (B) of insulin producing cells in the liver of a STZ-diabetic Wistar rat transduced with the viral vector containing the insulin cassette. Secretory granules (250-300 nm) are seen in the cytoplasm (A) The immunoelectron micrograph (B) shows secretory granules labelled with 10 nm gold particles to show the location of insulin. The gold label is seen only over the secretion granule, which is surrounded by a pale halo. The membrane of the secretory vesicle is unstained and the granule is pale, as only uranyl acetate staining was performed, identical to what is seen in the β cell line Nit-1 cells (positive control) (C).
Reversal of diabetes has been seen within five days of administering the vector (Fig. 3) and has been stable for over 8 months. There is thus far no evidence of autoimmune attack.

Figure 3. Serum glucose of STZ-diabetic Wistar rats treated with vector alone or the vector containing the insulin cassette (S. E. M. n=5). Blood glucose levels fell rapidly to subnormal levels on addition of the insulin cassette and by day 5 were not significantly different to non-diabetic controls, reversal of diabetes was maintained for 240 days. STZ-diabetic rats treated with the vector alone remained diabetic until sacrifice at day 60.

Reversal of diabetes has been seen within five days of administering the vector (Fig. 3) and has been stable for over 8 months. There is thus far no evidence of autoimmune attack.

Figure 4. Intravenous glucose tolerance test: after an overnight fast non diabetic control rats and STZ-diabetic rats treated with vector containing the insulin cassette were given an intravenous dose of glucose (0.5 g/kg body weight), blood glucose levels were measured over 120 min. There was no significant difference between control and treated rats (n=3).
Intravenous glucose response curves generated for the transduced animals also appear normal (Fig. 4).

There was no general pancreatic transdifferentiation observed in the liver tissue, which is likely to be desirable as it is very likely that the expression of an array of endocrine and exocrine function, may perpetuate autoimmune attack. Indeed it has been shown that autoimmune islet destruction in spontaneous Type I diabetes is not exclusive to β cells. By contrast insulin-secreting pituitary and liver cells were not attacked in non-obese diabetic (NOD) mice or in a culture system.

Albumin, aspartate transaminase and alanine aminotransferase levels were normal, indicating that the transduction and expression of insulin does not adversely affect normal liver function.

Thus we have accomplished the stable reversal of STZ-diabetes by the transduction of only the insulin cassette and maintained expression for over 8 months proving the concept that our strategy is a viable one. We are currently completing some experiments and our technique is being prepared for a provisional patent application prior to publication.

2. Glucose–regulated production of insulin:

We have seen in HEP G2ins/g and Huh7ins cells that when glucose reaches the cells, insulin is secreted on a minute-to-minute basis from the storage granules. It would appear from our preliminary results that we have induced significant storage in primary liver cells and regulated secretion of insulin occurs in the primary liver cell model, without any other intervention. However, using funding from ROTRF we have also tested a glucose- and insulin-sensitive promoter. This promoter has been constructed by inserting glucose-responsive elements from the rat L-pyruvate kinase (LPK) gene into the insulin-sensitive, liver-specific rat insulin-like growth factor binding protein (IGFBP-1) promoter, which may be useful in fine regulation of the level of glucose responsiveness. Its function has been verified in cultured liver cells (hepatocytes). Hepatocytes were transduced and cultured overnight in serum and insulin-free medium supplemented with lactate (10 mM) or increasing amounts of glucose (0–40 mM). Insulin in the conditioned medium was measured by radioimmunoassay (RIA). Confluent hepatocytes in a 30 mm dish cultured in the presence of lactate alone produced 2.3 ± 0.4 mU/ml insulin (n = 6, SEM). By comparison, cells exposed to 30 mM glucose produced 35 ± 3.9 mU/ml insulin during the same period. There was a correlation between glucose and insulin secretion with an ED50 of approximately 20 mM glucose. We have confirmed processing of proinsulin to mature insulin by measuring human c-peptide using a commercial RIA. Thus, the promoter is capable of conferring metabolic responsive insulin production from hepatocytes. As seen in a similar system, glucose (5–25 mM) stimulated and insulin (10^{-10}-10^{-7} M) inhibited insulin production in primary hepatocytes. In this study our constructs also increased protein synthesis in response to glucose within an hour. This is within the time frame that subcutaneous insulin
injections increase serum insulin. Lu et al\textsuperscript{12} reported stimulated insulin secretion following exposure to cAMP analogues within 15 min using transcriptional control with no storage present. However, we know that insulin-secreting cell lines are capable of much quicker responses when storage is present\textsuperscript{1,2}, and we could expect the same from primary hepatocytes that store insulin. This promoter is currently being patented.

We have thus completed a number of the aims for the above project in a short amount of time, and we wish to continue our studies in an autoimmune model of Type I diabetes in nonobese diabetic mouse.

\textbf{Publications:}


The objectives were:
1. To take advantage of the precise and hierarchical identification of clones with an altered repertoire in tolerant recipients to test the hypothesis that these clones are instrumental in the tolerance process in experimental models or in the clinic.
2. To understand the mechanisms underlying the regulation of $\beta$ chain mRNA in the direct allore cognition pathway.

A potential role for TCR-altered clones in the induction and maintenance of allograft tolerance.

A major aim of the original application was to study whether the V$\beta$ transcriptome topologies differ in a model of operationally tolerant drug-free patients.

1. Drug-free “tolerant” kidney recipients and patients under minimal immunosuppression present a strong and altered blood T cell clonal regulation. Immunosuppression exposure in transplantation is linked to an increased incidence in malignancies, infectious diseases and metabolic disorders. Therefore, interruption of immunosuppression would represent a major advance. Whereas most of the patients who discontinue immunosuppression reject their graft, a small cohort do not, suggesting that immune non-responsiveness can be achieved in the clinic. Because this “operationally tolerance” may result from an active regulation, we aimed at identifying T cell selection/activation in blood of “tolerant” patients. V$\beta$ transcriptome$^1$ was analysed [complementarity determining region 3 (CDR3) length distribution (LD), V$\beta$/HPRT transcript] in blood of “operationally tolerant” (Tol) patients [drug-free or under low dose of steroid monotherapy ($\leq 10$ mg/kg)] for more than 3 years and compared to normal individuals (NL), to patients with stable graft function under immunosuppression (Sta) or with chronic rejection (CR). Cytokine transcript accumulation was measured on sorted selected T cell
populations from the different groups by real-time PCR. We showed that blood of Tol recipients exhibited a very unusual TCR pattern combining strong Vβ transcript accumulation \((p < 0.001)\) and altered CDR3-LD \((p < 0.001)\). T cells from families with high Vβ/HPRT ratio and skewed CDR3-LD sorted from Tol patients using corresponding anti-Vβ antibodies were characterized by a lack of Th1/Th2 cytokine transcripts \((p < 0.05)\), suggesting a state of hyporesponsiveness or anergy\(^2\,^3\).

Figure 1. T cells from families with high Vβ/HPRT ratio and skewed CDR3-LD sorted from Tol patients using corresponding anti-Vβ antibodies were characterized by a lack of Th1/Th2 cytokine transcripts \((p < 0.05)\), suggesting a state of hyporesponsiveness.

2. Drug-free tolerant kidney recipients display a higher number of peripheral CD25\(^-\)CD4\(^+\) T cells expressing the CCR4\(^-\), CTLA4\(^-\), GITR\(^-\) and CD103-associated regulatory molecules and a lower number of CD45RA\(^-\)CD62L\(^-\) effector memory CD4\(^+\) T cells than patients with chronic rejection.

We hypotheze that several mechanisms are involved in this tolerance state, one of them being active suppression through so-called “regulatory cells”. We compared blood cell phenotype of drug-free tolerant kidney recipients (DF-Tol) with that of recipients with chronic rejection (CR), stable graft function under standard or minimal immunosuppression and healthy individuals. Despite DF-Tol patients display a normal Vβ distribution, they exhibited a higher number of CD25\(^-\)CD4\(^+\)T cells expressing CCR4/CTLA4/GITR/CD103 molecules, a decrease in CD45RA\(^-\)CD62L\(^-\) effector memory CD4\(^+\)T cells and an increase in B and cytotoxic CD160\(^-\)CD28\(^-\)CD8\(^+\)T cells compared with CR group. Interestingly, the CD25\(^-\)CD4\(^+\)T, CD160\(^-\)CD28\(^-\)CD8\(^+\)T and B cell numbers were similar between DF-Tol and healthy individuals. Finally, minimally immunosuppressed patients exhibited the same
phenotype as DF-Tol, suggesting that they may also be “operationally tolerant”. These results suggest that a T cell regulatory mechanism may be involved in the operational tolerance observed in DF-Tol patients and that a decrease in CCR4/CTLA4/GITR/CD103 CD25⁺CD4⁺T cells and increase in effector memory CD4⁺T cells may be associated with CR⁺.

Figure 2. T and B cell populations in the blood of DF-Tol and CR patients. The non-parametric Mann-Whitney test was performed to compare T and B cell values of DF-Tol (n=5) versus CR patients (n=8). The results are expressed as the percentage of CD4⁺ T cells (top panel) and cell numbers (bottom panel). Differences were statistically significant (p< 0.05).
Role of MHC/peptide interaction versus dominant MHC framework interaction in the allorecognition process:

If direct allorecognition mostly proceeds from interactions with MHC determinants, independently of the diversity of presented peptides, gaussian TcLand patterns were expected, irrespective of the MHC of the stimulating cells, since specific CDR3 peptide-specific interaction would not be involved. According to our project, to clarify the interpretation of the gaussian CDR3 length-distribution pattern in direct-type MLR and to address the question of the role of peptide diversity and/or dominant interactions with allogeneic MHC framework in the allorecognition process, the following studies have been performed.

We previously showed that purified naive T cells confronted, *in vitro*, with allogeneic APCs in a direct pathway-restricted MLR up-regulate their Vβ mRNAs without exhibiting skewing of
CDR3 length distribution. We now show that *in vivo* V\(\beta\) transcript regulation and CDR3 length distribution follow the same pattern during acute rejection of MHC incompatible heart allografts. In contrast, in tolerance induction by priming of recipients with donor cells, the vigorous V\(\beta\) mRNA accumulation with gaussian CDR3 length distribution is abolished, providing a possible explanation for the down-regulation of activated T cells in tolerant animals. In addition, tolerated grafts harbour T cells with a highly altered repertoire suggestive of self-restricted presentation with some patterns corresponding to previously identified regulatory cells\(^5\).

We initially aimed at exploring if the TcLandscape pattern could be specific of a genetic combination and if eventually different patterns could be obtained when different APCs were used *in vivo*. TcLandscape analyses have been performed in blood from rat recipients with long term survival graft induced by donor-specific transfusions. We showed that different V\(\beta\) families were mobilised in recipients sharing the same congenic strain and receiving the same treatment.

The data show that the V\(\beta\) CDR3 length of a given V\(\beta\) family characterize an animal rather than a MHC/combination. This indicates that the V\(\beta\) TCR of the recipient displays different private options to recognize similar allogenic MHC complexes and that public pattern are exeptional even in congenic combinations. However, the patterns observed in a given recipient were usually present (with same CDR3 length) at different time intervals. They teach us that *in vivo* derived-material is not appropriate to answer the question of the respective influence of MHC versus peptide on V\(\beta\) transcription pattern.
MLR have thus then been performed in in vitro conditions of strict "direct" interaction of T cells from LEW.1A [RT1^a (a, a, a)] with irradiated enriched allogeneic dendritic cells from rats sharing the same background but with different MHC: the LEW.1W [RT1^u (u, u, u)], LEW.1N [RT1^n (n, n, n)], LEW.1AR1 (a, u, u) or LEW.1WR2 (u, a, a) recombinant MHC. No significant alteration could be observed, whatever the MHC of the stimulating cells. This suggests a role for the MHC framework in direct allorecognition or/and a high number of peptides involved. Although these experiments cannot rule out a peptide-dependant recognition (i.e. the peptide is required for MHC conformation), they do not suggest that the recognition is peptide-specific (gaussian TcLand patterns). To respond to this point, we are actually performing experiments in collaboration with F. Sébille (R. Lechler, Imperial College London, UK). Human fibroblast cell line (M1) transfected with both HLA-DR1 and hCD80 molecules, was able to prime naive T cells in «direct» MLR. DR7 and DR4 positive T cells from different human donors have been used as responding cells. DR1 and DR4 molecules only differ in contacting peptide residues. In contrast, HLA-DR7 differs from DR1 not only in peptide contacting amino-acids but also in 5 additional amino-acids in the MHC framework. If HLA DR4 positive T cells are used as responding cells, the resulting Vβ transcriptome pattern should only depend on the interaction with the peptide through CDR3. In this case, the expected TcLand topology elicited by direct-type MLR, should be highly altered. Therefore, if HLA DR7 T cells are used as responding cells, the response should be different from that displayed by HLA DR4 cells with different Vβ mRNA species regulations. mRNA sample analysis is currently under investigation.

Publications:
Prof. Kathryn Wood, Principal Investigator

University of Oxford, Oxford, UK

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

The objectives of the project were:
1. To determine the phenotype, function and specificity of immunoregulatory CD4+ T cells responsible for the maintenance of tolerance to alloantigens *in vivo* following pretreatment with alloantigen in combination with either anti-CD4 or anti-CD154.
2. To develop *in vitro* assays that enable the functional activity of immunoregulatory T cells to be correlated *in vivo* and *in vitro*.

Work on the project was initiated in the laboratory in October 2000. The project terminated in July 2003. A research assistant, Gang Feng, was appointed to work on the project in October 2002. The training Gang received while employed to work on this project stimulated his interest in research. He has recently obtained a scholarship to enable him to study as a D.Phil student in Oxford.

Excellent progress was made during the funding period.

**Specific Aim 1: To determine if CD45RB<sup>+</sup>CD4+ cells are also responsible for immune regulation in mice following treatment with anti-CD154**

We have shown that in the maintenance phase of specific unresponsiveness to alloantigens, *in vivo* induced following pretreatment with donor alloantigens in combination with anti-CD4 therapy, that CD45RB<sup>+</sup>CD4+ cells contribute to long term graft survival. This work was then extended to determine whether anti-CD154 (CD40L) therapy had a similar capacity to facilitate the development of immunoregulatory T cells in the maintenance phase of the response.

Blocking CD40-CD154 interactions by administering anti-CD154 (MR1) at the time of transplantation was found to prolong the survival of cardiac allografts in a number of different donor-recipient combinations in mice. A short course of MR1 therapy enabled the development of CD4+ T cells with regulatory properties that had the ability to maintain specific immunological unresponsiveness to the allograft. The regulatory cells were characterised
phenotypically and functionally and were shown to express high levels of CD25 and have specificity for donor alloantigen. Thus we have shown that both anti-CD4 and anti-CD154 therapy can generate donor alloantigen specific regulatory cells in the maintenance phase of the response after transplantation.

As part of this study we also carried out an additional series of experiments not specified in the original application to investigate whether CD25+CD4+ induced as a result of anti-CD154 therapy could control the potential of CD8+ T cells to initiate rejection. This is an important question in this context, as previous work by ourselves and others has shown that anti-CD154 therapy does not impact the ability of CD8+ T cells to initiate rejection in the early phase of the response3-6. This could be because CD8+ T cells are refractory to control by regulatory T cells or that in naïve recipients there is an insufficient number of donor alloantigen-specific regulatory T cells to control the aggressive potential of CD8+ T cells.

To test the former possibility we investigated whether CD25+CD4+ induced as a result of anti-CD154 therapy could prevent rejection initiated by direct pathway CD8+ T cells expressing a T cell receptor specific for a donor MHC class I antigen. The data obtained show that once donor alloantigen-specific regulatory T cells have been established they are able to control the activity of aggressive CD8+ T cells. Moreover, CD25+CD4+ regulatory T cells induced by anti-CD154 therapy were shown to be very potent as they were able to suppress rejection initiated not only by the minimum number of CD8+ T cells but by 100 fold excess of the minimum number of cells required.

**Specific Aim 2: To refine the in vitro assay system used to detect immune regulation by CD45RBlow CD4+ cells**

Based on our findings that regulatory activity was enriched in CD25+CD4+ T cells, as outlined above, the objective to develop an in vitro assay system was updated to use CD25+CD4+ T cells. A number of different strategies for developing a reproducible assay system were investigated. Carboxy-fluorescein diacetate succinimidyyl ester (CFSE) based assay was found to be most informative. Responder T cells were labelled with CFSE before addition to the in vitro cultures in the presence of antigen presenting cells. Proliferation of the responder population in the presence and absence of CD25+CD4+ T cells was compared by FACS analysis. The assay was found to be robust in its ability to detect immune regulation. However, some mechanistic findings, such as the involvement of cytokines in regulatory activity, that had been confirmed in vivo could not be replicated in vitro. We are continuing to refine the in vitro assay system to try to develop an assay that replicates all of the in vivo findings. However, we accept that this may be very challenging as many groups have now reported that there is a disconnect between in vivo and in vitro assays in this respect.
Specific Aim 3: To determine the role of CTLA-4 in immune regulation by CD45RB\textsuperscript{low} CD4\textsuperscript{+} cells

We have established that CTLA-4 plays a key role in the functional activity of donor alloantigen-specific regulatory T cells \textit{in vivo}. When the CTLA-4 is blocked, we have shown that regulatory cells are unable to prevent allograft rejection\textsuperscript{7}.

Specific Aim 4: To investigate the migration, proliferation, functional activity and survival of CD45RB\textsuperscript{low} CD4\textsuperscript{+} cells from tolerant mice \textit{in vivo}

Based on our findings that regulatory activity was enriched in CD25\textsuperscript{+}CD4\textsuperscript{+} T cells, outline above, the objective was updated to use CD25\textsuperscript{+}CD4\textsuperscript{+} T cells. Two strategies for monitoring the migration of T cells \textit{in vivo} have been developed, real-time PCR using CD3 expression to normalise the data and labelling T cells before adoptive transfer to track them in the draining lymph nodes and graft \textit{in vivo}. As the \textit{in vivo} adoptive-transfer system uses small numbers of cells a number of technical difficulties were identified and have been overcome. The strategy for this aspect of the study has therefore been modified to use Campath-1 transgenic mice, kindly provided by Herman Waldmann, as cell donors. The validation experiments for this new approach have been performed since the end of the ROTRF grant and the actual studies are currently in progress.

Specific Aim 5: To use additional cell surface markers to refine the phenotype of CD45RB\textsuperscript{low} CD4\textsuperscript{+} regulatory T cells

In the first set of experiments carried out as part of this project we investigated whether CD25, the $\alpha$ chain of the IL-2 receptor, a marker that had been shown to be useful for enriching for regulatory T cells amongst CD4\textsuperscript{+} T cells that could respond to self antigens in unmanipulated hosts. The data we have obtained in 2 model systems clearly show that CD25 is a useful marker for enriching for CD4\textsuperscript{+} T cells with immunoregulatory activity specific for donor alloantigens in recipients previously exposed to donor alloantigens \textit{in vivo}\textsuperscript{2,7}. These data are important as they suggest that there may be a common set of molecules expressed by T cells with regulatory function.

In ongoing studies we have been examining the ability of other cell-surface markers to refine further the characterisation of regulatory T cells. To date we have ruled out CD122, the $\beta$ chain of the IL-2R, as providing a useful additional marker\textsuperscript{8}. Whereas, intracellular expression of high levels of CTLA-4 (CD152) has been found to be useful as a strategy for enriching for CD25\textsuperscript{+}CD4\textsuperscript{+} T cells with regulatory activity\textsuperscript{7}.
Publications:

Manuscripts and review articles arisen from the project:

Abstracts presented:
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