R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

April 2003

The Roche Organ Transplantation Research Foundation

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

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

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

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1. Preface

We are pleased to announce that F. Hoffmann-La Roche Ltd has decided to extend its support to the Roche Organ Transplantation Research Foundation (ROTRF) for another 2 years, with the donation of a further CHF 10 million. This brings the total amount of support to CHF 50 million over 10 years. Through this generous support from F. Hoffmann-La Roche Ltd, the ROTRF has been able to and will continue to distribute research grants of up to CHF 300 000 to scientists around the world.

In this latest cycle of funding, almost half of the Letters of Intent were received from European scientists (49% of the applicants), while over a third came from the USA. Grants totalling CHF 2.1 million were awarded to nine scientists from the USA, Canada, Australia, the UK and Germany.

Funding from the ROTRF helps scientists to explore the science of solid organ transplantation and the excellent work carried out by the investigators is demonstrated by the papers published in respected international journals. We are confident that, with the continued support of the ROTRF, scientists will continue to make further important progress in the science of solid organ transplantation, with the goal of improving the care of transplant recipients around the world.

Some of the outstanding research funded by the ROTRF will be presented during a satellite symposium in September this year at the European Society of Organ Transplantation (ESOT) Congress in Venice. The symposium, entitled “Advancing the Science of Solid Organ Transplantation”, will provide five scientists from Europe and the USA with the opportunity to present their findings from research projects funded by ROTRF.

To conclude, we would like to express our gratitude to F. Hoffmann-La Roche Ltd for its continuous support of the Foundation and to all the people involved, including the scientists who have served as Trustees and Advisors, the applicants, and the ROTRF office staff. The ROTRF remains a unique achievement in the field of organ transplantation, and a credit to the sponsor whose generous gift makes it possible.

On the behalf of the Board of Trustees

Philip Halloran

2. Facts and Figures

Funding Cycle IX – Letter of Intent Submission in October 2002

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that following its ninth cycle of grant review, nine research grants have been awarded to scientists around the world. The grants allocated in this cycle total 2.1 million Swiss francs (CHF). The Board of Trustees and the Scientific Advisory Committee of the ROTRF were once again very pleased with the high quality and innovation demonstrated in the applications received.

The ROTRF received 89 letters of intent from scientists around the world. Almost half of the applications came from Europe (49%), the major countries being the UK (16%), Germany (12%), France (8%), while 41% came from the United States (35%) and Canada (6%). Australia, Brazil and Colombia accounted for the remaining 10% of the applicants.

All the letters of intent were reviewed by the Scientific Advisory Committee for scientific quality and originality, and based on these reviews the Board of Trustees invited the 21 top-ranked applicants to prepare full paper submissions.

After thorough review, nine projects were awarded full or partial grants of up to a maximum of CHF 300 000: four from the United States, two from Canada and one each from Australia, Germany and the United Kingdom.

The abstracts of the novel and promising research projects that received funding in this ninth cycle are presented on the following pages. The research projects focus mainly on the improvement of long-term graft survival, the prevention of chronic organ dysfunction, the induction of tolerance and the improvement of donor organ preservation.

Statistics on Applications to the ROTRF

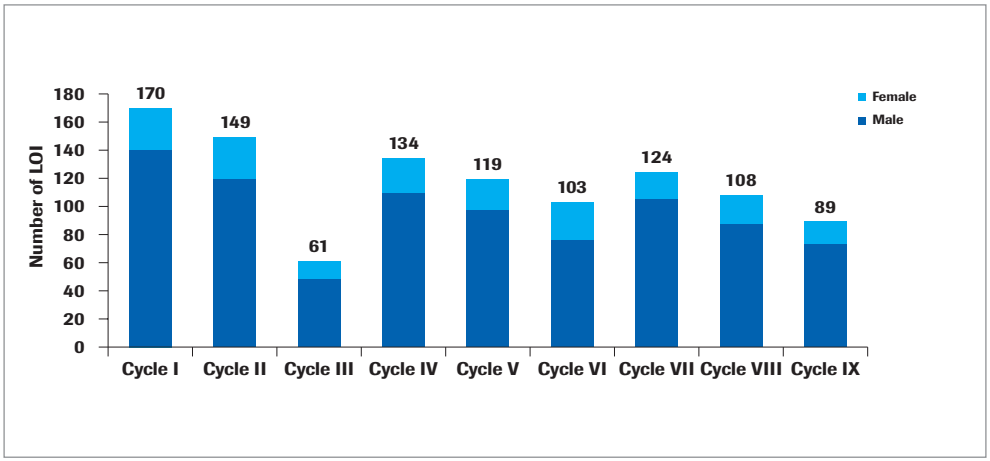


Figure 1. *Number and gender of applicants who submitted letters of intent (LOI) to the ROTRF during the first nine ROTRF funding cycles.*

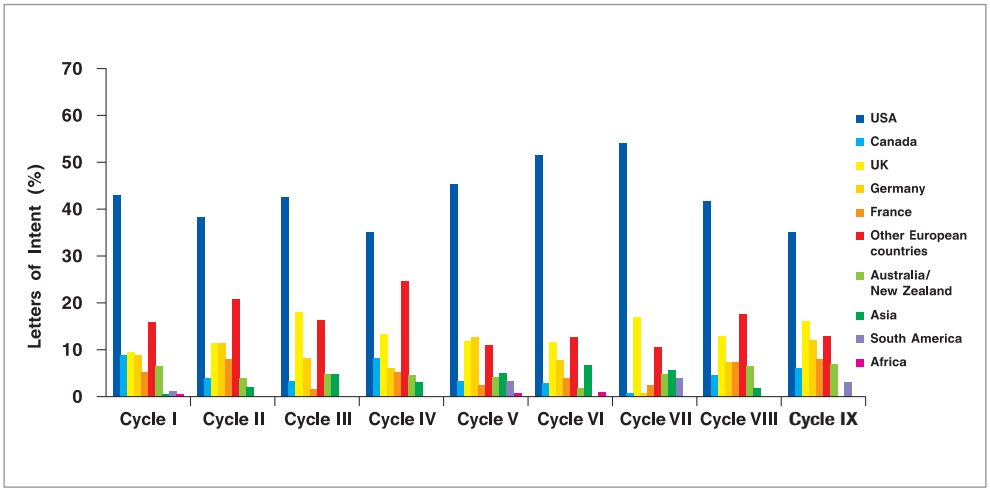
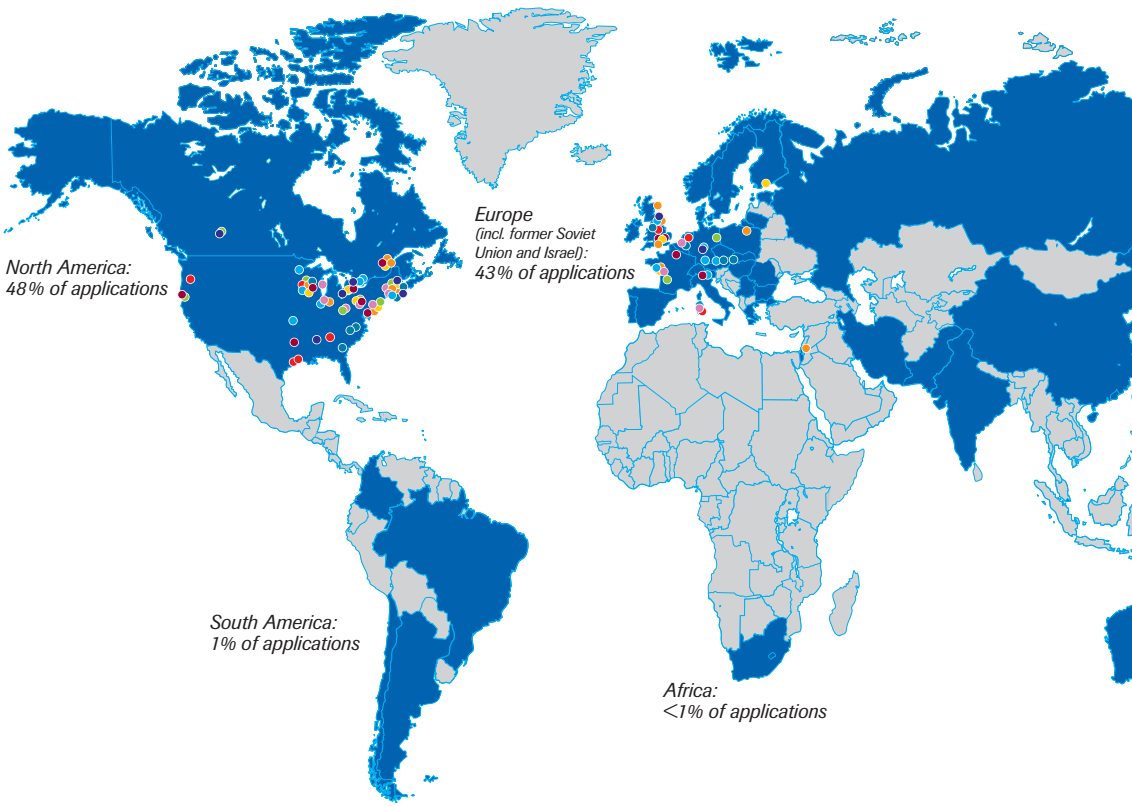
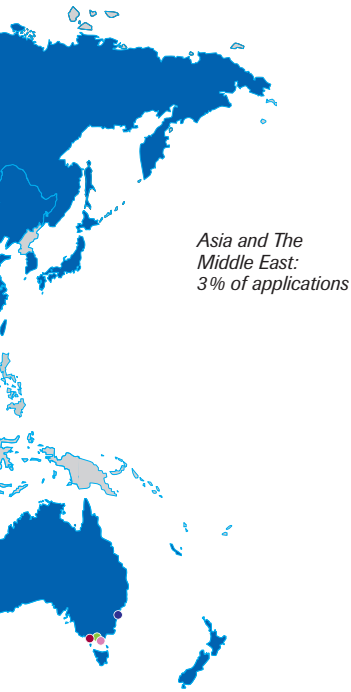


Figure 2. *Geographical distribution of the applicants who submitted letters of intent during the first nine ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF applications worldwide





Asia and The Middle East: 3% of applications

Australia: 5% of applications

at least one application ever received

no application received

Cycle I Grantees

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

Cycle II Grantees

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

Cycle III Grantees

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

Cycle IV Grantees

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

Cycle V Grantees

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

Cycle VI Grantees

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

Cycle VII Grantees

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

Cycle VIII Grantees

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

Cycle IX Grantees

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

3. Grant Awards in Cycle IX

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

Harvard Medical School, Boston, USA

Vascular Endothelial Growth Factor in Acute and Chronic Rejection

Transplantation has become widely accepted as the treatment of choice for end-stage organ failure. Unfortunately, transplanted organs eventually fail as a result of immunologic reactions resulting in two distinct processes called acute and chronic rejection. Acute rejection typically occurs early within the first few months following transplantation. In contrast, chronic rejection is more insidious and takes years to develop. Current treatments for rejection involve immunosuppressive medications and have been most successful at preventing acute rejection. There is currently no treatment for chronic rejection. In this research proposal, we have identified a molecule called vascular endothelial growth factor (VEGF) as a mediator of inflammation in the transplanted organ. VEGF is induced in tissues when there is a lack of oxygen and it promotes signals that result in immune cells being recruited into the tissue. Since transplantation characteristically involves organs that have been without oxygen for a period of time, we suggest that VEGF expression will also be a characteristic feature of transplantation. If VEGF is expressed, it may function to mediate immune rejection of the organ. However, this is entirely unexplored. We will first test whether VEGF is expressed and whether it is functional in organs as a result of a lack of oxygen or whether other factors associated with rejection mediate its expression. To block VEGF, we plan to administer an anti-VEGF antibody to mice following transplantation. We will also use this antibody with conventional immunosuppressive medications to determine whether it improves outcomes. We have several mice and reagents that will enable us to determine how VEGF promotes inflammation in the transplanted organ. Together, we believe these studies have the potential to identify VEGF as a therapeutic target for the future.

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

Ohio State University, Columbus, USA

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

Most current immunotherapeutic agents interfere with $CD4^+$ T-cell-dependent rejection. Work from our laboratory and others has identified an aggressive CD8-dependent rejection response which is CD4 independent and not susceptible to a variety of immunotherapeutic strategies that readily control CD4-dependent rejection responses. We have used a unique functional model of hepatocyte transplantation to separately assess the effect of various interventions on CD8- and CD4-dependent rejection processes. Recently, we have demonstrated that a novel immunosuppressive strategy, which targets lymphocyte function associated antigen 1 (LFA-1) and CD40/CD40L costimulation, suppresses both aggressive CD8-dependent and CD4-dependent rejection responses. This proposal will investigate how this strategy influences T cell recruitment and activation. In addition, we will determine whether this short-term immunosuppressive strategy results in the development of regulatory T cells, which permit long-term hepatocellular allograft survival. These studies involve the transplantation of purified hepatocytes into host mice, which are treated with monoclonal antibodies directed at LFA-1 and CD40L. Host tissue will be analysed by immunohistochemical means to determine the influence of the treatment strategy upon recruitment of host inflammatory cells to lymph nodes, spleen and liver tissue. Host inflammatory cells at these sites will also be isolated and analysed by flow cytometry for expression of various phenotypic and activation markers to determine the influence of the therapy upon cell subset recruitment, activation and function. Mice with long-term hepatocellular allograft survival induced by treatment with anti-LFA-1 and anti-CD40L mAbs will be assessed for the presence of regulatory T cells. In addition, host cells will be tested for evidence of immunoregulation in *in vitro* assays.

The successful regulation of immune damage of allogeneic hepatocytes will advance the therapeutic modality in patients with liver disease who could be cured or supported by successful hepatocyte transplantation.

Dr. Juan Contreras, Principal Investigator
Dr. Devin Eckhoff, Co-Investigator
Ms. Cheryl Smyth, Research Associate

University of Alabama, Birmingham, USA

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

Pancreatic islet transplantation (PIT) has been validated as a treatment for human type I insulin-dependent diabetes mellitus (IDDM). However, in the most successful clinical experience in PIT, two or more islet infusions were necessary to achieve sufficient engrafted islet mass for establishing normoglycemia without exogenous insulin therapy. While islet re-transplantation is effective, it lacks cost-effectiveness and is constrained by the shortfall of donor pancreatic tissue. Thus, new strategies for improving durable functional islet mass will be instrumental in facilitating PIT as a cure for IDDM. When islets are isolated and cultured before transplantation, the islet endothelium dedifferentiates or degenerates. A rapid revascularization is therefore crucial for islet function and engraftment after transplantation. Recent studies demonstrated that the vascular density in revascularized transplanted islets is markedly decreased compared with endogenous islets. The feasibility of revascularization of ischemic tissues by mobilizing bone-marrow endothelial progenitor cells (angioblasts) or transplantation of *ex vivo* expanded angioblasts has been demonstrated. Therefore, the long-term objective of this proposal is to evaluate the possibility of increasing islet revascularization and therefore preserving functional islet mass after islet transplantation by mobilizing or infusing angioblasts in combination with islet transplantation. Based on these considerations, we hypothesize that mobilized or infused angioblasts can increase revascularization and engraftment of pancreatic islets. The proposed studies are innovative and likely to yield important new information and new protocols to allow implementation of PIT as a routine therapy for diabetes.

Prof. Philip Halloran, Principal Investigator

University of Alberta, Edmonton, Canada

Mechanisms of Renal Allograft Rejection

Our aim is to identify the mechanisms of tissue injury and destruction in kidney transplant rejection. The mechanisms of graft rejection remain known and are important. Even though immunosuppressive drugs can now arrest rejection in many cases, these drugs have harmful effects. Rejection can be hard to recognize and treat, because we do not understand the mechanisms of destruction. The old methods of exploring mechanisms have been hampered because many mechanisms produce similar ultimate effects: destruction and failure of the graft. So failure alone is not a good way to look at mechanisms. To identify the important mechanisms for human rejection, our new approach is to focus on pathology. In other words, we study mechanisms in models that exactly mimic the processes seen in clinical rejection, particularly the process called tubulitis.

We have developed a mouse system in which the pathology is similar to that seen in rejecting human kidney transplants. We will study the mechanisms of tubulitis using mice that lack specific mechanisms. That way we can test which mechanism is needed for tubulitis to develop. We will study how the lymphocytes stick to the kidney tissue (the tubules), and whether they use a protein called CD103. We will explore how the glue that holds kidney units together – E cadherin – changes during rejection. Finally we will try to find the “fingerprint” of tubulitis by using gene “chip” methods.

If successful, the findings will have direct applications to human clinical transplantation. They will help us to diagnose rejection, to develop new drugs, and to find new ways of creating a favorable environment in the patient for the graft, to avoid rejection without drugs.

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

University of Newcastle, Newcastle upon Tyne, UK

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

Kidney transplantation remains the preferred treatment for end-stage renal failure but long-term graft survival is disappointing, with 35% failing within five years. A major feature of the chronically failing graft is the development of fibrosis, especially in the interstitium around the kidney tubules. Grafts that suffer the most severe acute rejection problems during the early period after transplantation tend to be those that subsequently develop fibrosis and fail. The link between these two processes is not understood and there is no effective treatment for chronic graft failure.

We have shown that recipient T cells accumulate within the kidney tubules during acute rejection and some of these T cells persist for long periods. In this project we will test the hypothesis that long-lived T cells generate a chronic stimulus, causing local tubular epithelial cells to change into fibroblasts. These fibroblasts then multiply around the tubules and produce extracellular matrix, which, over time, takes the place of functional tissue, causing graft dysfunction and eventually graft failure.

Loss of airway epithelium and fibrosis are also major features in obliterative bronchiolitis causing chronic lung graft failure, and vanishing bile duct syndrome occurs in late failure of around 10% of liver transplants. Therefore, there is the potential for similar processes to those described above to occur after transplantation, leading to dysfunction of lung and liver allografts. This will also be investigated.

An important consequence of our hypothesis is that elimination of intraepithelial T cells should prevent progression to chronic graft failure after initial acute rejection.

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

University of Western Ontario, London, Canada

Novel Strategy for Inducing Transplant Tolerance by Genetically Modifying Dendritic Cells with siRNA

Transplantation has saved the lives of many patients with end-stage organ failure. However, these patients have to take drugs that stop their immune system from attacking the graft. These drugs also stop the immune system from attacking other bacteria and viruses, and these medications must be taken for a lifetime. Therefore, transplant recipients are especially susceptible to developing many diseases, including cancers and infections. A safer and more natural way to block rejection of transplanted organs is to trick the immune system of the recipient into seeing the organ as part of the body and therefore not rejecting it. Playing this trick on the immune system is called "tolerance induction". A component of the immune system called the dendritic cell is essential for teaching the body what to attack and what not to attack. Dendritic cells that have low concentrations of immune-stimulating molecules usually instruct the body not to attack foreign tissue. It has long been believed that graft rejection can be prevented by decreasing the concentration of these immune-stimulatory molecules on dendritic cells. Unfortunately, limited success was achieved in previous studies because of the lack of efficient tools to suppress immune stimulatory molecules on dendritic cells. We have recently discovered a new method of blocking immune-stimulatory molecules on dendritic cells, which is far more potent and safer than previously used techniques. This new method is called "RNA interference" and operates by specifically destroying the genetic information that instructs the cell to produce immune-stimulatory molecules. We anticipate that our new method will successfully trick the immune system of the transplant recipient into accepting the foreign graft without the need to use immunosuppressive drugs.

Dr. Jeffrey Schechner, Principal Investigator
Prof. Jordan Pober, Co-Investigator

Yale University School of Medicine, New Haven, USA

Vascularization of Engineered Human Skin Equivalents

One solution to address the shortage of organs available for transplantation is to engineer synthetic living tissues. The most widely used example of such a strategy is living skin equivalents. Unfortunately, the available products do not contain blood vessels and, probably due to a resultant inadequate delivery of oxygen and nutrients, have not performed well. To overcome this deficiency, we have developed a methodology for perfusing human skin equivalents through blood vessels formed from cultured endothelial cells (the cells that normally line blood vessels). In the proposed studies, we will first optimize the formation of blood vessels in the grafts by evaluating the benefit of adding various growth supplements prior to implantation. Then, endothelial cells will be genetically altered to overexpress the gene Bcl-2, a modification that we have previously shown to improve the formation of mature blood vessels. We will also examine the relative performance of blood vessels formed with endothelial cells derived from different sources, such as skin, blood and umbilical veins, after transplantation into immunodeficient mice. This determination of optimal vessel function will include a comparison with vessels in similarly transplanted human skin grafts using the parameters of two- and three-dimensional structure and density, presence of surface marker characteristics typical of arteries, veins and capillaries, and permeability. In addition, potential adverse effects of vascularizing grafts, such as inducing immunologic rejection and the formation of vascular tumors as a result of genetically altered endothelial cells, will be evaluated. Finally, the benefit of these modifications on overall graft performance will be determined by assessing graft take, blood flow, and retention of an intact barrier, a critical function of skin. If the hypothesis that vascularizing skin equivalents enhances their performance is correct, these studies will provide a strategy for improving the clinical utility of skin equivalents and other bioengineered tissues.

Prof. Ann Simpson, Principal Investigator
Dr. Ming Wei, Co-Investigator

University of Technology, Sydney, Australia

Correction of Diabetes Using Insulin-Secreting Liver Cells

Type I diabetes mellitus is caused by the autoimmune destruction of the β cells of the pancreas that secrete insulin. Permanent correction of diabetes by pancreas transplantation is restricted by the availability of normal human pancreatic issue and by the potential for further autoimmune damage to the transplanted pancreas. Moreover, substantial morbidity, due to life-long immunosuppression, inevitably follows pancreas transplantation. These problems could theoretically be overcome by engineering from the patient's own cells an "artificial β cell", i.e. a non-islet cell capable of synthesising, storing and secreting mature insulin in response to metabolic stimuli, such as glucose. We have engineered two liver cell lines that store and secrete insulin in response to glucose. However, the ultimate goal of this technology is to deliver the insulin gene directly to a patient's own liver cells, which would regulate insulin secretion in response to glucose and other substances that stimulate insulin secretion, controlling blood glucose without the need for immunosuppression.

To accomplish this, it must be possible to deliver the insulin gene efficiently to primary liver cells (animal or human cells). Results from our laboratory using a non-pathogenic viral delivery system indicate that we can deliver genes to 90% of liver cells. We must also be able to engineer storage of insulin in primary liver cells. Based on preliminary studies, we have ascertained that certain β cell transcription factors (activating proteins, the expression of which is normally restricted to β cells), namely Neuro D and Neurogenin 3, promote storage of insulin in liver cell lines. In the current proposal we aim to express these transcription factors in primary liver cells. We also wish to look at the control of insulin expression in primary liver cells using a gene promoter that is glucose and insulin sensitive.

The results from this research proposal should result in the delivery of the insulin gene to large numbers of primary liver cells that store and secrete insulin in response to glucose. These cells would control blood glucose levels in patients without the need for immuno-supression and forestall the onset of the chronic complications of diabetes.

Dr. Peter Terness, Principal Investigator

University of Heidelberg, Heidelberg, Germany

Using the Immunosuppressive IDO Gene for Prevention of Allograft Rejection

When a foreign tissue or organ is transplanted into an unrelated person, the immune system of the recipient destroys the graft. This process is known as the rejection reaction. To suppress rejection, patients carrying transplanted organs receive immunosuppressive drugs. These drugs, however, not only suppress the unwanted immune reaction to the foreign tissue but also that to harmful aggressors, such as bacteria and viruses, which often leads to serious infections and other side effects. The ultimate objective of transplantation immunology is to specifically inhibit the response against the graft while leaving the remaining immune defence unaltered. Nature has provided us with at least one example of how this can be accomplished. Pregnant women carry fetuses, half of whose genes come from the mother and the other half from the father. The part inherited from the father is foreign to the mother. In spite of that, the expectant mother does not reject the fetus. It is the placenta, the organ located between the mother and the conceptus, which is made responsible for suppressing the lymphocyte attack. Recently, a gene harboured by placental cells that suppresses the activity of lymphocytes has been identified. In the current project, we plan to insert this gene into the prospective donor organ. In order to test the action of the gene, a rat heart transplant model has been chosen. It is expected that, after expression of the suppressive gene in the transplanted heart, the infiltrating lymphocytes will be inactivated. After successful completion of animal experiments, first clinical trials will be considered.

4. Progress Reports of ROTRF Grantees

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

University of Newcastle, Newcastle upon Tyne, UK

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

Acute transplant rejection is dependent on patterns of leukocyte migration and activation within donor tissue. The vascular endothelium plays a central role in the recruitment of blood-borne cells to the subendothelial tissues during rejection by facilitating a cascade process involving intravascular arrest of certain cell types and directed extravasation of responsive cells. It is becoming increasingly clear that this process is regulated principally by specific cytokine molecules termed chemokines.

Work started in first year and finished during the 2nd year

Chemokines have a variety of functions, including the selective recruitment of leukocytes, which are mediated through activation of seven-transmembrane-spanning receptors during inflammation. It has been suggested that an important component of this process is the formation of a haptotactic gradient by immobilisation of chemokines on cell surface glucosaminoglycans (GAG), which are normally expressed within the proteoglycan component of the cell surface and extracellular matrix. However, the significance of this interaction for the regulation of inflammation remains unclear. In this study, confocal microscopy was used to examine the relative expression of GAG species including heparan sulphate (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. Significantly, the potential of HS on these cells to bind RANTES/CCL5 was increased following stimulation with IFN-γ and TNF-α. 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 RANTES was associated with HS-rich tubular basement membrane during rejection but the distribution of RANTES at this site was not uniform, providing evidence for focal accumulation. This suggests a mechanism for formation of the static chemokine concentration gradients required for vectorial leukocyte migration during the development of rejection-associated tubulitis. Thus, chemokines and their interaction with GAGs may

significantly contribute to the localisation of leukocytes beyond migration patterns defined by chemokine receptor interactions[2].

Work initiated during the 2nd year

In the second year we further sought to investigate the nature of chemokine binding to endothelial cells and the selectivity of different chemokines for the various GAGs during cardiac rejection compared with normal heart. We have so far stained 10 myocarditis sections with antibodies specific for various GAGs, i.e. HS, C4S and C6S. Furthermore we have also optimised staining for the chemokine RANTES, and work is underway to optimise protocols for staining of the chemokine IP-10 and receptors CCR5 and CXCR3, as they are implicated as having an important role in Th1-type response/rejection. We have identified cases ranging from mild to severe cardiac rejection, hence future work will include staining consecutive paraffin-embedded human biopsy sections of normal and rejecting heart tissue with antibodies specific for GAGs, chemokines and chemokine receptors (optimised during the 2nd year).

We have further created a panel of mutant RANTES molecules containing neutral amino acid substitutions within putative basic GAG binding domains. Studies are underway to express them and analyse them. We will determine the *in vivo* role of chemokine-GAG binding using these variants engineered for reduced ability to bind GAGs.

Publications

1. Ali S, Fritchley SJ, Chaffey BT, Kirby JA. Contribution of the putative heparan sulphate binding motif BBXB of CC chemokines to trans-endothelial migration. *Glycobiology.* 2002; 12: 535–543.
2. Ali S, Malik G, Robertson H, Kirby JA. Renal transplantation: Examination of the regulation and localisation of chemokine binding during acute rejection. Manuscript submitted.
3. Ali S, Hardy LA, Kirby JA. Transplant immunobiology: a crucial role for glucosaminoglycans. *Transplantation* 2003; in press.

Dr. Michel Y. Braun, Principal Investigator

Université Libre de Bruxelles, Brussels, Belgium

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

We have recently validated the indirect pathway of T-cell allorecognition in a mouse TcR transgenic model. The transgenic TcR specifically recognizes the peptide NAGFNSNRANSSRSS, derived from the male antigen encoded by gene *Dby,* presented in an I-A^b-restricted fashion. The T cells are not alloreactive to the H-2^k haplotype, because the transgene was originally isolated from a T-cell clone derived from an H-2^{bxk} F1 female. Moreover, they are not activated when adoptively transferred into RAG2$^{-/-}$ $\gamma c^{-/-}$ double-mutant H-2^k male or female mice. However, skin from H-2^k males, but not from H-2^k females, is acutely rejected by RAG2$^{-/-}$ TcR-transgenic female recipients. Taken together, these results show that the indirect recognition that triggers rejection in this model is due to the recognition of H-Y antigens shed from H-2^k male allograft and presented by the recipient's own I-A^b APC to transgenic CD4 T cells. The purpose of this project is to understand the mechanisms that ensure the specificity of tissue destruction when rejection occurs in the complete absence of direct physical recognition of the graft by T cells. More specifically, we are trying to identify the role played in this process by recipient-derived endothelial cells colonizing the graft.

Bone-marrow-derived precursor elements have the potential to induce angiogenesis of ischemic tissues. Several studies have addressed the question of endothelial cell replacement in solid organ transplantation. A strong correlation was found between the percentage of recipient endothelial cells in rejected organs and the type of rejection. These cells were particularly detected among patients whose graft suffered chronic vascular rejection. It was postulated that endothelial cells of the recipient can replace those of the donor and that this replacement was associated with rejection. This is an important issue, because it is generally assumed that chronic rejection results exclusively from the activity of indirect pathway T cells. Recipient-derived endothelial cells could well represent the main cell target within the graft for indirect T cells.

If the hypothesis that recipient-derived vascular structures trigger graft destruction by indirect T cells is correct, then one has to postulate an essential role for endothelial cells in the alloantigen-specific recognition of grafted tissues by T cells. Experiments were

conducted to test this possibility. Male H-2^b skin from CD3$^{-/-}$ γc$^{-/-}$ double-mutant mice was transplanted onto female H-2^k CD3$^{-/-}$ γc$^{-/-}$ recipients reconstituted with purified TcR-transgenic T cells. In this model, T cells can only recognize autologous APC of donor origin present within the grafted skin. H-Y-specific I-A^b-restricted TcR-transgenic T cells transferred in H-2^k recipients were able to mediate the rejection of male H-2^b grafts. In this setting, T-cell priming was the result of encountering I-A^b-positive dendritic cells (DC) that had migrated from the graft into the local draining lymphoid organs. Remarkably, immunostaining of rejected grafts with I-A^b- or I-E^k-specific monoclonal antibodies revealed that expression of I-A^b was strictly restricted to the endothelium of the rejected graft (Fig. 1). Moreover, all MHC class II antigen-positive cells infiltrating the graft expressed recipient's MHC class II molecules I-E^k and, thus, could not stimulate T cells for rejection (Fig. 1). These observations strongly support the critical role played by endothelium in the cognate recognition of the graft by T cells. They also substantiate the hypothesis that, while T-cell priming results from the alloantigen-specific recognition of donor DC in lymphoid organs, graft rejection depends on the alloantigen-specific recognition of endothelial cells within the graft.

Whether endothelial cells from recipient origin have the potential to induce angiogenesis of ischemic allografts is under investigation. If this is indeed the case, then we shall be able to determine whether rejection of H-2^k male allografts by indirect pathway T cells depends on the presence of recipient I-A^b endothelium within the graft.

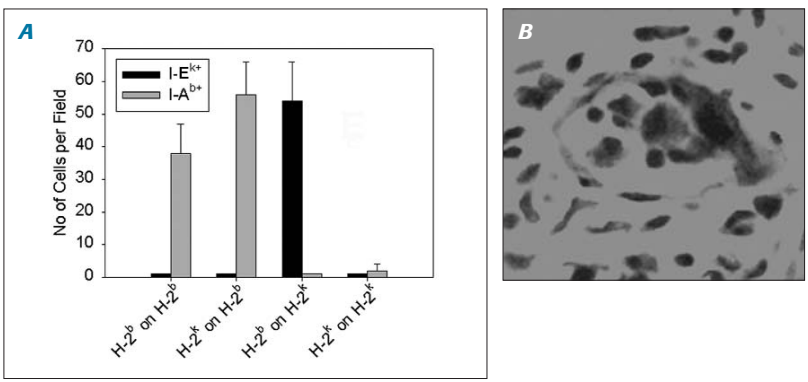


Figure 1. Restricted expression of I-A^b on endothelial cells of H-2^b male graft rejected by H-2^k RAG2$^{-/-}$ γc$^{-/-}$ female recipients reconstituted with H-Y-specific TcR-transgenic T cells.

A. *H-2^b or H-2^k CD3$^{-/-}$ γc$^{-/-}$ recipient mice were reconstituted with 7×10^6 RAG2$^{-/-}$ Tg T cells, then transplanted with skin from H-2^b or H-2^k CD3$^{-/-}$ γc$^{-/-}$ donor mice. Eleven days later, grafts were taken and tissue sections were immunostained with anti-mouse I-A^b- (clone 25-9-17) or anti-mouse I-E^k- (clone 14-4-4S) specific monoclonal antibodies. Graft-infiltrating mononuclear cells expressing I-A^b or I-E^k were then counted. Results are expressed as the mean ± SD of counts from three different sections.*

B. *Expression of I-A^b on vascular structures from H-2^b CD3$^{-/-}$ γc$^{-/-}$ skin rejected by H-2^k CD3$^{-/-}$ γc$^{-/-}$ recipient reconstituted with Tg T cells.*

The second part of this study analyzes the nature of the effector mechanisms of graft destruction triggered by indirect pathway T cells. We have observed that male skin allografts rejected by H-Y-specific Tg T cells contains mRNA of Th1 as well as Th2 cytokines, suggesting that both Th1- and Th2-dependent pathways of rejection operate in our system. The possible involvement of Th2 cytokines was confirmed by histology as numerous eosinophils were detected within the rejected skin. However, preliminary experiments involving cytokine neutralization by antibody treatment failed to show a role for Th1 or Th2 cytokines in the rejection process. Moreover, grafting Fas-deficient allograft did not modify the capacity of H-Y-specific TcR-transgenic recipients to reject H-2^k male skin, discarding a role for Fas-dependent cytotoxicity in our model of rejection. We are in the process of generating genetically modified IFN-γ-deficient RAG2$^{-/-}$ TcR-Tg mice. The effect that IL-4 or IL-5 neutralization, as well as Fas invalidation, could have on male H-2^k skin survival in these animals will be analyzed.

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Interaction of OX2 with its Receptor Controls Organ Rejection

My laboratory has described and begun the characterization of the novel molecule OX2 (now referred to as CD200) and its receptor, CD200R, which are implicated in immunoregulation in transplantation, autoimmunity, infection and tumor immunity. Our research proposal funded by the ROTRF concerned experiments designed to investigate this immunoregulation further, following development of the following new reagents, anti-CD200R(s), and knockout or transgenic mice for CD200/CD200R.

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

Rationale: While we reported that CD200 is important in immunoregulation *in vivo* and *in vitro,* its molecular structure implied that it acted primarily following engagement of the receptor CD200R on target cells[1,2]. In preliminary studies we showed that both whole and Fab anti-CD200R blocked graft prolongation following increased CD200 expression, while soluble CD200Fc was a potent immunosuppressant[3]. Interestingly, $CD200R^+$ cells were identified in an LPS-stimulated macrophage population, and in ConA-activated T cells[4], and we showed that $F4/80^+$ $CD200R^+$ splenic macrophages are immunosuppressive in concert with CD200Fc[4]. Based on these data we planned to prepare and investigate the properties of mAbs against CD200R expressed on different tissues/cells.

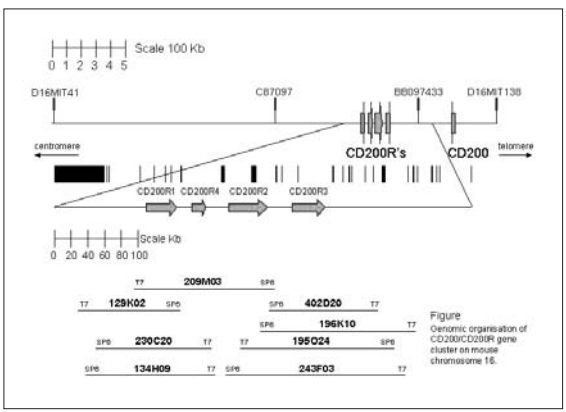

Figure 1. *Genomic organization of CD200/CD200R gene cluster on mouse chromosome 16 located between markers D16MIT41 and D16MIT138. CD200 is located downstream from its receptors (CD200Rs) and has an opposite direction. RPCI-22 BAC clones 209M03, 402D20, 195O24 and 196K10 spanning this region are indicated.*

Results to date: In association with Dr. P. Mardsen we have defined, by molecular cloning, considerable heterogeneity in both the mouse and human CD200R gene family. At least 4 major murine CD200Rs have been 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 in Fig. 1.

Analysis of tissue expression of the products of these various gene members has been performed, and this has helped explain some of the controversy in the literature regarding the relative tissue expression of CD200R – some tissues (notably thymus/bone marrow) do not transcribe products of certain members of the gene family. We have preliminary data on the functional effects of mAbs against different CD200Rs on allosensitization *in vitro* (a tissue culture model used to detect immune responses potentially of relevance in graft rejection, showing the expected suppression by anti-CD200Rs).

2. Analysis of transplantation of organs from CD200-transgenic mice ($CD200^{tg/tg}$) 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 an increased susceptibility to spontaneous autoimmune disease[5]. We predicted that after constructing a mouse CD200-transgenic line, we could show that organs from CD200-transgenic mice ($CD200^{tg/tg}$) would be less easily rejected than organs from wild-type (+/+) mice.

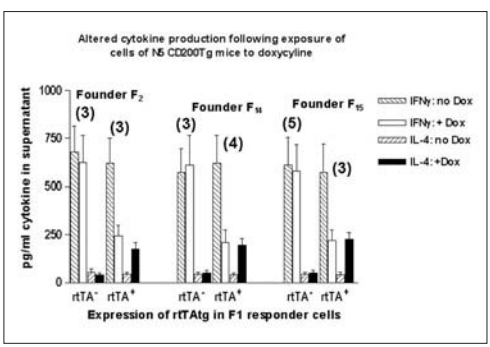

Figure 2. *Altered induction of cytokine production (increased IL-4, decreased IFN-γ) measured by ELISA in 40-h cultures following allostimulation of spleen cells from doubly transgenic ($rtTA^{tg}$ and TRE-CD200-GFP^{tg}) F1 mice in the presence of doxycycline. MLR cultures were initiated using BALB/c 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 $rtTA^{tg}$. 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-γ, IL-4) were seen for IL-2 and IL-10 respectively (data not shown for clarity).*

Results to date: We have almost completed breeding through 10 backcross generations to C57BL/6 (currently at N8) transgenic mice expressing full-length mouse CD200 under control of a Tet-on promoter. F1 hybrids made by crossing with an $rtTA^{tg}$ line (again on a C57BL/6 background) show inducible expression of CD200 in multiple tissues in the presence of doxycycline. When we performed allo-MLRs using spleen cells from doubly transgenic mice as responder cells, we observed profound suppression of immunity, as defined by decreased induction of CTL and type-1 cytokines *in vitro* (see Fig. 2, using N6 F1 mice, with suppression of IFN-γ, and increased IL-4).

In a very preliminary study, using skin allografts only, we have confirmed the expectation that grafts from $CD200tg^{tg/tg}$ mice survive longer than grafts from non-tg littermate controls, and are less able to induce allogeneic immunity as defined by reduced CTL induction *in vivo*.

3. Analysis of transplantation of organs from $CD200^{tg/tg}$ vs littermate non-tg controls into normal or CD200RKO recipients, in the presence/absence of anti-CD200 (or anti-CD200R) mAbs and CD200Fc.

Rationale: As noted earlier, the CD200 KO mouse, has, as predicted, an increased susceptibility to spontaneous autoimmune disease[5]. Since immunoregulatory signaling only follows from engagement of cell surface CD200R, we predicted the following: (i) that defects in CD200 KO mice could be "rescued" by infusion of CD200Fc (currently under investigation by DNAX), and (ii) in contrast, the phenotype of CD200R KO mice would be similar to that of the CD200 KO, but "rescue" by CD200Fc would not be possible. Thus organs from CD200-transgenic mice ($CD200^{tg/tg}$) and wild-type (+/+) recipients would show equal tendency for rejection in +/+ and CD200R KO mice (-/-). Rejection of all grafts in CD200R KO mice would be unaffected by infusion of anti-CD200R mAbs, though this would improve survival in +/+ mice. Similarly, infusion of CD200Fc into either +/+ or CD200R KO mice would lead to immunomodulation of graft rejection only in +/+ mice, and this would be modified by a decoy receptor, CD200RFc.

Results to date: Our original strategy to construct a CD200 KO mouse was based on our earlier (erroneous) supposition that there was only one gene for CD200R. As noted above, this is a complex gene family, with evidence of considerable redundancy in the expression/function of various gene members. Thus, while we temporarily considered the potential value of developing mice with deletion of only one discrete family member, we have now redesigned our approach, and have planned vector construction which will lead to complete deletion of the entire family (under control of temporally and tissue-restricted expression of lox/p sites). This work is being performed in the ES facility in our research division, in collaboration with Drs Cybulski, Marsden and Levy, who have experience in this technology. 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 in more detail the functional potential of individual CD200R gene products in the absence of expression of other members of the family.

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Towards a Human Hepatocyte-Based Bioartificial Liver

The overall aim of the present proposal is to exploit novel approaches for the potential use of isolated hepatocytes as a therapeutic tool. Within this context, our research group has developed a model of massive liver repopulation via cell transplantation. Our basic observation is that exposure of rats to retrorsine (RS) sets the stage for liver repopulation to occur, following transplantation of normal hepatocytes[1,2]. RS is a naturally occurring pyrrolizidine alkaloid known to block the hepatocyte cell cycle. While several agents have the capacity to transiently inhibit hepatocyte proliferation, the persistent nature of the cell-cycle block imposed by RS is rather uncommon and appears to elicit profound perturbations in liver growth control mechanisms. Thus, gaining insights into the molecular bases of these perturbations may help to elucidate general pathways related to liver repopulation. Accordingly, the specific aim of these studies is to characterize molecular mechanisms involved in liver repopulation by hepatocyte transplantation in the RS model.

As mentioned above, the specificity of the RS effect lies in its long-lasting block of the endogenous hepatocyte cycle. For example, virtually no mitotic events were observed in liver exposed to RS for two weeks following 2/3 partial hepatectomy (PH)[3]. We have proposed that such a persistent mitotic block may form the basis for the selective growth of transplanted cells in this system.

However, this hypothesis has been questioned by recent data reported by Gordon *et al.,* describing complete restoration of liver mass within 4 weeks post-PH in rats treated with RS (*Am J Pathol* 2000; 156: 607). Under these conditions, no growth stimulus for transplanted cells would persist over an extended period of time, and alternative mechanisms would have to be considered to explain the process of liver repopulation by host cells. Given the relevance of this issue, we have decided to perform a long-term study on the kinetics of response to PH in rats treated according to the RS protocol for liver repopulation.

Fischer 344 rats (five to six weeks old) were given RS (two doses of 30 mg/kg each, two weeks apart), while control animals received 0.9% NaCl solution. PH was performed 4 weeks

later in both groups and animals were killed at different time points thereafter (up to 4 months). The results (Table 1) confirmed and extended our previous observations: restoration of liver mass was severely impaired in RS-treated animals throughout the experimental time considered. For example, relative liver weight was still about 35% lower in the RS group compared with controls at 16 weeks post-surgery. This was associated with a parallel reduction in total liver DNA and protein content, indicating that functional liver mass was effectively reduced.

Table 1.

The long-term kinetics of liver regeneration in rats treated with retrorsine. Values are mean ± SD of 3–4 animals.

	Weeks after 2/3 partial hepatectomy			
	2	**5**	**8**	**16**
Liver weight/b.w. (%)				
Control	2.97±0.19	3.21±0.08	3.08±0.06	3.07±0.09
Retrorsine	1.50±0.21	1.98±0.40	1.54±0.30	1.99±0.13
Liver DNA (mg/liver/100 g b.w.)				
Control	8.33±1.42	8.77±0.68	10.21±0.40	8.95±0.57
Retrorsine	4.73±0.47	6.08±1.37	4.68±1.02	6.68±0.49
Liver protein (g/liver/100 g b.w.)				
Control	0.64±0.09	0.87±0.05	0.79±0.08	0.80±0.14
Retrorsine	0.27±0.07	0.38±0.10	0.37±0.09	0.52±0.04

The discrepancy between our data and those by Gordon *et al.* has no obvious explanation at present. Our study has been conducted using the experimental protocol associated with liver repopulation. Therefore we can safely conclude that this protocol causes a long-lasting inability of endogenous hepatocytes to restore liver mass, which in turn may trigger the selective growth of transplanted cells. Relevant to this point, the process of liver repopulation is largely completed (>90%) within 4 months post-PH[1].

A second set of studies has been devoted to the analysis of possible molecular targets in the process of liver repopulation. A starting point in this analysis has been the previous observation that transplanted cells can repopulate RS-treated livers in the absence of exogenous growth stimuli (such as PH)[2]. This indicates that treatment with RS *per se* sets the stage for the selective expansion of transplanted cells. Thus, both the biological driving forces and the molecular alterations relevant to liver repopulation must be already present in rat liver exposed to the RS protocol.

Based on this consideration, we have examined the expression of cell-cycle-related genes and their products in the liver of Fischer 344 rats treated with RS (30 mg/kg, two doses, as above) and killed 4 weeks later.

Two main issues appear to emerge from the results obtained. There is a set of inhibitory proteins (namely TGF-β and p27) that are over-expressed in RS-treated animals. Given their known role in cell-cycle regulation, they could be related to the persistent block in the cell cycle imposed by RS. It is noteworthy that they are over-expressed even 4 weeks after the last exposure to RS, documenting the long-lasting effect of this treatment at molecular level. The CDK inhibitor p27 is thought to inhibit the cell cycle after cyclin D activation. Our results support this conclusion: in fact, RS-treated hepatocytes were blocked despite the high expression of cyclin D, both at the mRNA and the protein levels.

The second issue pertains to the molecular nature of the stimulus sustaining the growth of transplanted cells in this system. At least mRNA and/or protein species associated with cell cycle stimulation were found to be up-regulated 4 weeks after exposure to RS, including TNF-α, PCNA and cyclin D. Each of these proteins, alone or in combination, could provide the appropriate stimulus for transplanted cells to respond. It appears difficult at the moment to delineate a precise sequence of events. Furthermore, these results have been obtained by homogenizing the entire liver. Having these data at hand, the next step is now to perform *in situ* analysis in order to identify specific cell types expressing each mRNA and/or protein product. These studies will also be conducted in the presence of cell transplantation. Since transplanted cells can be easily located using the dipeptidyl-peptidase type IV (DPP-IV) marker enzyme (as described in our previous studies), relevant information can be obtained on the interaction between donor-derived and resident cells during the entire process of liver repopulation.

The identification of molecular mechanisms related to this unique effect of RS (its ability to induce an environment which allows for the selective growth of transplanted cells) could offer the opportunity to devise a general strategy for liver repopulation with possible clinical applicability[4].

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

This project is directed at elucidating the possible role of the "innate" immune system in the production of proliferative atherosclerotic lesions in transplanted organs. Our experimental system makes use of hearts transplanted between members of selected inbred strains of mice. We had previously found that such vascular lesions were regularly produced in hearts transplanted to recipients that had been rendered fully tolerant of donor tissues by two different methods, as well as to recipients genetically incapable of adaptive immune responses (SCID and RAG1$^{-/-}$).

With ROTRF support we have made considerable progress in focusing upon the possible participation of natural killer (NK) cells as the most likely component of the innate immune system to produce arteriopathy in this setting. We have studied transplants from parental strain donors (C57BL/6, hereafter B6) to hybrid recipients between the donor strain and another unrelated strain (C57BL/6, H-2^{b} X BALB/c, H-2^{d}F1, hereafter CB6F1). These transplanted hearts continue to beat normally and appear to be in excellent condition when removed at about 60 days. No immune suppression of any kind is used. Parental-to-F1 transplants should not be opposed by any conventional immune reactivity on the part of their recipients but NK activity should be retained, producing the phenomenon of "hybrid resistance" that has been extensively studied with bone marrow transplants. In the above strain combination, 12 heart transplants have been followed for the prescribed two months. These hearts survived very well and continued to beat normally throughout this period, appearing to be well vascularized at the time of their removal. Nevertheless, all 12 developed advanced coronary arteriopathy (Fig. 1). Using this convenient experimental combination we are now attempting to establish the contribution of NK cells to this process by suppressing NK cell activity in heart

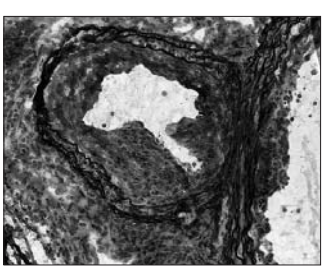

Figure 1. *Coronary artery of C57BL/6-to-CB6F1 heart transplant at 60 days.*

transplant recipients. This is being done by treating recipients with a monoclonal antibody (mAb, anti-NK 1.1) reactive with these cells.

We have also set up appropriate *in vitro* tests of NK cell numbers and cytotoxic activity as a means of following the effects of treatment on NK cells compared with control animals. These ongoing studies are expected to be part of experiments in which purposeful alterations in NK cell numbers and activity are carried out.

In the first series of experiments, 11 B6 hearts have been placed into CB6F1 recipients that are receiving injections twice weekly of anti-NK1.1 mAb using the large dose of 400 µg antibody protein. Treatment was begun 4 weeks prior to heart transplantation and will be continued throughout the 2-month observation period. At the conclusion of this experiment the transplanted hearts will be removed for immunopathological evaluation and the spleens of recipient mice will be examined for NK cell activity. In order to check the efficacy of antibody treatment in reducing NK cell activity, the peripheral blood of one of these recipients was tested. Those results indicated a considerable, though incomplete, reduction in activity.

This work is still at an early stage. Nevertheless, we feel that all our evidence so far are consistent with the conclusion that NK cells can play a significant role in the production of proliferative vasculopathy in transplanted hearts in mice. This conclusion must be thoroughly verified. Should it prove to be the case, it would have useful implications for the management of this condition in patients.

In a separate series of experiments we are exploring the effects of incompatibilities determined by genes outside of the major histocompatibility complex (MHC), i.e. non-H-2 antigens, in the mouse. The first such antigen(s) to be evaluated are those determined by the Y chromosome. Thus, 7 transplants from male C57BL/6 donors to female recipients of the same strain have been performed. Recipients received no immunosuppression and hearts were removed at about 60 days; they appeared to be in excellent condition. Control transplants employing female donors have been performed as well. Impressive lesions have formed in the coronary vessels of all of the male hearts but in none of the female hearts (Fig. 2). This example of arteriopathy is probably mediated mainly by the conventional adaptive

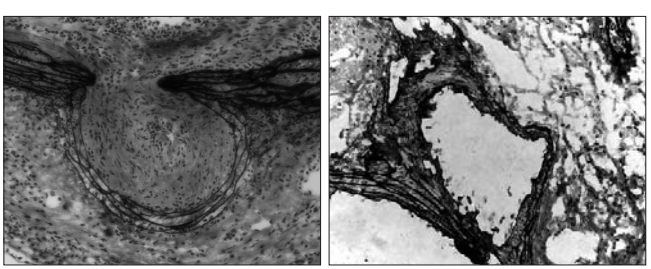

Figure 2. *Coronary arteries near takeoff from the aorta of (left) C57BL/6 male-to-female and (right) C57BL/6 female-to-female heart transplants, both at 60 days.*

immune response. It will be of interest, however, to determine the effects of a few additional non-MHC-determined antigens to find out how prevalent their effects are, and we plan to do this with the collaboration of Dr. Derry Roopenian of the Jackson Laboratory. He will make available combinations of congenic mice differing only in respect of the H-4 or H-60 specificities. Each of these specificities has been determined to have some attractive attributes for elucidating features of the pathogenesis of transplant arteriopathy. The H-60 antigen is a particularly strong one and appears to engage NK cells particularly well. It may not be represented, however, on myocardial or endothelial cells, although it should be present on dendritic cells in the heart. H-4, on the other hand, appears to be ubiquitous and of moderate strength.

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Source of Intimal Smooth Muscle like Cells in Aortic Allograft Arteriopathy

Despite excellent therapies to prevent acute allograft rejection, successful long-term solid organ transplant survival continues to be limited by transplant-associated arteriosclerosis. The arteries of allografted organs typically develop severe, diffuse intimal hyperplastic lesions leading eventually to lumenal stenoses and to ischemic graft failure. The intimal lesions, variously called chronic rejection, allograft arteriopathy, graft vascular sclerosis, transplant-associated arteriosclerosis, or graft arterial disease (GAD), result from an initial alloimmune response, although the entire set of effector mechanisms remain to be elucidated. The lesions consist primarily of smooth muscle cells (SMC) and associated extracellular matrix, mixed with infiltrating mononuclear leukocytes.

Until recently, the working hypothesis was that low-level endothelial or perivascular immune responses induced persistent allograft vascular damage. In turn, inflammatory cells and/or activated, dysfunctional endothelial cells (EC) and medial SMC would secrete growth factors that induced the migration of SMC from the donor media into the arterial intima, and stimulated their proliferation and matrix synthesis. This hypothesis has now been modified by the recent demonstration in aortic and heart experimental models, as well as in human renal transplantation, that intimal SMC (SMLC, smooth-muscle-like cell) are actually host-derived. The major elements that direct the recruitment of T cells, monocytes and other leukocytes, as well as SMC into grafts are adhesion molecules and chemokines. Recent clinical and animal studies have demonstrated the presence of specific chemokines in allografts during acute rejection and during the development of GAD lesions. Moreover, in animal models, blockade of chemokine and chemokine receptor interactions have attenuated leukocyte recruitment into allografts and diminished the development of GAD, pointing towards novel and important therapeutic strategies for this disorder. Thus, we hypothesize that endothelial injury or dysfunction caused by a variety of vascular insults induces a chronic healing response characterized by the recruitment and activation of SMLC. Proliferation of these cells with ongoing matrix synthesis forms the basis of the GAD lesions. Whether these SMLC derive from donor SMC or from host cells has important implications for targeting therapeutic intervention in GAD, as well as in more conventional atherosclerosis.

Specific Aim 1.
To test the hypothesis that the SMLC in TxAA lesions are recruited by pathways different from those used by inflammatory cells that mediate acute allograft rejection, including distinct chemokine and/or chemokine receptor usage.

Results

1. Direct anti-inflammatory mechanisms contribute to attenuation of experimental allograft arteriosclerosis by statins.

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

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

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

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

Recruitment of activated macrophages is critical to the development of GAD. Cellular signaling from the TGF-β superfamily occurs through the formation of a heterodimeric receptor complex consisting of two transmembrane receptor serine/threonine kinases,

type I and type II. After ligand activation, the type II receptor phosphorylates and activates the type I receptor. The activated type I receptor may then phosphorylate the intracellular signaling mediators, termed Smads, which translocate to the nucleus where they direct transcriptional responses. Three classes of Smads are responsible for coordinating the downstream signaling effects. TGF-β/activin receptors phosphorylate the pathway-restricted Smads, Smad2 and Smad3, whereas bone morphogenic protein receptors activate Smads 1, 5, and 8. Upon phosphorylation, these pathway-restricted Smads may hetero-oligomerize with Smad4, the only common Smad, and translocate to the nucleus where they may participate in regulating transcriptional events. Smad6 and Smad7, known as inhibitory Smads, are structurally divergent from other Smads and function to block TGF-β signaling by preventing activation of pathway-restricted Smads.

The importance of Smad proteins in regulating inflammatory events by TGF-β1 is highlighted by the phenotype of mice deficient for Smad3. These mice exhibit spontaneously activated T cells, impaired mucosal immunity, and abnormal wound healing. However, the role of Smad3 in regulating macrophage activation in these mice and its role in vascular inflammation *in vivo* had not been previously examined.

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

Smad3$^{-/-}$ mice. These data indicate a novel mechanism by which TGF-β1 via Smad3 can suppress inflammatory responses in the vascular system. We are investigating whether similar Smad3-mediated pathways can influence SMLC recruitment.

Specific Aim 2.

We hypothesize that intimal SMLC in TxAA derive from both host bone marrow (BM) as well as host non-BM precursor cells. Although we have demonstrated that intimal SMLC are essentially all host-derived, it is not yet known whether the SMLC of BM origin derive from hematopoietic or mesenchymal stem cell precursors. Moreover, BM origin of such cells is responsible for at most 20% of the total, and ingrowth from the edges of anastomses likewise does not account for the presence of the host cells in TxAA lesions. Therefore, the exact anatomical origin of the majority of the apparently non-BM-derived SMLC is unclear. Since the various precursors may utilize distinct pathways to access grafts and have different growth requirements, identifying all of the potential precursor cells is critical to effectively develop strategies to prevent their recruitment and activation.

In the next year of the study, we will use adoptive transfer experiments with hematopoietic and stromal cell-fractionated BM-derived cell populations derived from green fluorescence protein (GFP) transgenic mice to identify which could be the precursor BM population for the intimal SMLC in TxAA. Similarly, we will use the GFP animals as sources of circulating stem cells, and for generating cultured medial SMC and intimal SMLC for adoptive transfer into wild-type recipients of allografts, to identify which cell populations can contribute to TxAA intimal lesions.

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Vβ Transcriptome Regulation during Allograft Rejection and Tolerance

A major aim of the original application was to study whether Vβ transcriptome topologies differ by tolerance induction regimens in the same rat strain and in a model of operationally tolerant drug-free patients. During this first year, we focused principally on this second part:

Detecting "operationally tolerant" state indices in long-term kidney recipients with stable function.

To date, long-term allograft survival relies on the administration of a combination of efficient immunosuppressive drugs. However, long-term treatment is associated with severe side effects, including nephrotoxicity and increased susceptibility to opportunistic infection[1] and malignancies, which have been shown to be proportional to immunosuppressive drug exposure[2]. This apparent need for continuous immunosuppression (IS) has made the induction of immune tolerance to the graft one of the main research objectives in the field of transplantation. For reasons that are not understood, whereas it is easy to induce immune tolerance in rodents, tolerance has been difficult to achieve in primates and humans. Acute or chronic graft rejection usually occurs when IS is interrupted. However, a minority of patients can accept a graft indefinitely without IS, demonstrating that a tolerant state can exist in humans. The actual percentage of operationally tolerant (Op-Tol) kidney recipients may be underestimated since no study has explored the possibility of IS withdrawal in kidney recipients. However, if drug-free Op-Tol kidney recipients are extremely rare, patients with minimal immunosuppression (steroid monotherapy, $<$10 mg/day) are more common. In addition, in a substantial proportion of patients with post-transplant lymphoma disease (PTLD) in whom IS has been interrupted, rejection does not occur, despite rapid IS withdrawal[3]. Finally, although liver transplantation represents a different situation from kidney transplantation, it is important to note that about 30% of long-term liver recipients can be weaned from IS[4]. Therefore, it is important to consider the hypothesis that a

substantial proportion of long-term kidney graft recipients could be in a stage of (masked) Op-Tol.

Preliminary data obtained during this first year

Ten Op-Tol kidney recipients were studied for TCR Vβ transcriptome topologies (using TcLandscape) and were compared with 15 healthy subjects and 7 patients with chronic rejection. CDR3 length-distribution alteration of the Vβ chain of the TCR and Vβ transcript accumulation (Vβ/HPRT transcript ratios) were significantly higher in Op-Tol patients than in healthy individuals ($p<0.01$ for both) and in patients with chronic rejection ($p<0.01$ for both, see Fig. 1 and Table 1).

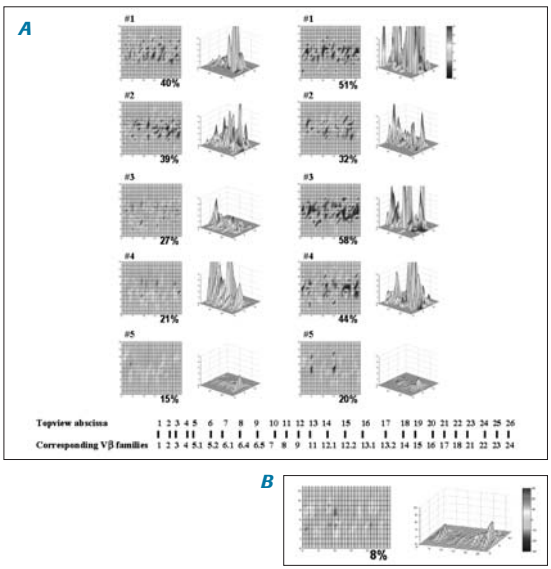

Figure 1. Vβ transcriptome alterations in tolerant patients. *Panel **A** shows the integrated % variations of CDR3 length distribution (LD) and Vβ/HPRT ratios of blood T cells of tolerant kidney recipients (mean of CDR3 LD % x Vβ/HPRT). Patients considered to be tolerant (without treatment or with low-dose steroid monotherapy) have been pooled. Panel **B** shows a representative TcLandscape of blood T cells of a normal individual. The individual T-cell landscape of drug-free patients and patients under minimal doses of immunosuppression are displayed on the left and right-hand side of panel **A**, respectively. In the TcLandscape profiles, the X axis displays the 26 Vβ families analyzed. The Y axis shows the ratio of the number of Vβ transcripts to the number of HPRT transcripts. The Z axis gives the 13 possible CDR3 lengths. TcLandscape Topview (the X axis displays the 26 Vβ families analyzed; the Y axis gives the 13 possible CDR3 lengths) allows an easy representation of the global CDR3 LD alteration. The global percentage of CDR3 LD alteration for each individual compared with the Gaussian reference (mean of the values of the 15 normal individuals tested) are given below each Topview.*

A

CDR3-LD (%)	<30	>50
N^L	93	1
DF-Tol	55	9
Ster	32	38
$StaCNI^+$	63	6
$StaCNI^-$	63	0
CR	54	11
DF-CR	59	1

B

Vβ/HPRT ratio	<5	>15
N^L	83	1
DF-Tol	75	8
Ster	69	8
$StaCNI^+$	84	1
$StaCNI^-$	99	0
CR	89	1
DF-CR	99	0

Table 1. CDR3 LD alteration and Vβ/HPRT transcript ratios in normal individuals and kidney recipients. *For the different clinical groups of kidney recipients tested (N^L: healthy individuals; Op-Tol: drug-free (DF-Tol) and under low-dose steroids (Ster); Stable: stable with (CNI^+) and without (CNI^-) calcineurin inhibitor; CR: chronic rejection with (CR) or without (DF-CR) treatment), the distribution of Vβ families according of CDR3 LD alteration (<30%, >50%) (A) and the Vβ/HPRT transcript ratios (<5, >15) (B) are given. The MANOVA test was performed on CDR3 LD alterations and Vβ/HPRT transcript ratio values. Significant differences compared with other groups ($p<0.01$) are highlighted in blue.*

The patients from these different groups have been tested for values related to qualitative alterations (CDR3 LD) and quantitative values (Vβ/HPRT transcript ratios) of the 26 human Vβ families of the TCR Vβ transcriptome: normal individuals ($n=15$), Op-Tol patients ($n=10$), stable patients ($n=7$) and chronic rejection ($n=7$).

Statistical tests were used to compare CDR3 LD and Vβ/HPRT transcript ratio values of the different groups, including possible correlation (MANOVA), with a global test (F-test) and related pair-wise comparisons. Type I error was not controlled for multiple comparisons.

CDR3 length distributions of TCR and Vβ transcript accumulation (Vβ/HPRT transcript ratios) were significantly higher in Op-Tol patients than in healthy individuals ($p<0.01$) and patients with chronic rejection ($p<0.01$; see Table 1). Paired comparisons of the different groups (patients with stable graft function, chronic rejection and tolerant patients) show that they all have significantly more altered CDR3 LD ($p<0.01$) than normal individuals. Among them, Op-Tol patients (drug-free and low doses of steroids pooled) displayed significantly more altered CDR3 LD than recipients with chronic rejection ($p<0.01$). Vβ/HPRT transcript ratio values were also significantly higher in this group ($p<0.01$ vs patients with chronic rejection). These patterns reflect a strong T-cell selection and alteration in normal clonal cell "regulation" in the peripheral blood of tolerant patients. These data were particularly surprising because they suggest that the blood is a very informative compartment in these patients. Interestingly, rats in which tolerance to an MHC-mismatched heart allograft is obtained using donor-specific transfusion (DST) have similar long-term (100 days) blood patterns[5]. Thus, whereas until now blood mononuclear cells have been considered *a priori* considered to be not very informative, our preliminary data in long-term tolerant rats show strong clonal alteration in blood but not spleen. Note that the literature does not contain (as far as we know) any blood TCR study in a classical tolerance models in rodents. Finally, we showed that most of the affected blood T cells belong to the $CD8^{+ve}$ subset. Furthermore, sorted T cells of Vβ families with altered CDR3 LD and high Vβ/HPRT transcript ratios from Op-Tol recipients did not accumulate IL-2 or IFN-α and did not present a Th2 shift. Such a pattern of positive selection, combined with low Th1/Th2 cytokine transcripts, suggests a state of anergy.

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Tolerance Through Hematopoietic Cell Transplantation with Costimulation Blockade

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

The highest numbers of hematopoietic stem cells (HSC) can currently be obtained through the collection of mPBSC from a living donor. mPBSC would thus be an attractive source of HSC for use in tolerance regimens intended for living-donor organ transplants. Since little is known about the effects of mPBSC in non-myeloablative tolerance protocols, we first investigated the engraftment potential of murine mPBSC in a CD45-congenic model. Levels of chimerism after conditioning with different non-myeloablative doses of total body irradiation (TBI) were compared after transplantation of 20 million mPBSC or the same number of bone marrow cells (BMC). As shown in Fig. 1, mPBSC led to substantially lower levels of hematopoietic chimerism in all tested lineages [e.g. 76% vs 37% myeloid donor chimerism 17 weeks post-hematopoietic cell transplantation (HCT) and 2 Gy TBI]. This difference was sustained for the length of follow-up.

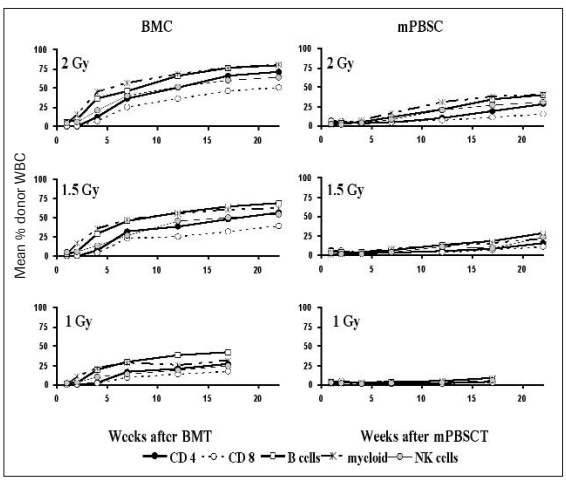

Figure 1. *Hematopoietic chimerism after transplantation of the same number of CD45-congenic mPBSC or BMC following different doses of TBI.*

These preliminary data suggest that under non-myeloablative conditions, unseparated mPBSC have a lower engraftment potential on a per cell basis than BMC, and thus support the argument that mPBSC might not be simply interchangeable for BMC in tolerance protocols using mixed chimerism. Together with our previous data showing that approx. 200 million BMC are required for the induction of lasting allogeneic macrochimerism without cytoreductive conditioning, these results made it appear unlikely that a clinically feasible cell dose (in the order of 75 million per mouse) of fully allogeneic

mPBSC would engraft without any cytoreductive conditioning. We therefore aimed to determine the minimum dose of TBI (2 Gy, 1.5 Gy or 1 Gy) necessary to allow engraftment of 75 million fully allogeneic mPBSC under costimulation blockade (Balb/c → B6), using single doses of MR1 (anti-CD154 mAb) and CTLA4-Ig). Unexpectedly, not even 2 Gy TBI allowed the induction of mixed chimerism. As early as 1 week post-HCT, chimerism was undetectable in most of the mPBSC recipients. mPBSC recipients also showed preserved donor-reactivity in MLR assays, while control chimeras induced with BMC typically demonstrated hyporesponsiveness towards donor-type stimulators. Early data from a subsequent experiment indicate that chimerism was still not reliably induced after transplantation of 200 million allogeneic mPBSC and 3 Gy TBI. These data thus suggest that 1) mPBSC have a lower potential than BMC to induce macrochimerism under non-myeloablative conditions irrespective of alloreactivity; and 2) a regimen of costimulation-blocking reagents used successfully in allogeneic BMT protocols does not overcome alloreactivity when given together with a very high dose of mPBSC and 3 Gy TBI. Additional studies will attempt to overcome alloresistance after mPBSC transplantation by increasing the dose and duration of treatment of costimulation blocking reagents, and by adding immunosuppressive drugs that are compatible with the transplantation of BMC plus costimulation blockade (see Aim 2).

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

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

Given the beneficial effect of these immunosuppressive drugs, we evaluated whether their use would improve the reliability of our previously published radiation-free model of high-dose BMT under costimulation blockade (200 million BMC per mouse, no cytoreductive conditioning). This regimen leads to lasting macrochimerism and tolerance in approximately

60% of recipients. An ongoing experiment revealed that 5 of 5 mice transplanted with 200 million BMC plus immunosuppressive drugs, and also 5 of 5 mice transplanted with 100 million BMC plus immunosuppressive drugs, but only 2 of 5 transplanted with 100 million BMC without drugs, developed lasting multilineage hematopoietic chimerism (Fig. 2). All long-term chimeras accepted donor skin grafts for more than 100 days, while promptly rejecting third party grafts. These preliminary data suggest that a one-month course of rapamycin, mycophenolate mofetil and steroids improves the rate of long-term chimeras and allows a reduction in the cell dose required for the induction of mixed chimerism without cytoreduction. Models inducing durable macrochimerism without cytoreduction have used BMC doses that cannot be obtained clinically. Modifications of such protocols allowing success with lower numbers of BMC are thus a significant step towards clinical applicability of this approach.

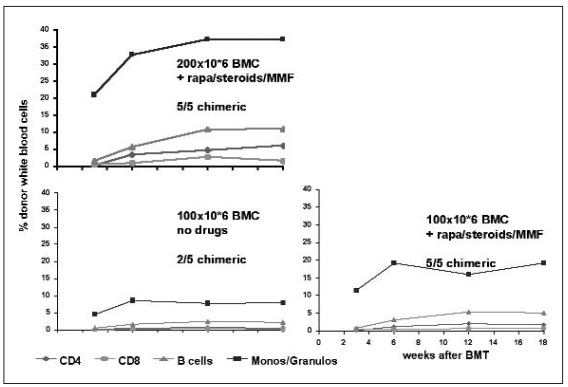

Figure 2. *Hematopoietic chimerism after high-dose BMT without cytoreductive conditioning.*

AIM 3: To increase the reliability of HCT with costimulation blockade by improving peripheral deletion of donor-reactive cells through the additional infusion of selected donor populations.

Towards this goal, we followed the deletion of relevant superantigen-reactive $CD4^+$ PBL ($V\beta5^+$ or $V\beta11^+$) after treatment with anti-CD154 mAb and transplantation of separated allogeneic cell populations (Balb/c → B6). In the initial experiments performed we did not see deletion after the transplantation of either selected B cells or dendritic cells. Since this approach has not identified the sought population, additional studies are planned in which the candidate population is eliminated from the transplanted inoculum to investigate whether deletion is inhibited in this way.

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5. Final Reports of ROTRF Grantees

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Human Hepatic Dendritic Cells Induce Tolerance via Notch Signalling

Abstract

Liver-derived dendritic cells (DC) may have an enhanced capability to induce tolerance in allogeneic T-cells. We investigated the phenotype and function of human liver-derived DC to understand how they may induce tolerance. The phenotype is consistent with a myeloid-tissue-derived subset of DC. We found no evidence to suggest that liver DCs are plasmacytoid or DC2-type. The cells express MCP-1 and IL-10 and compared with skin, pure liver DCs induce a lower rate of proliferation of pure peripheral blood T cells.

It has been suggested that the Jagged/Notch system is a mechanism for inducing peripheral tolerance in T cells. We show that both Jagged 1 and 2 are expressed by pure liver DCs, and up-regulated in co-culture with T cells. However, T cells alone also express the Notch receptors, so that there may be an element of autocrine stimulation. To understand the significance of this interaction on a cellular level we used immuno-fluorescent staining of co-cultures.

We show for the first time clear differences in phenotype and function between DC from different human tissues, and expression of Jagged 1 that is consistent with its proposed role in tolerance induction.

Hypothesis

Human liver DCs express higher levels of Notch ligand than equivalent skin DCs, and activate Notch signalling in allogeneic T-cells leading to the generation of regulatory cells and a less aggressive immune response.

Aims

1. To determine the expression and regulation of Notch ligands on comparable human DCs isolated from liver and skin.
2. To investigate Notch pathway activation in naive T-cells co-cultured with tissue-specific DCs by quantifying Notch/Notch ligands and downstream components of the Notch signalling pathway.
3. To correlate activation of Notch signalling with expression of immune regulatory cytokines and tolerance induction.

Methods

Isolation of liver dendritic cells and skin dendritic cells

Normal liver tissue is obtained from liver resected surgically either during hepatectomy for the removal of liver tumours or from surplus donor tissue that is left after donor livers have been reduced in size for transplantation into paediatric recipients. Diseased liver is obtained from liver explants of patients undergoing transplantation. The tissue is cut into small pieces, rinsed and cultured overnight in media supplemented with 10% FCS. Low-density cells migrate spontaneously from the tissue and are collected from the media for analysis. Skin DCs were isolated from normal skin pieces in the same way. Pure DCs were isolated using density gradient separation and immunomagnetic negative selection (residual T cells and neutrophils were removed).

Flow cytometry and immunohistochemistry used to phenotype cells

During the last two years, several new DC markers have become available. We have used DC-sign, CD1c, BDCA-2 and CD123 to define the phenotype of the liver DCs. Several markers were also used to stain liver tissue.

Functional comparison of liver and skin DCs

Pure dendritic DC were used to test function. Cytokine expression was assessed by real-time PCR and detection of protein in a multiplex assay. Luminex cytokine quantification was carried out on media from DC cultures. Liver-, skin- and monocyte-derived cells were studied.

We were particularly interested in determining how liver DCs direct T cell responses and we set up several co-culture experiments to investigate this aspect of DC function. The ability of tissue-specific DCs to induce T cell proliferation was tested in a modified mixed lymphocyte reaction. T cell cytokine production in the co-culture was assessed by intracellular staining and two-colour flow cytometry.

Jagged molecule expression

In a preliminary experiment with Edinburgh, we showed that Jagged receptors were differentially regulated in co-cultures of T cells with DCs from liver and skin. During this project we developed a reliable method of isolating RNA, which produced useful quantities of high-quality RNA. This banked RNA/cDNA will also be used for gene array experiments. We set up real-time PCR to quantify expression of Jagged molecules in DCs after isolation and following activation in culture, as well as in co-cultures up to 5 days. To define more clearly the role of Jagged in co-cultures, immunofluorescent staining of cytospins was used to determine patterns of protein expression on T cells and DCs at different times.

Results

Phenotype of liver DCs

Immunocytochemistry and flow cytometry of cells isolated by overnight migration from normal human liver confirms that human liver DCs express DC-sign and low levels of CD123 (Fig. 1). CD123 expression was detected on class II positive cells, and on further analysis a proportion of these cells were negative for CD14. The cells were again negative for BDCA-2. Recent publications suggest a human DC subset that expresses CD11c, with low level CD123 in peripheral blood[1]. We therefore looked at the marker for this subset, CD1c/BDCA-1, and found that all liver-derived DCs express this marker. As of yet, none of the markers has been specific for DCs in whole liver tissue by immunohistochemistry (Fig. 2). Thus, it has not been possible to isolate tissue DCs directly from the tissue and we have had to rely on the system we developed based on the characteristic ability of DCs to migrate out of tissue during an overnight incubation.

The phenotype of liver- and skin-derived cells was similar except that skin DCs express CD1a, which is not expressed by liver DCs in tissue or following isolation. We plan to use electron microscopy to gain more specific information about morphology, such as the presence of Birbeck granules, characteristic of Langerhans cells.

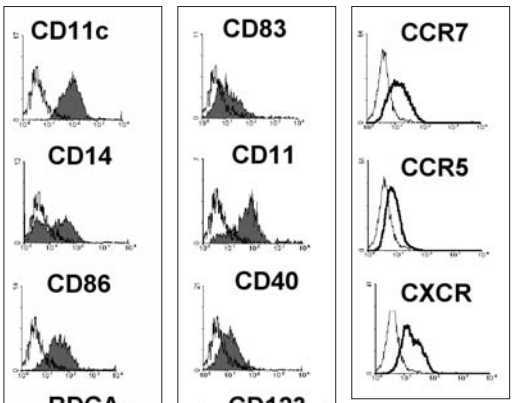

Figure 1.
Liver dendritic cells were isolated by migration and density gradient centrifugation. Overnight migrated liver DCs were characterised by dual colour flow cytometry. Cells shown were gated on scatter characteristics and positive staining for class II. The control staining is shown as an unfilled histogram. The cells conform to a myeloid DC type.

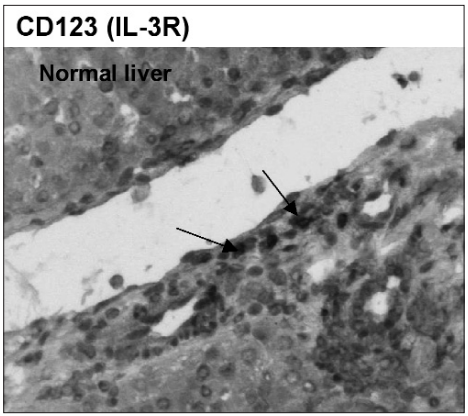

Figure 2.
Immunohistochemistry has been used to find a specific marker for DCs in liver. Many markers are shared with Kupffer cells, and in this case with endothelium. An APAAP technique was used to stain normal liver for CD123. Portal tract DCs have been arrowed.

DC cytokine production

Using ELISA we found that liver-derived DCs preferentially secrete IL-10 in response to activation and fail to secrete IL-12, whereas skin-derived DCs fail to secrete IL-10. We used real-time PCR to look for expression of IL-12 subunits by liver DCs and found that both IL-12p40 and IL-12p35 mRNAs were expressed by liver DCs despite the lack of cytokine secretion. In addition, the luminex immunofluorescent microparticle-based system was used to assay multiple cytokines from DC conditioned media (Fig. 3). This confirmed the differential expression of IL-10 and demonstrated that liver DCs secrete high levels of MCP-1, also implicated in down-regulation of immune responses[2].

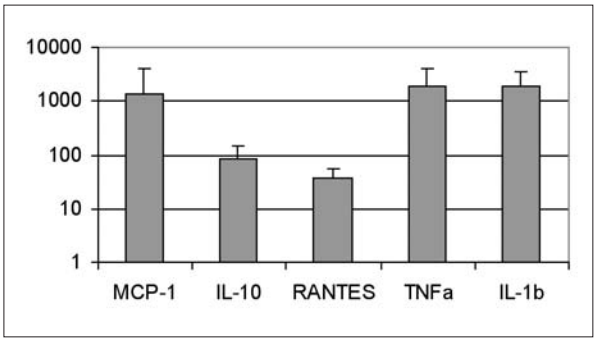

Figure 3.
Pure liver DCs were isolated after overnight migration and then cultured for a further 8 hours in plain media. The media was collected and cytokines assayed using the Luminex fluorescent immuno particle system. The results shown are in pg/ml, and SEM is shown.

T cell stimulation by skin and liver DCs

When pure liver and skin DCs isolated in the same way were compared for their ability to stimulate allo-proliferative responses using adult T cells as responders, the liver DCs were markedly less efficient inducers of T cell proliferation (Fig. 4).

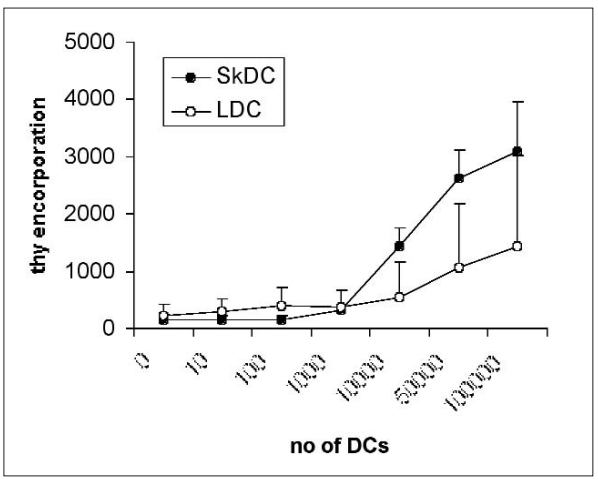

Figure 4.
The ability of DCs from skin (n=3) and liver (n=5) to stimulate allogeneic T cells was tested in a modified MLR in which highly purified, negatively selected allogeneic T cells were used as responder cells and DCs that had been completely purified using negative selection as stimulators. The skin DCs were significantly better stimulators of allogeneic T cells than liver-derived DCs.

T cell cytokine production in response to activation by DCs

Pure T cells from adult peripheral blood were tested in an MLR experiment with pure DCs from skin and liver (Fig. 5). Skin DCs stimulated 10% of the responding cells to secrete IFNγ and IL-4, but no or few IL-10 positive T cells were detected ($n=2$). In contrast, liver DCs stimulated 2% of the responder T cells to secrete IFNγ and IL-4 and a similar percentage of T cells secreted IL-10 ($n=3$). The results were similar for parallel experiments using cord blood T cells as responders.

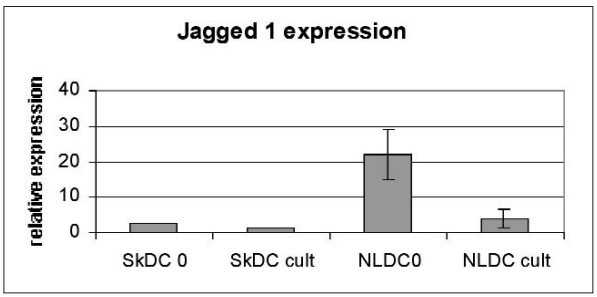

Figure 5.
Pure populations of skin and liver DCs were assessed before culture and after culture for 8–24 hours. Total RNA was isolated and expression of molecules assessed by real-time quantitative PCR, using multiplexed bactin as a control. All the cell populations showed expression of Notch receptors, but only uncultured liver DCs expressed high levels of this proposed pro-tolerance molecule.

Comparison of Notch ligand expression and function on liver- and skin-derived DCs

We compared Notch receptor expression on liver- and skin-derived DCs. Using real-time PCR, Jagged 1 and 2 expression was quantified. Jagged 1, which has been associated with induction of tolerance, was expressed at 11× greater levels on liver DCs than skin DCs (Fig. 6). Maturation of liver DCs in culture resulted in down-regulation of Jagged 1, suggesting that liver DCs may lose their immunomodulating properties as they mature. We also found that T cells up-regulate Notch receptors in culture. Previously we found that Notch receptors were up regulated in co-cultures of liver DCs and T cells.

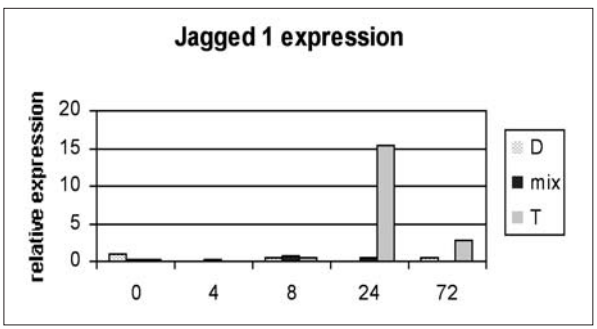

Figure 6.
T cells, DCs and co-cultures were sampled at time points in culture. We found that T cells reliably up-regulated Jagged 1, suggesting that increases that occur in co-cultures (not marked in this example) may be as a result of T cell up-regulation of Jagged 1. This would not be surprising as T cells may regulate immune responses via autocrine signalling.

Discussion

In the first year of the project we were able to make use of several new DC antibodies that became available. This enabled us to define more accurately the subset of DCs found in the

liver. This subset is characterised by expression of CD11c, CD1c, and low levels of CD123, but no BDCA-2. This corresponds with a myeloid cell type that has been described in blood. This is relevant, because reports in mice have suggested that a number of plasmacytoid-type cells in the liver were responsible for down-regulating liver immune responses. There is no evidence for this in man. We are still looking for specific markers in whole human liver tissue to enable the direct isolation of DC, and to allow further study of cells in tissue.

We have shown a complete absence of Langerhans-type cells ($CD1a^+$) in the liver and furthermore have demonstrated marked functional differences between DCs isolated by the same technique from human skin and liver tissue. There is very little information about the function of human tissue-derived DCs and no previously published studies directly compare DCs from different tissues. We found that liver-derived DCs were less effective at inducing proliferation of adult blood T cells compared with skin-derived DCs. Furthermore, skin-derived DCs induce expression of IFN-γ, but little IL-10 in responding T cells, whereas IL-10 dominates in T cell stimulated by liver DCs. This implies that skin DCs stimulate a more vigorous Th1 type response compared with liver DCs, which may help to explain differences in the immune responses between these two tissues.

We hypothesised that expression of Jagged 1 by liver DCs would induce T cells to differentiate into pro-tolerance cells. In support of this we have shown that liver DCs express more Jagged 1 than skin DCs and that on activation in culture Jagged 1 is down-regulated whereas in co-cultures of DCs and T cells Jagged 1 is up-regulated. When we looked at cultured T cells separately, we noted similar findings. We are now determining whether the Jagged 1 up-regulation in co-cultures is expressed by regulatory T cells or by liver DCs.

Publications

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2. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. *Nat Immunol* 2:102–107.

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Gene Therapy-Based Treatment for Insulin-Dependent Diabetes Mellitus

Insulin-dependent diabetes mellitus (IDDM) is caused by selective autoimmune destruction of insulin-producing ß cells in the islets of Langerhans present in the pancreas. Currently there are two therapies available: commonly used periodic injections of insulin, and transplantation of the pancreas or pancreatic islets. Both treatments have serious limitations. Among patients using commonly employed insulin therapy, physiologically optimum blood sugar control has been difficult to achieve and consequently patients suffer from many complications associated with long-term hyperglycemia. Pancreas or pancreatic islet transplantation can achieve precise blood glucose control, but the usefulness of these options is limited because of a severe shortage of donor organs and the side effects of the necessary lifelong treatment with immunosuppressive drugs. This project represents our continued effort towards the long-term goal of providing a replacement ß cell in the form of the recipient's own cells, engineered for glucose-regulated insulin secretion, as a much needed alternative therapy for IDDM.

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

Our original proposal included modifications in the insulin gene construct and optimizations in the procedures of gene delivery to improve the overall insulin production. A number of new insulin gene constructs were produced, their integrity was confirmed by DNA sequencing, and they were then cloned into a replication-defective adenovirus vector for the purpose of *in vivo* functional analysis. The method of adenoviral *in situ* transfection of liver cells was modified for further improvements, such that the blood glucose levels of Ad.3SAM-treated diabetic rats after an overnight fast were statistically indistinguishable from those of the normal rats (84±15 mg/dl in treated versus 76±5 mg/dl in normal, whereas the diabetic controls remained at 430±46 mg/dl). The reduction in fasting blood glucose levels was Ad.3SAM-dose-dependent.

An important aspect of this study was to confirm that biologically active insulin, which causes lowering of blood glucose in insulin-gene-treated diabetic rats, is produced in the liver cell, and does not originate from the remaining pancreatic ß cells that may have escaped destruction due to suboptimal streptozotocin treatment. The inability of immunohistochemical methods to detect insulin-producing cells in the pancreas of streptozotocin-treated diabetic rats supports the above contention. However, this observation alone does not exclude the possibility that the remaining ß cells were depleted of stored insulin (degranulated) due to increased demand, and therefore could not be detected. If this is true, then as part of their normal compensatory mechanism these remaining ß cells under hyperglycemic challenge would overproduce insulin mRNA and secrete insulin. We generated rat and human insulin sequence-specific primers and used them to amplify insulin cDNA from the reverse transcribed mRNA isolated from the liver, the pancreas and other control tissues of normal, diabetic, and insulin-gene-treated diabetic rats. The results of RT-PCR analysis showed that among insulin-gene-treated diabetic rats, only the liver contained human insulin mRNA, and the pancreas lacked the rat insulin I and II transcripts. None of the other tissues tested showed the presence of human or rat insulin transcripts, confirming that the insulin gene treatment caused a liver-specific human insulin production, and that streptozotocin completely ablated pancreatic ß cells.

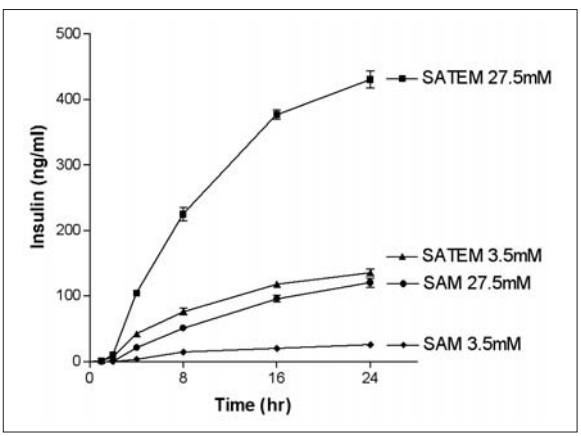

Figure 1.

Human insulin was detected by radioimmunoassay in the serum of diabetic rats treated with Ad.3SAM and not in the diabetic sham-treated, control diabetic, and the normal control rats. There was a 3.02±0.37-fold increase in the level of human insulin when fasted Ad.3SAM-treated diabetic rats were fed *ad libitum*. Additionally, the rat C-peptide levels, assessed by radioimmunoassay, reached a readily measurable level (19±2 µU/ml) only in a group of normal control rats fed *ad libitum,* whereas the levels in all diabetic rats, regardless of treatment, remained undetectable. Collectively, these observations show that the reduction in hyperglycemia in insulin-gene-treated diabetic rats was a direct consequence of glucose-dependent human insulin production in their liver cells. The majority of the preceding data have been published[1].

The insulin construct Ad.3SATEM, containing a translational enhancer (TE) from VEGF, significantly increased (>3.5-fold) insulin production in hepatocytes *ex vivo,* while retaining glucose responsiveness, as shown in Fig.1. Preliminary studies using Ad.3SATEM *in vivo* showed an improved correction of hyperglycemia. Thus, a shorter duration of fasting (9hr, instead of 16hr) was sufficient for achieving euglycemia. Blood glucose levels among the rats fed *ad libitum* also decreased 70–100 mg/dl (p=0.006) compared with the levels in diabetic rats treated with the construct lacking TE.

In summary, we have completed most aspects of the original proposal and made progress on others as planned. We are optimistic that use of the newly generated insulin construct with translational enhancer and further optimization of gene delivery procedures will yield improved output as well as a longer duration of insulin expression.

Publications

1. Alam T, Sollinger H. Glucose-regulated insulin production in hepatocytes. *Transplantation* 2002, 74: 1781–1787.

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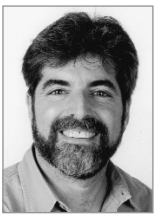


Immunosuppression and Tolerance Induction by Genetically Engineered Dendritic Cells Expressing Cell-Surface Anti-CTLA-4 mAb

Introduction and Project Aims

Among the best characterized negative regulatory pathways is that mediated by the interaction of CTLA-4 (CD152) on T cells with a pair of ligands [B7-1 (CD80) and B7-2 (CD86)] expressed by antigen-presenting cells (APCs) such as dentritic cells (DCs), macrophages, and B cells. A large body of literature now exists to show that CTLA-4 plays a central role in the generation of immune tolerance to peripherally expressed antigens. The strategy of recruiting additional CTLA-4/B7 interactions during alloantigen presentation is complicated, however, by the fact that CTLA-4 shares specificity for its natural ligands with a homologous T-cell receptor (CD28) that transmits a potent activation signal when it is engaged along with the T-cell receptor (TCR). We have developed and characterized an artificial surface-bound ligand for CTLA-4 that has no ability to bind to CD28. We initially hypothesized that this engineered ligand – derived from a single-chain anti-CTLA-4 antibody – would deliver a negative signal when expressed on the same cell surface as a TCR ligand (anti-CD3 or peptide/MHC complex). *In vitro* studies confirmed the predicted function for this cell-surface anti-CTLA-4 protein[1] and led us to develop the hypothesis and specific aims upon which the current project is based. The overall hypothesis for the project was that expression of a CTLA-4-specific surface ligand on professional APCs presenting alloantigen will promote subsequent immune tolerance to allografted organs and tissues expressing the same alloantigens.

The specific aims of the project were as follows:

Specific Aim 1: To generate transgenic mice expressing a surface-linked CTLA-4-specific ligand on DCs.

Specific Aim 2: To utilize a retrovirus-based gene transfer system to engineer anti-CTLA-4-expressing APCs for tolerance induction.

Specific Aim 3: To compare the effects of control and anti-CTLA-4-expressing DCs in allotransplantation.

Significant results during the grant period

Specific Aim 1

CTLA-4 has been shown to play a critical role in the negative regulation of immune responses. Since CTLA-4 expression on activated T cells negatively regulates activation events and favors tolerance, we hypothesize that the use of tolerogenic APCs which express a membrane-bound single-chain anti-CTLA-4 molecule will alter or disrupt T-cell activation and thus provide a more potent tolerogenic reagent. The expression of single-chain anti-CTLA-4 scFv on a definable APC population allows for a selective negative signal via CTLA-4 without the complications of binding to CD28.

Initial studies showed that we could make a membrane-bound form of anti-CTLA-4 that inhibited T-cell responses[1] (Fig. 1). However, the affinity of the antibody was suboptimal. Thus, for the *in vivo* experiments, attempts were made to increase affinity. Yeast display mutagenesis was used to overcome the limitations decreased stability or antigen-affinity of mAbs. As shown in Fig. 2, the selected mutations occurred in framework regions of the V_L sequence rather then in the CDR loops. These mutants were shown to bind the ligand, CTLA-4, more effectively[2].

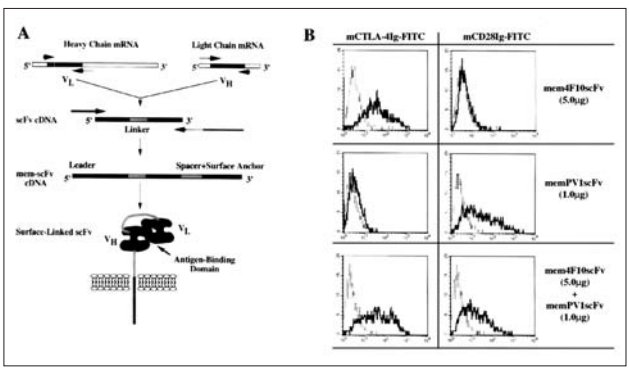


Figure 1.
Schematic of membrane bound scFv.
A. *The general strategy used to develop the membrane bound single-chain Fvs.*
B. *Flow cytometric analysis of HEK293 transfected cells with expression vectors containing anti-CD28 and anti-CTLA-4 scFvs. Fluorochrome-labeled soluble ligands, CD28-Ig and CTLA-4-Ig demonstrated binding specificity. Cells expressing anti-CD28scFv bind CD28-Ig but not CTLA-4-Ig, and cells expressing anti-CTLA-4 scFv bind CTLA-4-Ig but not CD28-Ig.*

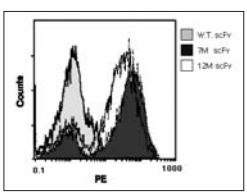
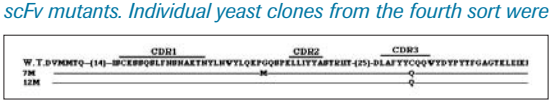


Figure 2.
A. *Flow cytometric analysis of two mutant anti-CD152 scFvs with improved yeast surface display expression compared with the parent (W.T.) construct. Yeast surface-displayed anti-CD152 scFv was detected with biotinylated rabbit anti-mouse IgG2a followed by streptavidin-PE. The yeast clones bearing selected mutant anti-CD152scFv (7M and 12M) demonstrated significantly enhanced surface binding of CTLA-4-Ig. The amplification product from an error-prone PCR of the anti-CD152 scFv gene was cloned into the yeast display vector pCT302 (1) and used to transform E. coli, resulting in ~2×10^5 colonies. Plasmid isolated from the E. coli library was used to transform Saccharomyces cerevisiae-EBY100 (3) to generate ~10^6 yeast transformants. Mutant anti-CD152 scFvs were isolated from the yeast library after four flow cytometric sorts with successively decreasing concentrations of the CTLA-4-IgG2a fusion protein: 160 , 40 , 0.8 , 0.08 μg/ml.* ***B.*** *Amino acid sequence alignment of the two selected anti-CD152 scFv mutants. Individual yeast clones from the fourth sort were analyzed for improved binding to the CTLA-4-IgG2a ligand and the plasmid rescued and sequenced. The complementarity determining regions of the variable light gene is overlined.*



Development of cell-surface-linked single-chain Fvs (scFv)

Utilizing an Igμ promoter and enhancer to drive constitutive expression on mature B cells, we have generated a mouse expressing anti-CTLA-4 mem scFv on about 50% of $B220^+$ B cells. The CTLA-4-Ig-FITC binding was specific as no staining was observed on the control or Tg^- mice (Fig. 3). Studies demonstrated the inhibitory effects of these transgenic B cells in T-cell-dependent B-cell antibody responses including antibody class switching and antigen-specific antibody production. Anti-TNP responses were measured from serum of mice receiving LPS-activated, TNP-OVA-loaded B cells from either wildtype FVB or 7MBS transgenic B mice. Serum responses were measured on days 0, 7, 14, 21 following the transfer of these B cells, a priming event. Recipient mice were boosted with 100 μg TNP-OVA/CFA 7 days following the transfer of the LPS-activated TNP-OVA loaded B cells. Results indicate that the anti-CTLA-4 B cells decrease antigen-specific T-cell-dependent B-cell class switching to IgG_{2a} when challenged with hapten-protein conjugates (Fig. 4).

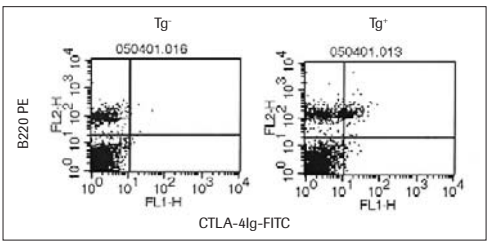
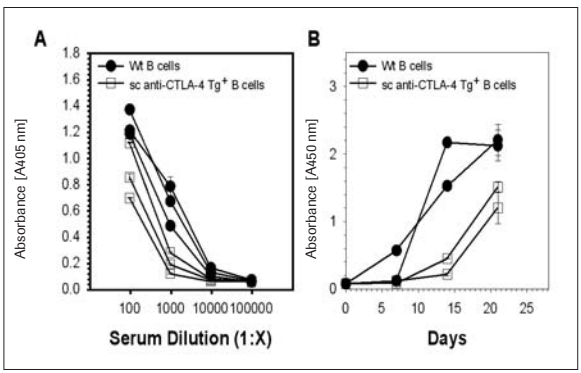


Figure 3.

Figure 4.
Anti-CTLA-4 scFv transgenic B cells decrease anti-TNP-OVA IgG_{2a} antibody production.
$B220^+$ (CD45R) B cells were purified from anti-CTLA-4 scFv transgenic and FVB control mice using a high speed cell sorter and cultured for 72 hours in the presence of 1μg/ml LPS. Following culture, transgenic or control B cells were loaded with TNP-OVA for 3 hours. Cells were washed and transfered intravenously to naïve FVB recipients. 7 days later, both groups of mice were boosted with 100μg TNP-OVA/CFA. Serum was collected days 0, 7, 14, 21 following boost. ***A.*** *Anti-TNP IgG2a-specific ELISAs from day 7 post antigen boost at logarithmic dilutions.*
B. *Anti-TNP IgG_{2a} specific ELISA at 1:1000 dilution for days 0, 7, 14, and 21 following adoptive transfer of purified B cells.*



Specific Aim 2

Although retroviral transfection vectors expressing surface-linked anti-CTLA-4 have been successfully generated and result in high-level expression of the construct in a variety of murine cell lines, it has not been possible to achieve high-efficiency transfection of cultured murine DCs using these vectors. For this reason experiments have focused on alternative techniques for achieving this aim. In addition, modifications to the single-chain construct have been carried out with the goal of improving surface stability and allowing detection of

the surface-expressed protein on B7-expressing APCs. These goals have been met as follows:

(a) The mutated anti-CTLA-4 construct developed by yeast display technology in collaboration with Dr. David Kranz (University of Illinois, Urbana, USA) was used in an adenoviral vector that has been generated and has been shown to transduce a variety of murine cell lines and primary cells resulting in high-level expression of the protein. Furthermore, a protocol for high-efficiency adenoviral transfection of murine cultured DCs has been developed. Adenovirally mediated gene expression in cultured murine DCs has been shown to persist for at least one week following transduction, allowing an ample duration of expression for functionally meaningful interactions with T cells to occur *in vitro* and *in vivo.*

(b) A system for the generation of significant numbers of anti-CTLA-4-expressing DCs for use in *in vitro* and *in vivo* experiments has, therefore, been developed.

Specific Aim 3

In previous studies to determine the functionality of the anti-CTLA-4 scFv reagent, T-cell suppression was assessed using mem4F10scFv-transfected 293 cells. These anti-CTLA-4-transfected cells effectively suppressed *in vitro* proliferation and cytokine production of CD4+ and CD8+, Th1 and Th2 T cells when co-expressed with an antigen in the context of MHC (see below). This construct was also found efficacious in an allogeneic tumor graft rejection model. Tumor cells bearing the membrane-bound anti-CTLA-4 scFv on their surface were effective at neutralizing the T-cell clearing response and increased in size, whereas control cells were rejected and the tumors resected and diminished in size[2]. Given the impressive inhibitory effects of this reagent *in vitro* and *in vivo,* we generated a transgenic mouse expressing the high-affinity mutant (7M) anti-CTLA-4 membrane-bound single-chain Fv, 7M-4F10scFvB7.1, in pBSEuPu which contained an immunoglobulin IgH enhancer and promoter to limit expression to B cells. We have continued to pursue the use of anti-CTLA-4 membrane-bound scFv to suppress allogeneic responses. Using the newly developed mutant form of the anti-CTLA-4 mAb we now see robust inhibition of T-cell responses[2]. $RAG^{-/-}$ mice transplanted with P815 cells transfected with the 7M grow equally to normal P815 T cells. However, when 10^4 2C T cells (that recognize H-$2L^d$) were co-transferred, the control vector transfected P815 but tumors expressing membrane-bound anti-CTLA-4 are rejected. The difference is also seen in the survival (or lack thereof) of mice bearing the vector versus anti-CTLA-4-transfected tumor cells.

B cells isolated from 7M transgenic mice demonstrated decreased mixed lymphocyte reaction (MLR) proliferation and effector cytokine production *in vitro* (Fig. 3). Expression of anti-CTLA-4 scFv resulted in 42% lower T-cell proliferation in MLR reactions and an 80% reduction in IFN-γ secretion.

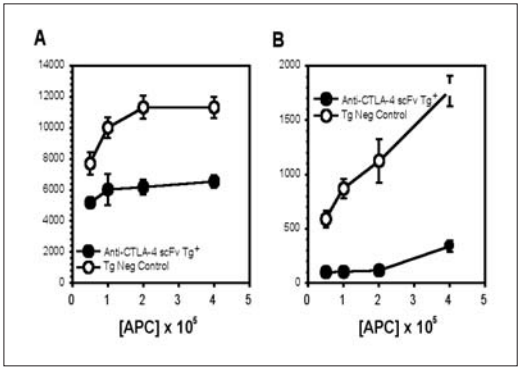


Figure 5.
Membrane-bound anti-CTLA-4 scFv transgenic B-cell expression attenuated allogeneic T-cell proliferation and cytokine production in a mixed lymphocyte reaction.
B220+ (CD45R) B cells were purified from anti-CTLA-4 scFv transgenic and FVB control mice and cultured for 72 hours in the presence of LPS. Transgenic or control B cells were co-incubated with purified resting C3H T cells.
A. *Proliferation was assessed by tritiated thymidine incorporation between 72 and 84 hours of culture.*
B. *IFN-γ cytokine levels in the culture supernatant was determined after 48 hours of culture.*

In summary, we have overcome the challenge of developing high-level expression systems for the anti-CTLA-4 surface-linked construct and have shown that it can inhibit alloimmunity. The immune phenotype of mice expressing anti-CTLA-4 on B cells will continue be characterized. Anti-CTLA-4-expressing B cells from these transgenic animals will also be used isolated and compared with wild-type B cells for their ability to induce primary or secondary T-cell activation or tolerance *in vitro* and *in vivo* using allogeneic and TCR transgenic T-cell model systems.

Publications

1. Griffin MD, Ashourian N, Hong DK, et al. Surface-linked single chain antibodies deliver T cell receptor and co-stimulatory signals to resting T lymphocytes *in vitro* and *in vivo*. *J Immunol* 2000, 164:4433–4442.
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3. Kwang WH, Sweatt WB, Brown IE, et al. Cutting edge: Targeted ligation of CTLA-4 *in vivo* by membrane-bound anti-CTLA-4 antibody prevents rejection of allogeneic cells. *J Immunol* 2002, 169: 633–637.
4. Starwalt SE, Masteller EL, Bluestone JA, et al. Directed evolution of a single chain Class II MHC product by yeast display, *Prot Biochem, in press.*

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Dr. Maria-Cristina Cuturi, Co-Investigator
Dr. Ignacio Anegon, Research Associate
Mr. Cédric Lovet Research Associate
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The Role of TRANCE/RANK Interaction during Allogenic Immune Responses

The major goal of this study was to understand the role of a recently described member of the tumor necrosis factor (TNF) superfamily called TRANCE (TNF-related activation induced cytokine) and its receptor RANK (receptor activating NF-κB) during allogeneic immune responses *in vivo.* In the immune system, TRANCE is expressed by activated T cells and RANK is mostly expressed by mature dendritic cells (DC) that are professional antigen-presenting cells. TRANCE induces survival and activation of mature DC. Despite the critical role of the CD40L pathway of costimulation in antigen-presenting cell functions, the TRANCE pathway can mediate CD40L-independent T-cell activation. Our hypothesis is that TRANCE is involved in DC-mediated T-cell priming during allograft rejection. In preliminary experiments supported by the ROTRF grant, we have shown that TRANCE mRNA is strongly upregulated in acutely rejected heart allografts in rats and that the inhibition of the TRANCE-RANK pathway led to enhanced allograft survival.

Aim 1. To extend our study on the regulation of expression of TRANCE and RANK during acute and chronic rejection.

We have studied the expression of TRANCE and RANK mRNA in acutely rejected heart allografts in rats. We found that both molecules were strongly upregulated during acute rejection. The upregulation of TRANCE mRNA was transient, similar to that observed for CD40L. In contrast, RANK was strongly upregulated as soon as day 1 after grafting and its expression remained stable during the first week. The expression of TRANCE and RANK protein was assessed by immunofluorescence on tissue section. As expected, TRANCE was expressed by both $CD4^+$ and $CD8^+$ T cells but not by antigen-presenting cells. Surprisingly, we found that 25% of TRANCE-expressing cells were not T cells or antigen-presenting cells. Future experiments will be designed to identify the nature of these cells. RANK was expressed by antigen-presenting cells in acutely rejected allografts. We have also generated biological tools to study the function of the TRANCE-RANK interaction, such as stable transfectants for rat TRANCE and RANK, soluble rat TRANCE and antibodies against rat TRANCE.

Aim 2. To analyze immunological mechanisms of allograft enhancement by the RANK.Fc fusion molecule.

We have shown that TRANCE blockade using the RANK.Fc fusion molecule induced prolongation of heart allograft survival in rats (Table 1). The prolongation was modest but similar to that observed by CD40L blockade in the same model. Optimal survival was obtained by two IV injections of 1 mg protein. The effect of TRANCE blockade was also assessed in a mouse model of heart allograft (Balb/c → C57Bl/6). In this model, TRANCE blockade also induced allograft enhancement, with 3 out of 10 animals exhibiting long-term survival (>100 days). To analyze the effect of prolonged TRANCE blockade we have generated a recombinant adenovirus coding for RANK.Fc. Via *in vitro* control experiments, we have shown that transduced COS cells expressed the recombinant protein (immunohistology) and produced RANK.Fc in their supernatants (ELISA). Hearts from LEW.1W donors were transduced with recombinant adenoviruses by slow injection into the apex and ventricular walls at four points. A strong expression of RANK.Fc was observed in transduced allografts on day +5 and high levels of RANK.Fc were detected in the sera of these animals one month after grafting (>100 µg/ml). Similar to allograft recipients injected with RANK.Fc protein, rats that received an allograft transduced with RANK.Fc adenovirus exhibited a prolonged graft survival compared with control Addl324 adenovirus (Table 1). These results indicate that prolonged TRANCE blockade does not appear to improve allograft survival further than that achieved by short-term blockade.

Table 1. *Allograft survival.*

Groups	Survival (days)	Survival (MST ± SD)	*p* value
Untreated	5, 5, 6, 6, 6, 7, 7, 7, 9, 10	6.8 ± 1.6	
0.5 mg human IgG on days 0, +2, +4, IP	7, 7, 7, 7	7 ± 0	NS (vs untreated)
0.5 mg TR.Fc on days 0, +2, +4, IP	10, 11, 20, 45	21.5 ± 16	0.01 (vs hIgG)
1 mg TR.Fc day 0, IV	8, 8, 9, 34	14.8 ± 12.8	NS (vs hIgG)
1 mg TR.Fc on days 0, +2, IV	14, 21, 25, 44	26 ± 12.8	0.0003 (vs hIgG)
Addl324-transduced allografts	6, 6, 7, 7, 7, 7, 7, 8, 10, 11	7.6 ± 1.6	
Ad.RANK.Fc-transduced allografts	10, 11, 11, 12, 13, 14, 15, 16, 16, 18, 21	14.3 ± 3.3	< 0.0001 (vs Addl324)

We investigated the mechanisms of allograft enhancement by TRANCE blockade in recipients of Ad.RANK.Fc-transduced allografts. RANK.Fc-transduced allografts exhibited a strong leukocyte infiltrate similar to that observed in control allografts. Immunostaining revealed a substantial decrease in both the numbers and intensity of staining of CD11b-positive cells. Other markers were not modified (MHC class II, CD4, CD8, CD25). TRANCE blockade did not inhibit anti-donor alloantibody production or anti-RANK.Fc humoral response. We did not observe any decrease in the expression of IFN-γ, IL-2, IL-4, IL-10, TNF-α or TGF-β mRNA in RANK-Fc-transduced allografts compared with controls on day

5 after grafting. However, the expression of CD40L mRNA was strongly increased in RANK-Fc-transduced allografts. This suggests that TRANCE blockade might upregulate CD40L on activated T cells which could compensate for the lack of TRANCE/RANK signaling in mature DCs and therefore eventually lead to acute rejection. Moreover, the strong humoral response against RANK.Fc could also inhibit the effect of the fusion molecule.

Aim 3. To optimize the effect of TRANCE blockade on acute and chronic allograft rejection.
We have focused on the effect of combined TRANCE and CD40L blockade on chronic heart allograft rejection. Indeed, the strong upregulation of CD40L expression in heart allografts from RANK.Fc-treated animals could impair the beneficial effect of TRANCE blockade and we therefore assessed the effect of combined TRANCE and CD40L blockade. Our group has previously shown that long-term CD40L blockade can induce long-term or indefinite allograft survival; however it did not inhibit chronic allograft rejection, suggesting CD40L-independent allogenic immune response in this setting. We hypothesized that this response could be TRANCE-dependent. To test this hypothesis, heart allografts were transduced with both AdCD40.Fc and Ad.RANK.Fc adenovirus and compared with grafts transduced with Ad.CD40.Fc alone. Comparable long-term survival was obtained in both groups. Cotreatment with CD40.Fc significantly increased serum levels of RANK.Fc and inhibited the production of anti-RANK.Fc and anti-adenovirus antibodies. Long-term allografts were then examined for the presence of chronic allograft rejection. We found that TRANCE blockade significantly decreased chronic allograft rejection lesions found in allografts from CD40.Fc-treated animals as assessed by the decrease in fibrosis, leukocyte infiltration, vessel occlusion and lesions and of mast cells. The residual humoral response found in CD40.Fc-treated animals was significantly reduced in the sera of CD40.Fc + RANK.Fc-treated animals, suggesting that TRANCE-RANK interaction could play a role in B-cell function when the CD40 pathway is inhibited. Finally, we showed that combined CD40L and TRANCE blockade did not inhibit systemic T-cell-dependent antibody response against sheep red blood cells in allograft recipients, arguing against the possibility of a general immuno-suppression in these allograft recipients.

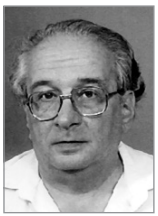
Conclusions

The ROTRF funding has been critical to support our project and therefore to decipher the role of TRANCE-RANK interactions during allogenic immune response *in vivo.* Our results indicate that this interaction plays an important role both in acute and chronic allograft rejection, and that CD40L-independent allogenic response can be, at least in part, mediated by the TRANCE-RANK pathway. Further experiments will be necessary to analyze the mechanism of action of TRANCE-blocking reagents *in vivo,* to assess the effects of the association of TRANCE-blocking reagents and classical immunosuppressants and to determine the mechanism by which TRANCE blockade could upregulate the CD40L-CD40 pathway of costimulation.

Publications

1. Guillonneau C, Louvet C, Renaudin K, Heslan J-M, Heslan M, Vignes C, Guillot C, Choi Y, Turka LA, Cuturi M-C, Anegon I, Josien R. The Role of TNF-related Activation-Induced Cytokine (TRANCE) – Receptor Activating NF-κB (RANK) Interaction in Acute Allograft Rejection and CD40L-Independent Chronic Allograft Rejection. *Submitted for publication.*

Prof. Peter Lonai, Principal Investigator
Dr. Marat Gorivodsky, Research Associate



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Can ES Cells Form a Specific Complex Organ?

We will report two research efforts. The first is a direct continuation of our original proposal and describes the effect of ES cells on outgrowths observed in mutant mouse embryos that lack *Fgfr2b* function and display no limb outgrowth. The second effort is based on a new finding from our laboratory, which is that the *Fgfr2c* alternative is a positive regulator of bone development and its gain of function mutation activates numerous aspects of bone formation. We wish to utilize this observation for an ES cell therapy of osteoporotic or osteoarthritic bone loss. The idea is to prepare ES cells carrying a homozygous gain of function mutation in *Fgfr2c* to influence the healing of bone fractures.

Part 1

The recessive *Fgfr2* mutation used in these experiments is defective in limb and branching morphogenesis and dies at mid-gestation[1–3]. Our mutation affects the entire *Fgfr2* locus. Since the specific *Fgfr2b* limb phenotype arises earlier than the viable craniosynostosis and dwarfism due to loss of *Fgfr2c,* this program analyzes a defect connected to the IIIb alternative. As described in the original proposal we study cultures of trunk fragments of these limbless embryos. The culture system employed allows embryo fragments at the pre-limb outgrowth stage (9.5–10.0 d.p.c) to develop limb buds with distinguishable falangeal rays in four days in culture[5]. We inject normal ES cells into the trunk fragments and study their growth, investigate their histological architecture, the origin of the cells in the outgrowth and study the genes expressed during this process.

We have established in this project period that most injected embryos display a more or less amorphous outgrowth at the injected side. The outgrowth consists of chondroblasts, chondrocytes and primitive mesenchymal elements. Introduction of a β-galactosidase cassette into the mutant ES cell clone allowed tracking the origin of the induced outgrowth. Interestingly both recipient-type mutant and donor-type wild-type chondrocytes could be found. This suggests that the graft differentiating towards the chondrocyte lineage induces differentiation in mutant stem cells, which normally would form no limb cartilage, to follow suit. This finding suggests that the mutant cells are capable of differentiation into chondroblasts and chondrocytes provided that wild-type-derived environmental factors are present. This important observation requires further investigation.

Teratomas formed by subcutaneously injected ES cells frequently form cartilage, although this usually takes several days. It is therefore crucial to know whether cartilage that differentiates in the presence of wild-type ES cells follows the structure of the limb skeleton. Although some preparations look encouraging, we need better whole mount and histological evidence and a statistical evaluation of this problem before a decisive conclusion can be reached.

It was essential to know at this stage whether the ES-cell-induced growths express characteristic limb development genes. Most important are genes such as *Fgf8,* which are normally expressed in the apical ectodermal ridge (AER), an epithelial growth center of the limb bud. The AER is responsible for proximal-distal limb outgrowth. Untreated embryo fragments of mutant origin do not express FGF8. Upon injecting wild-type ES cells into mutant embryos, the cultured fragments expressed FGF8 within two days of incubation. Closer inspection reveals two ridge-like areas of strong FGF8 expression of limb outgrowth. Additional experiments will have to clarify whether the FGF8 label is associated with normal AER structure. At face value this result is an important advance towards validating our hypothesis. It suggests that the outgrowth observed includes genuine characteristics of limb development.

To sum up, two main observations were made in this project period. We have shown that wild-type ES cells induce cartilage proliferation in *Fgfr2*$^{-/-}$ embryo fragments and that this process is associated with the expression of bona fide AER genes, such as *Fgf8.*

Difficulties of the project: A major recurrent difficulty is the sensitivity of the culture system. Whereas in most experiments considerable development was observed when wild-type (and not when mutant) ES cells were injected into mutant embryo fragments (Table 1), there are periods when no development can be observed. Modification of the culture medium may overcome this problem.

#	R2 –/– ES	Remarks	#	Wt ES cells	Remarks
1	+		11	++	Deformed
2	++		12	+++	Deformed
3	+/-		13	+++	Extra growth
4	+		14	++	
5	++		15	+++	
6	+		16	+++	Extra growth
7	+/-	Extra growth 1 side	17	+++	Extra growth + digits
8	+		18	++	Extra growth
9	++		19	+++	
10	+/-		20	+++	Extra growth 1 side

Table 1. *R2 mutant ES cells transplanted into the normal forelimb create extra growth compared with wild-type ES cells.*

Part 2
Use of mutant ES cells expressing a positive regulator of bone growth for the treatment of bone fractures.

The planned stem cell therapy experiment is built on results obtained in a separate project, which is now in evaluation. In this genetic study we investigated the function of the IIIc transcriptional alternative of *Fgfr2*. This alternative is expressed in mesenchymal cells and during later development in the skeleton[6,7]. Mutations of FGFR2c that create intra-chain disulfide bonds or other changes stabilizing receptor dimerization are connected to craniosynostosis and limb defects (fused fingers and/or toes) in man[8].

We created two *Fgfr2c* mutations. In the first, a stop codon and a new restriction enzyme site was created in exon 9, which inactivated this receptor variant, without affecting the other, IIIb variant in the *Fgfr2b* variant[4]. This mutation caused a loss of function phenotype, which manifested as recessive viable dwarfism with skull base craniosynostosis and late ossification of the appendicular and axial skeleton. Significantly, this loss of function mutation down-regulates *Spp1, Cbfa1, ihh and PTHrP,* which are bone development genes of the osteocyte and chondrocyte lineages. In the second "knock in", the S-S bond-forming cystein of exon 9 was mutated to glutamine. This change, typical for Crouzon-type craniosynostosis in man, causes ligand-independent activation of the FGFR2c receptor; hence it is a gain of function mutation. Heterozygous mice are viable with skull vault craniosynostosis and up-regulated bone development genes. The homozygote, which is unknown in man, is perinatally lethal with cleft palate, agenesis of the knee, carpal and tarsal joints, connected with strong up-regulation of the above-mentioned bone development genes (in preparation).

We interpret these results to suggest that FGFR2c is a positive regulator of bone development. Its effect is opposite to that of another FGFR isotype, FGFR3, which inhibits chondrocyte and osteoblast development[9,10]. Since both genes are epistatic to most bone development genes, it is reasonable to assume that they affect osteogenesis from its early stages.

Based on these findings we would like to develop a stem cell therapy approach. We assume that ES cells homozygous for the gain of function allele of *Fgfr2c* should support fracture healing. Preparation of ES cell clones homozygous for the mutation and a simple bone fracture healing protocol could be the first test of this hypothesis. Defective bone reorganization is a major clinical problem. Outstanding examples are incomplete healing of osteoporotic fractures and the bone and cartilage loss observed in osteoarthritis. These significant diseases have no definitive, hypothesis-driven therapy for the time being.

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Potentiation of Dendritic Cell Tolerogenicity with NF-κB Antagonist Phosphorothioate Oligonucleotide

The ability of dendritic cells (DC) to induce immunity or tolerance is related to their state of functional maturation. We proposed that treatment with short double-stranded oligodeoxyribonucleotides (ODN) with consensus NF-κB binding sites might maintain DC in an immature state, enhancing their tolerogenicity.

The proposal was funded for two years as a proof-of-concept study (Aim I and Aim II) with a one-year extension to define the mechanisms of tolerance induction by NF-κB ODN-modified DC (NF-κB ODN DC), and to potentiate tolerogenicity of NF-κB ODN DC by genetic engineering (Aim III). In three years, we have made significant progress towards achieving the goals intended in the proposal.

Aim I. Devise an effective strategy to deliver NF-κB ODN to dendritic cells (DC) to arrest DC maturation and activation.

In Aim I, the "therapeutic window" of NF-κB ODN, i.e., the most appropriate dose that yields the maximal effect with minimal toxicity, and the duration of action of NF-κB ODN on DC were determined. DC propagated from bone marrow (BM) with GM-CSF+IL-4 (IL-4 DC) efficiently took up ODN, with FITC-ODN detectable in DC as early as 2 h after exposure, and persisting within the cells for more than 14 days without apparent toxicity (Fig. 1).

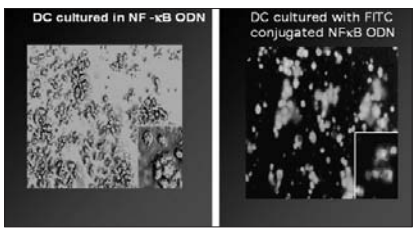

Figure 1.
DC propagated from mouse BM efficiently take up FITC-conjugated NF-κB ODN.

NF-κB ODN specifically block NF-κB DNA binding activity:

1. Gel shift assays of nuclear/cytosolic protein fractions of cultured DC revealed that NF-κB ODN bind specifically to cytosolic NF-κB, blocking nuclear translocation of NF-κB and subsequent DNA binding. Mutant ODN controls had no such effect (Fig. 2).

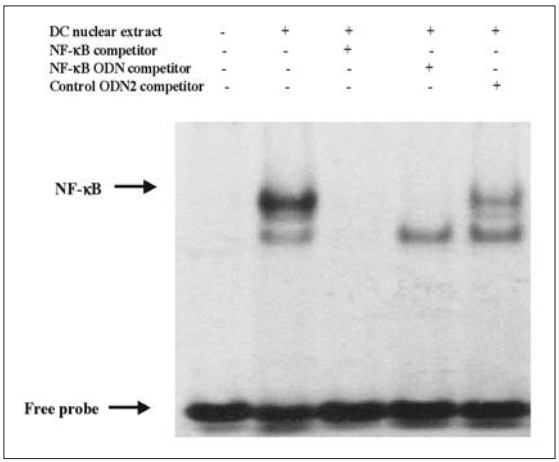

Figure 2.
DNA binding activity for NF-κB.

2. Transient transfection with a luciferase transgene expressed under the influence of a NF-κB promoter element revealed that transcription and transgene expression in response to NF-κB-inducing stimuli were inhibited by NF-κB ODN, but not by mutant control ODN.
3. The production of nitric oxide (NO) in response to LPS, dependent upon NF-κB activation, was completely inhibited by NF-κB ODN.
4. Expression of costimulatory molecules (CM) (CD80, CD86 and CD40) and allostimulatory capacity were significantly reduced in NF-κB ODN DC. This was resistant to maturation stimuli, such as IL-4 or adenoviral vector transfection (Fig. 3).

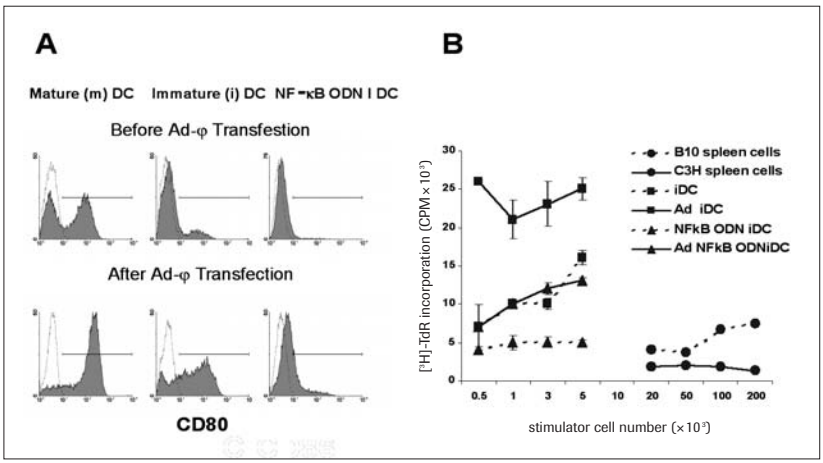

Figure 3.

However, costimulatory molecule inhibition required addition of ODN at the initiation of culture. NF-κB ODN do not alter CM expression once DC become mature. Compared with mutant ODN DC or untreated DC, the proliferation of allo-T cells stimulated by NF-κB ODN

DC was reduced 80% in MLR, and the levels of proinflammatory cytokines, such as IFN-γ and TNF-α were significantly reduced in the supernatants of MLR as determined by ELISA. In summary, double-stranded NF-κB decoy ODN are efficiently taken up by DC under defined conditions, inhibit NF-κB binding in the nucleus, and arrest DC maturation without toxicity.

Aim II. Assess the influence of NF-κB ODN DC on immune responses *in vivo* in allogeneic mice.

The immunomodulatory effect of donor-derived DC treated with NF-κB ODN administered to unmodified recipients was compared with that of IL-4 DC (immunostimulatory DC) and TGF-β DC (transiently tolerogenic DC, propagated with GM-CSF+TGF-β). The fate of NF-κB ODN DC after *in vivo* administration was determined by immunohistochemical staining with mAbs to anti-donor MHC class II and CD86. NF-κB ODN did not alter the migration pattern of DC in allogeneic recipients (Fig. 4). Increased numbers of NF-κB ODN DC with low CM expression on their surface were seen in T-dependent areas of host secondary lymphoid tissues.

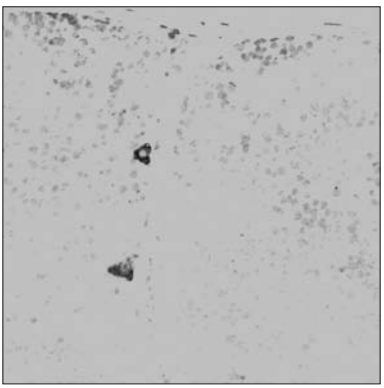

Figure 4.
Identification of donor BM-derived NF-κB ODN DC (H-2^b, red) in allogeneic recipient's spleen (H-2^K).

Cytokines released by NF-κB ODN DC at rest and after activation were analyzed by RNase protection assay and ELISA. IL-12 production of NF-κB ODN DC was totally inhibited (Fig. 5 A and B). In contrast, the expression of IL-12 mRNA in IL4-DC (mature DC) was high, and inducible in TGF-β DC (immature DC) by allostimulation or LPS.

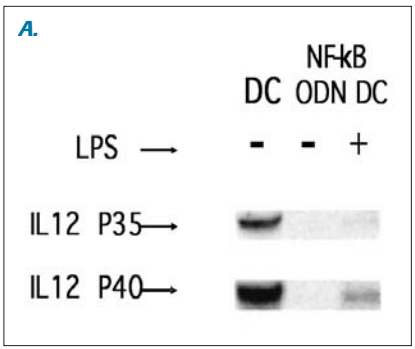

Figure 5A.
NF-κB ODN suppressed IL-12 mRNA expression of DC induced by LPS stimulation.

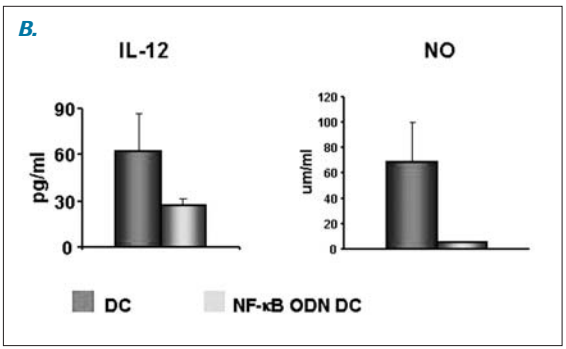

Figure 5B.
NF-κB ODN inhibited IL-12 and NO production by LPS-stimulated DC.

Administration of donor-derived NF-κB ODN DC before heart transplantation significantly prolonged survival of cardiac allografts in a donor-specific manner, and was associated with dramatically reduced IFN-γ production in heart grafts and draining lymph nodes, as determined by RNase protection assay and ELISA. Anti-donor CTL responses were inhibited in recipient spleen T cells.

In summary, our results show that NF-κB ODN decoys inhibit CM expression and IL-12 production of DC activated by LPS or adenoviral transfection, resulting in inhibition of T-cell immune responses *in vitro* and *in vivo.*

Aim III. To potentiate tolerogenicity of NF-κB ODN DC by genetic engineering.

The ability of NF-κB ODN to prevent LPS- and rAd-induced DC maturation is potent; however, there was a modest but progressive increase in CD80/CD86 expression by NF-κB ODN DC over time in the DC and allo-T-cell co-culture (Fig. 6). This finding suggested that an additional NF-κB-independent signaling pathway existed whereby allogeneic T cells could enhance their stimulatory potential. It was concluded that further genetic modification of NF-κB ODN DC with immunosuppressive transgenes might maximize their tolerogenicity. In Aim III we assess the effect of NF-κB ODN DC transfected with Ad-CTLA4Ig (NF-κB-AdCTLA4Ig DC) on allo-immune responses *in vitro* and *in vivo* in the context of allogeneic heart transplantation.

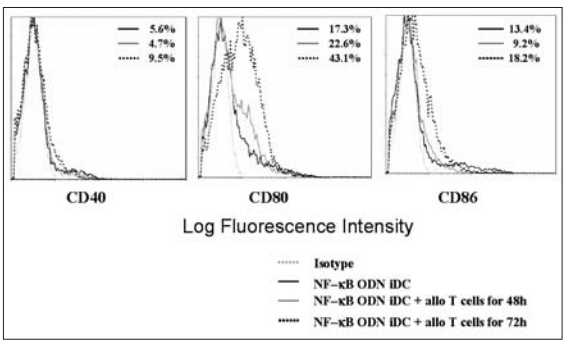

Figure 6.
Increase in costimulatory molecules expression of NF-κB ODN DC by allogeneic T cells.

1. Exposure to NF-κB ODN prevents DC activation induced by Ad-transfection, and does not interfere with transgenic gene expression. Most of DC or NF-κB ODN DC transduced with AD-EGFP fluoresced within 2 days of gene transfer (Fig. 3A and 7). Similarly, Ad-CTLA4Ig-transduced, NF-κB ODN-treated DC secreted similar amounts of transgenic CTLA4Ig compared with Ad-CTLA4Ig DC that had not been exposed to NF-κB ODN.

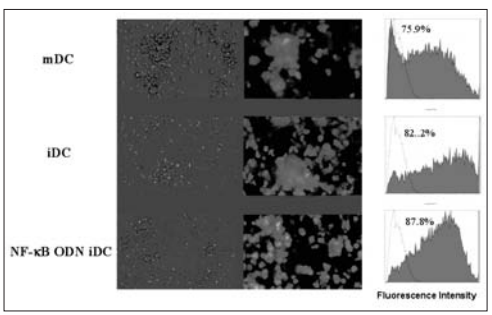

Figure 7.
Exposure to NF-κB does not interfere with transgenic expression of EGFP.

2. Treatment of DC with NF-κB ODN combined with Ad-CTLA4Ig is more effective than either agent alone in reducing T-cell allostimulatory ability.
3. Combined treatment of DC with Ad-CTLA4Ig and NF-κB ODN promotes apoptosis of activated T cells. Con A-activated, alloactivated, or naive T cells were cultured in the presence of either NF-κB ODN/Ad CTLA4Ig DC, Ad CTLA4Ig DC, Ad CTLA4Ig DC, or Ad-φ DC. Significant levels of T-cell apoptosis were promoted by NF-κB ODN/Ad CTLA4-Ig DC, Ad CTLA4Ig DC, Ad CTLA4-Ig DC as determined by two-color staining of CD3 and TUNEL. By contrast, mature control DC (Ad-φ DC) appeared to protect alloactivated T cells from apoptosis (Fig. 8).

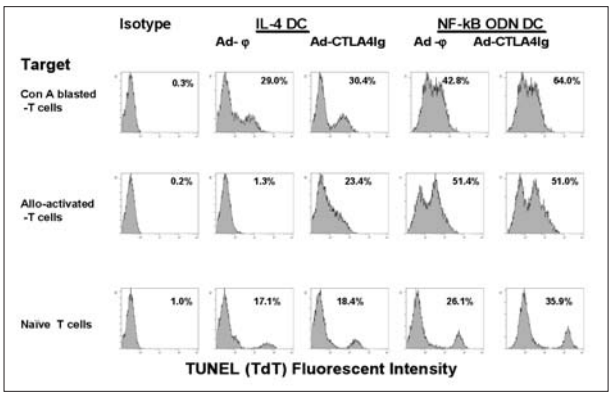

Figure 8.

The greatest degree of apoptosis was noted in T cells exposed to NF-κB ODN/Ad CTLA4-Ig DC, suggesting that concomitant inhibition of both CM expression (by NF-κB ODN) and functional blockade of CM (by CTLA4-Ig) was more effective than either treatment alone in promoting activated T-cell apoptosis.

4. NF-κB ODN/Ad CTLA4-Ig DC promote long-term donor-specific cardiac allograft survival. Pretreatment of recipients with donor-derived NF-κB ODN/Ad CTLA4-Ig DC extend heart allograft survival MST to 71 days. More than 40% of the animals exhibited indefinite (>100 day) graft survival. The effects were achieved in the absence of circulating CTLA4Ig in the serum.
5. NF-κB ODN/Ad CTLA4-Ig DC suppress Th1 and Th2 cytokine gene expression within cardiac allografts. IL-2, IFN-γ and IL-10 gene transcription was readily detected in grafts of untreated animals. Although there was evidence that ODN-treated DC promoted Th skewing toward Th2, no unequivocal evidence of immune deviation was obtained from analysis of hearts from NF-κB ODN/Ad CTLA4-Ig DC-treated recipients (Fig. 9).

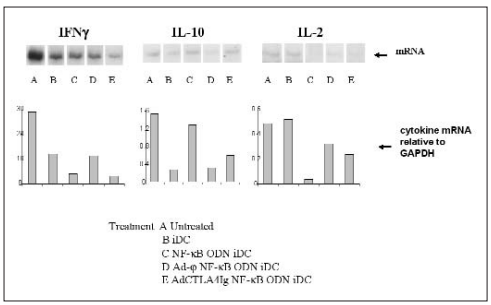

Figure 9.

The results of this project are extremely encouraging and strongly supportive of the conception that blockade of NF-κB signal transduction in DC with NF-κB ODN decoys is a powerful approach to promoting antigen specific tolerance. Further elucidation of the impact of NF-κB ODN DC on alloreactive T-cell apoptosis will aid us in designing a molecular therapy for prevention of allograft rejection and for inhibition of autoimmune disease.

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Effect of Costimulatory Blockade with Bone Marrow Transplantation on Organ Transplant Rejection

Previous work demonstrates that intestinal allografts elicit an immune response that is both quantitatively and qualitatively different than that observed with most other transplanted organs. The robust recipient immune response to intestinal allografts results clinically in outcomes inferior to those associated with other types of organ transplants and experimentally is associated with the failure of many immunosuppressive strategies that are effective in other transplant models. Although numerous agents have been shown to promote long-term survival or tolerance in murine models of heart and skin transplantation, we have yet to identify an approach that results in the long-term survival of intestinal allografts in wild type recipients. These observations suggest both that intestinal transplantation would benefit from the development of new approaches for modulating the recipient immune response and that the intestinal transplant model is a useful tool for evaluating new immunotherapies.

The development of hematopoietic macrochimerism has long been recognized to promote the development of donor-specific tolerance to organ allografts. One of the major factors limiting the broader clinical application of strategies seeking to induce donor-specific tolerance via the creation of hematopoietic macrochimerism has been concern over the toxicity of the conditioning regimens necessary for the engraftment of donor bone marrow. Recently an approach has been described that avoids the need for recipient irradiation or T-cell depletion. Treatment with the alkylating agent busulfan, which preferentially depletes early hematopoietic stem cells, in combination with anti-CD154 and CTLA4-Ig permits the engraftment of allogeneic bone marrow and the donor-specific acceptance of skin or heart allografts. Last year we reported that this regimen also resulted in the long-term survival of intestinal allografts in mice. Findings from our study that are consistent with results obtained using the skin and heart transplant models include the following:

- The majority of long-term surviving intestinal allografts did not display histologic evidence of chronic rejection.
- T cells from treated recipients bearing intestinal allografts were hyporesponsive to donor but not third party alloantigen *in vitro.*
- Donor-reactive recipient T cells were deleted *in vivo.*

However, the results of experiments performed using the intestinal transplant model varied from those using other transplant models in several significant ways, including the following:

- The observation that donor-responsive T cells persist *in vivo* at least at intermediate time points in mice engrafted with donor bone marrow following treatment with busulfan and costimulation blockade. This conclusion is based on the finding that recipient T cells from treated mice proliferated in a graft-versus-host disease model after adoptive transfer into recipients that expressed donor alloantigen.
- Although the survival of donor-strain skin grafts placed on treated recipients bearing long-term surviving intestinal allografts was significantly prolonged relative to third party skin grafts, nearly all chimeric mice eventually rejected donor-strain skin grafts. Interestingly this did not result in the rejection of the original intestinal allograft in most recipients.
- Unlike the results of experiments using skin or heart transplant models where allografts transplanted on the day of initiation of the chimerism-inducing regimen survived indefinitely, all intestinal allografts transplanted on the day that the treatment regimen was initiated were promptly rejected.

These findings have several important implications. First, although this regimen promoted the development of multi-lineage hematopoietic chimerism and the long-term survival of intestinal allografts, recipient mice were not tolerant by the strictest definition as they remained capable of rejecting subsequently placed donor strain skin grafts, albeit with delayed kinetics. Secondly, the clinical application of this strategy for the transplantation of highly immunogenic organs like the intestine may be limited by the inability to perform the transplant prior to the completion of the chimerism-inducing regimen, which requires at least six days.

The next set of experiments undertaken was designed to more fully characterize the mechanisms responsible for the prolonged allograft survival associated with the regimen of busulfan, bone marrow infusion and costimulatory blockade. We hypothesized that a process of active immune regulation contributed to the ability of this regimen to promote the long-term survival of organ allografts. This hypothesis was based on several observations. First, substantial deletion of donor-reactive T cells required 35 or more days and was not complete for periods of 50 to 60 days. The observation that control mice that received combined costimulation blockade (anti-CD154 + CTLA4-Ig) plus donor bone marrow but no busulfan failed to develop chimerism and all rejected intestinal allografts by day 18 demonstrated that costimulation blockade alone was not sufficient to allow intestinal allografts to survive for the 1 to 2 months necessary for deletion to occur. Second, early depletion of $CD4^+$ T cells, a potential population of regulatory cells, prevented the development of chimerism and tolerance. Lastly, the demonstration that donor alloantigen-reactive

T cells persisted for at least 4 to 6 weeks, as shown using the GVHD model, suggests the presence of a mechanism such as regulation that is capable of preventing allograft damage by the persistent donor-reactive T cells.

In collaboration with Dr. Charles Orosz of Ohio State University, experiments were designed to directly test this hypothesis. For these experiments a murine model of heart transplantation was used. This model offered the advantage that Dr. Orosz's group has demonstrated that mice treated with a short course of gallium nitrate (GN) accept cardiac allografts for more than 100 days, although chronic rejection characterized by fibrosis and neointimal hyperplasia is a characteristic finding of this group. Importantly, allograft survival in mice treated with GN is associated with immune regulation, which is mediated by recipient T cells that produce TGF-β as demonstrated using the *trans-vivo* DTH assay. Thus, GN-treated mice bearing heart allografts could be used as a comparison group in which regulation could be demonstrated. All heart allografts in mice treated with either GN or combined costimulation blockade, busulfan, and donor bone marrow survived for the duration of the study (90 days). However, by day 60 the majority of heart allografts in GN-treated mice displayed histologic evidence consistent with severe chronic rejection. In contrast, heart allografts from mice treated with the chimerism-inducing regimen demonstrated normal histology without evidence of either acute or chronic rejection at all time points examined. T cells from GN-treated mice bearing heart allografts displayed regulatory properties as detected by the *trans-vivo* DTH assay. This regulatory effect was dependent upon TGF-β, as indicated by the return of a DTH response following the addition of an anti-TGF-β monoclonal antibody. This response was well developed by day 60 and persisted beyond 100 days. A similar regulated immune response was observed in heart allograft recipients treated with costimulation blockade, busulfan, and donor bone marrow. However, regulation occurred earlier (day 30) and was lost by day 60.

These are the first data to directly demonstrate the presence of regulatory T cells in allograft-accepting mice rendered chimeric by the infusion of bone marrow cells. Our data support a model in which allograft acceptance following treatment with costimulation blockade, busulfan, and donor bone marrow is the result of two sequential and partially overlapping mechanisms – the early development of a transient population of regulatory T cells, and deletion which eventually eliminates both alloaggressive and regulatory donor-reactive T cells. In this model the ability of recipient mice to reject second donor-strain allografts at certain time points may reflect the waning of regulation before the deletion of other donor-reactive T cells is complete. Finally, comparison of the outcome of allografts from GN-treated recipients, where allograft acceptance is associated with the long-term persistence of regulatory cells, and the chimerism-inducing regimen, where regulatory cells are only transiently present, supports the hypothesis that persistence of regulatory populations of T cells that produce TGF-β may contribute to the fibrosis and neointimal

changes characteristic of chronic allograft damage. Future experiments will use these models to directly test this hypothesis.

Publications

1. Guo Z, Wang J, Dong Y, Adams AB, Sirasugi N, Kim O, Hart J, Newton-West M, Pearson TC, Larsen CP, Newell KA. Long-term survival of intestinal allografts induced by costimulation blockade, busulfan and donor bone marrow infusion. *Am J Transplant, in press.*

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Role of Viral Chemokine Receptors in Cytomegalovirus-Accelerated Transplant Vascular Sclerosis

Transplant vascular sclerosis (TVS), the vascular lesion associated with chronic allograft rejection, is characterized by concentric neointimal smooth muscle cell (SMC) accumulation resulting in vessel narrowing and ultimate graft failure. Cytomegalovirus (CMV) accelerates the development of TVS in human and rodent solid organ transplant recipients, and is associated with an earlier onset and increased severity of TVS, and ultimately higher rates of graft rejection. The mechanisms behind CMV's link to TVS may involve recruitment of inflammatory cells and SMC by chemokines. Chemokines are a group of inducible, small molecular weight cytokines that promote cellular migration and activation. These molecules and their receptors play major roles in the development of vascular disease as they are important stimuli for the infiltration of inflammatory cells and the migration/proliferation of SMC. The expression of CC-chemokines, which include MCP-1, MIP-1α and RANTES, is upregulated in the vascularized graft during ischemia/reperfusion injury, acute rejection, and chronic rejection. Other investigators have demonstrated that the chemokines fractalkine and IP-10 are also crucial mediators of acute and chronic rejection. By contrast, long-term allograft acceptance has been attributed to the absence of chemokine expression, thus substantiating a major role for chemokines in allogeneic graft rejection and during the development of TVS. Importantly, CMV encodes a CC-chemokine and chemokine receptors and is capable of modulating host chemokine expression *in vivo.* The focus of our ongoing study is to determine the mechanisms of CMV's link to TVS and allograft rejection.

Aims / Results

Specific Aim 1: Does RCMV induce rat SMC migration *in vitro,* and is the R33 gene required for virally induced SMC migration?

We have demonstrated that HCMV infection of human arterial SMC resulted in significant cellular migration *in vitro.* This migration event was dependent upon expression of the HCMV-encoded chemokine receptor US28, and the presence of RANTES or MCP-1. Using adenovirus vectors expressing US28 we have recently determined that US28 induces rat aortic SMC migration in a ligand-dependent manner (Fig.1A). We then extended these *in vitro* studies to rat CMV (RCMV) and rat SMC, because the rat model forms the basis for our *in vivo* studies. We found that RCMV infection of rat SMC induced significant migration

(Fig. 1B), which is similar to the results observed with HCMV-US28. To determine the contribution of the RCMV-encoded chemokine receptor R33 in cellular migration, rat SMC were infected with RCMV R33 deletion mutant (RCMV-ΔR33; a generous gift from Dr. C Vink at the University of Maastricht, The Netherlands) and subjected to our migration assay. Infection with RCMV-ΔR33 failed to induce SMC migration compared with wild-type RCMV-infected cells (Fig. 1B). These data suggest that rat SMC are capable of migrating in response to the expression of viral chemokine receptors and that R33 is necessary for RCMV-induced SMC migration.

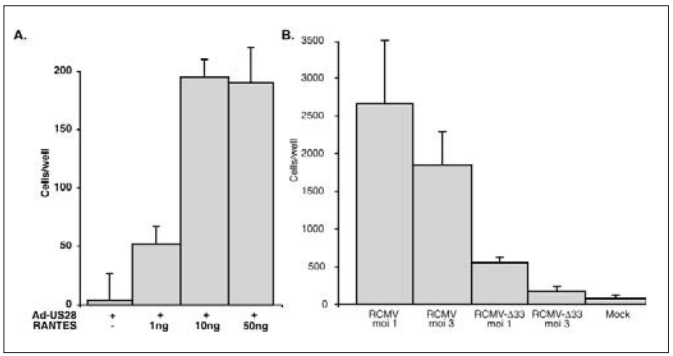

Figure 1.
A. *Ad-US28 induces rat aortic SMC migration which is enhanced by recombinant human RANTES.*
B. *RCMV induced rat aortic SMC migration is dependent upon expression of the R33 gene. Rat AoSMC were infected with either wild type RCMV or RCMV-ΔR33 and subjected to our migration assay.*

Specific Aim 2: Using an *in vivo* rat model of heart transplantation, what is the dynamic interplay of factors involved in RCMV acceleration of TVS?

We developed a rat heart transplantation model that exhibits accelerated TVS in allograft vessels from RCMV-infected recipients. We used this RCMV/rat heart transplant model to determine the mechanisms involved in CMV-accelerated TVS and allograft failure. First, in order to determine the timing of RCMV-accelerated TVS formation, we studied the kinetics of TVS in graft hearts with or without RCMV infection. For these studies, F344 hearts were transplanted heterotopically into Lewis recipients, treated with low dose CsA, and acutely infected with RCMV. Syngeneic transplant recipients and uninfected allograft recipients served as controls. Animal grafts and native hearts were harvested at days 7, 14, 21, 24, 28, 32, 35, 45 (45 days is the mean time to develop chronic rejection (CR) and TVS in CMV-infected allograft recipients). The transplanted hearts were evaluated histologically, and TVS was assessed morphologically as the mean percentage of vessel occlusion (neointimal index, NI). Graft vessels showed endothelialitis in the RCMV-infected, but not in the uninfected, allogeneic recipients at 7 and 14 days post-op (Fig. 2). TVS was detected at 21 days post-op with little difference between infected and uninfected allogeneic recipients (NI=38 vs NI=35, p=ns, Fig. 2). However, at days 28, 35 and 45 post-op, RCMV-infected recipient heart graft vessels showed a dramatic increase in the severity of TVS (NI=48, 70 and 82) compared with uninfected allografts (NI=31, 30 and 43, $p<0.001$, Fig. 2). These data suggest that the effect of RCMV on acceleration of TVS is manifested between 21 and

28 days post-op. Using quantitative PCR (TaqMan) specific for the viral polymerase sequences, we looked for the presence of RCMV DNA. The viral DNA was detected in both the graft and native hearts at all times tested, but it was only found in the blood up until 14 days post-op (Fig. 3A). Using antibodies directed against RMCV-immediate-early protein, we confirmed by immunohistochemical staining the presence of virus in the heart allografts (Fig. 3B). We have shown previously that the alloreactive environment is crucial for the RCMV-mediated acceleration of TVS.

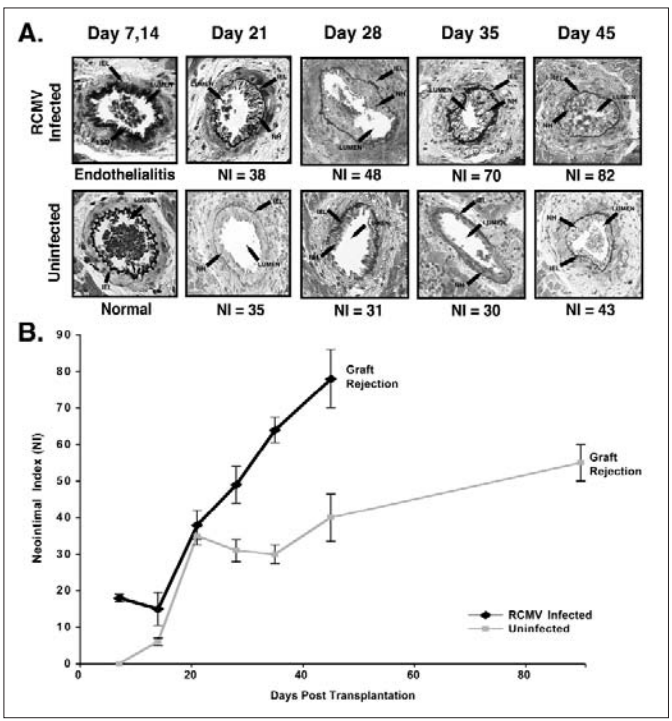

Figure 2. RCMV infection accelerates the time to develop TVS in graft hearts.
A. *Graft vessels stained with elastin showed endothelialitis in the RCMV infected, but not in the uninfected, allogeneic recipients at 7 and 14 days post-op. TVS was detected at 21 days post-op with little difference between infected and uninfected allogeneic recipients. However, at days 28, 35 and 45 post-op, RCMV infected recipient heart graft vessels showed a dramatic increase in the severity of TVS compared with uninfected controls. These data suggest that RCMV accelerates the time to graft rejection between 21 and 35 days post-op, however, this might be due to events occurring within the first 7–14 days post-op.*
B. *Comparison of mean neointimal indices of graft heart vessels of uninfected and RCMV-infected animals.*

We therefore wanted to determine whether the RCMV-induced acceleration was due to an enhancement of immune-cell infiltration into the graft vessels. The subset and distribution of infiltrating leukocytes was analyzed by immunohistochemical staining of either frozen or paraffin sections of allograft hearts harvested at POD 24, 28, and 32. Serial sections were stained for ED1 (macrophage/monocyte marker), CD4 (T-helper cell marker), or CD8 (cytotoxic T-cell marker) (Table 1, Fig. 4). As expected, native hearts from both infected and uninfected recipients failed to stain for ED1, CD4, or CD8. Vessel staining for the macrophage marker ED1 was minimal in sections from uninfected allografts until day 32 post-transplantation. However, grafts from RCMV-infected recipients showed faint staining for ED1 as early as 24 days post-transplantation with moderate to intense staining for macrophages at days 28 and 32.

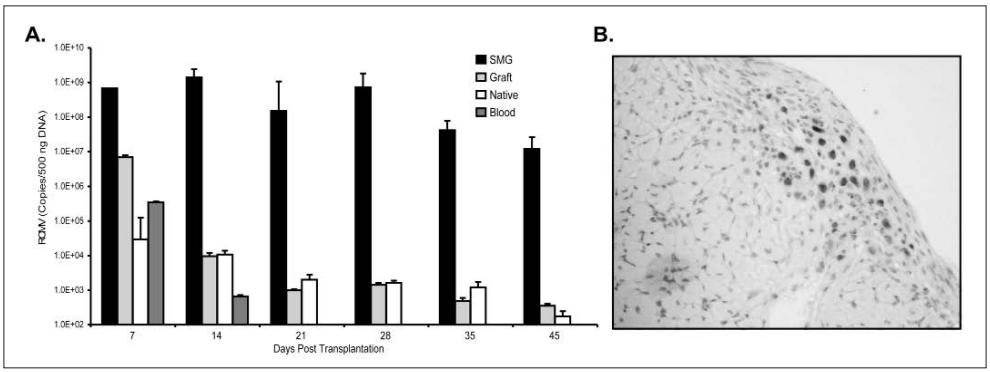

Figure 3. A. Detection of RCMV in heart allografts, blood and submandibular glands.
Total DNA was prepared from heart allografts, SMG and blood at 7, 14, 21, 28, 35, and 45 days post-op. Viral DNA was quantitated using TaqMan PCR techniques specific for RCMV DNA polymerase. This data suggests that RCMV is present within the graft tissues during all of the stages of RCMV-accelerated TVS.
B. *Immunohistochemical staining of heart allograft tissues using antibodies directed against RMCV-IE at day 7 post-transplantation.*

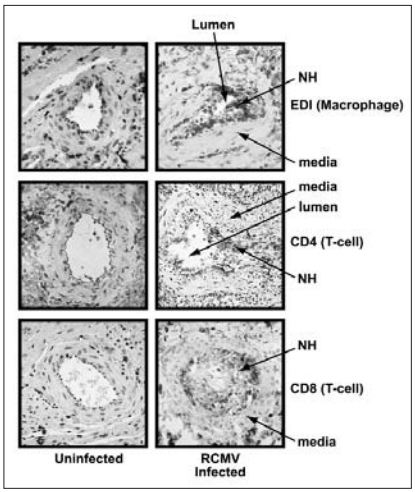

Figure 4. Host cellular infiltrates are higher in RCMV-infected graft tissues.
Heterotopic heart allografts, with or without RCMV infection, were harvested at the times of RCMV-accelerated TVS formation (24, 28, and 32 days post-op), and subsequently tissue sections were immunohistochemically stained to determine the levels and types of infiltrating cells. Immunohistochemical staining of graft heart tissues for the macrophage marker ED1, T-helper cell marker CD4, cytotoxic T-cell marker CD8 at 28 post-transplantation. RCMV infection increases the proportion of cellular infiltrates in both the intima and media of graft vessels, which corresponds to the timing of virus-enhanced chemokine expression.

Similarly, uninfected controls demonstrate little to no CD8 T-cell staining at days 21 and 28 post-transplantation but at day 32 there is a slight increase in CD8 T-cell staining, which was less intense compared with vessels from RCMV-infected allografts. The infected grafts demonstrate the presence of $CD8^+$ T cells at days 24, 28, and 32 (staining ranges from faint to moderate, Table 1). Little to no vessel $CD4^+$ T cell staining was observed at all times post-transplantation in the uninfected allografts. However, at day 32 the $CD4^+$ T-cell staining of vessels from the infected allografts was moderate to intense. These findings demonstrate that RCMV-infected allograft vessels contain increased cellular infiltration compared with uninfected controls.

Table 1. *Immunohistochemical staining of heart allograft frozen or paraffin sections for analysis of vessel intimal and medial infiltrating leukocyte subsets.*

EDI (macrophage/monocyte)					CD4 (T helper cells)					CD8 (Cytotoxic T-cells)				
	Intima		Media			Intima		Media			Intima		Media	
POD	+RCMV	Mock	+RCMV	Mock	POD	+RCMV	Mock	+RCMV	Mock	POD	+RCMV	Mock	+RCMV	Mock
24	0.2±0.1	0.1±0	**0.2±0.1***	0	24	0	0	0	0	24	**0.6±0.2***	0	**0.4±0.2***	0
28	**1.7±0.5***	0	**1±0.4***	0.2±0	28	0	0	0	0	28	**0.7±0.3***	0	**0.2±0.1***	0
32	**1.8±0.3***	1.1±0.6	**1.4±0.4***	0.4±0.2	32	**1.4±0.2***	0	0.4±0.2	0.3±0.2	32	**0.9±0.2***	0.3±0.6	**0.7±0.2***	0.2±0.2

In order to determine whether the RCMV-induced enhancement of immune-cell infiltration into graft vessels was due to infection-mediated alterations in chemokine expression, we used quantitative RT-PCR (TaqMan) techniques to compare mRNA levels of the host chemokines in the grafts and native hearts from infected and uninfected transplant recipients. Chemokine profiles in graft hearts differ dramatically with or without RCMV infection. In the infected animals, allografts showed high expression of the CC-chemokines RANTES, MCP-1 and MIP-1α between 21 and 32 days post transplantation (Fig. 5). Interestingly, the CXC chemokine IP-10 and the cytokine interferon-γ, which is the cytokine that drives IP-10 expression, were both increased about twenty-fold at day 28 in the infected allografts compared with uninfected controls (Fig. 6). Expression of two other chemokines, lymphotactin (C chemokine) and fractalkine (CX3C chemokine), was also increased between days 21 and 32, corresponding to the timing of RCMV-accelerated TVS (Fig. 7).

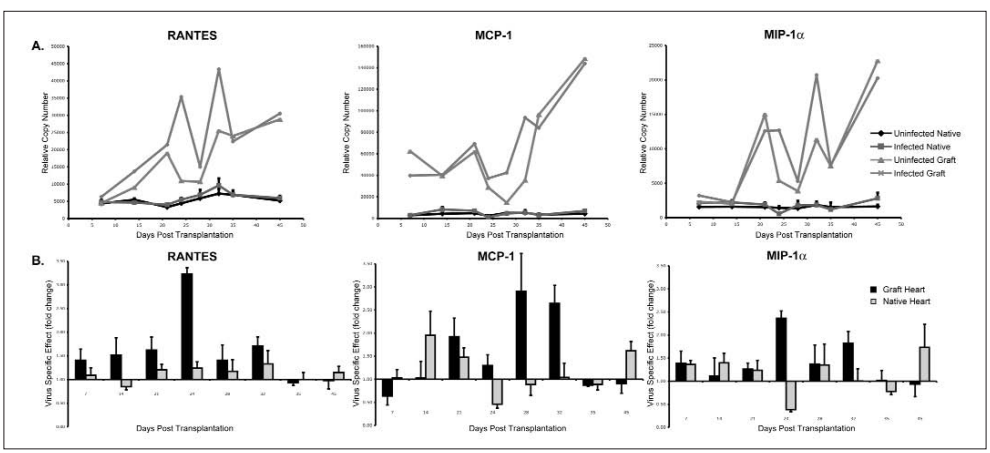

Figure 5. RCMV specifically induces the production of CC-chemokines in heart allografts.
RT-PCR TaqMan was used to detect expression of chemokines (Rantes, MIP-1α and MCP-1). The relative increase in chemokine expression in infected vs uninfected recipients (virus-specific effect) is shown graphically. RCMV specifically induces the expression of CC chemokines. Graft hearts from RCMV-infected and uninfected animals were analyzed at 7, 14, 21, 28, 32, 35 and 45 days post-transplantation (n=4). RT-PCR TaqMan detection of L32 was used as a control.

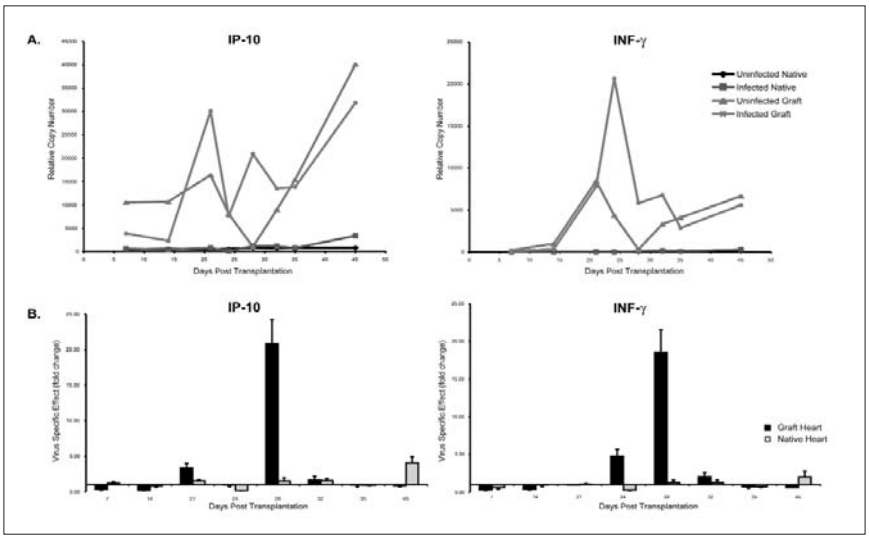

Figure 6. RCMV specifically induces the production of IP-10 in heart allografts.
RT-PCR TaqMan was used to detect expression of IP-10 and IFN-γ. The relative increase in chemokine expression in infected vs uninfected recipients (virus-specific effect) is shown graphically. RCMV specifically induces the expression of IP-10 and IFN-γ. Graft hearts from RCMV-infected and uninfected animals were analyzed at 7, 14, 21, 28, 32, 35 and 45 days post-transplantation (n=4). RT-PCR TaqMan detection of L32 was used as a control.

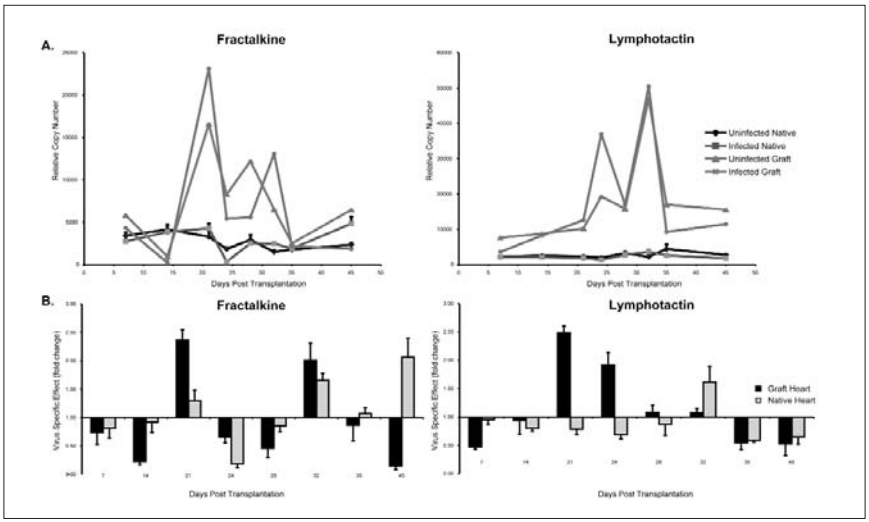

Figure 7. RCMV specifically induces the production of chemokines in heart allografts.
RT-PCR TaqMan was used to detect expression of chemokines Fractalkine and Lymphotactin. The relative increase in chemokine expression in infected vs. uninfected recipients (virus-specific effect) is shown graphically. Graft hearts from RCMV-infected and uninfected animals were analyzed at 7, 14, 21, 28, 32, 35 and 45 days post-transplantation (n=4). RT-PCR TaqMan detection of L32 was used as a control.

Specific aim 3: Using an *in vivo* rat model of heart transplantation, what is the contribution of CMV-encoded chemokine receptors to the acceleration of TVS?

Since the RCMV-ΔR33 virus has been shown to replicate to lower levels than WT RCMV in the rat, we are currently developing the bacterial artificial chromosome (Bac) system so that we can reliably mutate R33. The goal is to construct R33 mutants that prevent SMC migration while retaining their ability to replicate to WT levels *in vivo*. Once we have constructed a RMCV-Bac we will be able to mutate the other RMCV-encoded chemokine receptor, R78, and other related genes in order to evaluate their contribution to the RCMV-accelerated TVS in our rat model of heart transplantation.

In summary, we have shown that RCMV induces rat SMC migration and that this migration is mediated by the RCMV-encoded chemokine receptor R33. We have extended our *in vitro* studies to an *in vivo* model and have shown, through kinetic analysis of TVS, that RCMV infection of allograft recipients induces early events (endothelialitis), as well as upregulation of chemokine expression throughout the development of TVS leading to increased allograft recruitment of inflammatory cells. These events culminate in the RCMV-acceleration of this disease process. Allograft chemokine expression profiling demonstrates that RCMV infection dramatically enhances host chemokine expression in the allografts with kinetics that parallel the timing of the virus-mediated acceleration of TVS observed in this model. This enhancement of chemokine expression promotes an increase in macrophage and T-cell infiltration during the process. The results presented here support the idea that RCMV-accelerated TVS results from the virus-enhanced host chemokine expression and inflammatory cell infiltration. Future therapeutic strategies aimed at targeting this host response to the virus may be helpful in preventing the development and progression of TVS in solid organ transplant recipients.

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Mechanisms of T-Cell Mediated Injury After Renal Ischemia Reperfusion

Aim 1

a) Investigate renal ischemia-reperfusion injury (IRI) in signal transducer and activator transcription (STAT) 4 knockout mice (in which the Th1 response is blocked) with or without adoptive transfer of wild-type $CD4^+$ T cells.
b) Investigate renal IRI in STAT6 knockout mice (in which the Th2 response is blocked) with or without adoptive transfer of wild-type $CD4^+$ T cells.

We performed renal IRI in STAT6 knockout mice and found a marked functional worsening of renal IRI and enhanced structural injury compared with wild-type mice. Examination of the renal tissue neutrophil infiltration in STAT6-deficient mice revealed no correlation between neutrophil infiltration and the enhanced protection. We examined tissue myeloperoxidase levels to measure macrophage and PMN influx, and found this not to be the mechanism by which STAT6-deficient mice display a worsening of renal IRI.

Measurements of mRNA of IL-1, ICAM-1, TNF and IL-6 did not find this to be the mechanism by which STAT6 deficiency leads to worse renal IRI. We then assumed that IL-4 deficiency in the STAT6-deficient mouse was the cause of the worsening of renal IRI. We studied mice deficient in IL-4, and were excited to find that their phenotype was similar to STAT6 deficiency. We performed adoptive transfer of STAT6-deficient T cells into nude mice, and did not find a very marked worsening of renal IRI.

We conclude that STAT6 is a protective pathway in renal IRI, and that IL-4 is likely to be the mechanism by which this occurs. It is likely that IL-4 production may be occurring in other cells besides T cells in this disease. Our novel findings have unveiled a new protective pathway for renal IRI.

We then performed renal IRI in STAT4 knockout mice and found a modest functional protection in renal IRI, and similar structural injury compared with wild-type mice. Examination of the renal tissue neutrophil infiltration in STAT4-deficient mice did not reveal a correlation between neutrophil infiltration and the enhanced protection. Tissue myeloperoxidase levels did not change with STAT4 deficiency. mRNA of IL-1, ICAM-1, TNF and IL-6

did not change in the kidney with STAT4 deficiency. Adoptive transfer of STAT4-deficient T cells into nude mice did not result in protection from renal IRI, compared with transfer of wild-type T cells.

We conclude that the STAT4 deficiency, though it has a minor functional role in renal IRI, does not have the major effect that the STAT6 pathway has.

Publications

Yokota N, Burne-Taney M, Racusen L, Rabb H. Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2003; 285: F319–25.

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Donor Peripheral Blood Mononuclear Cells Homing the Thymus of Recipients to Induce Graft Tolerance

Aims

The goal of the project was to explore the possibility that enhancing migration of donor MHC class II$^+$ cells into the host thymus would favour the development of donor-specific transplantation tolerance.

This was originally designed as a three-year project, however only two years were funded by the ROTRF. The results of the first two years are reported below.

Results

1. Growth and characterization of mature and immature rat dendritic cells

To obtain mature dendritic cells (DCs), rat BN bone marrow cells were grown for 11 days in RPMI with rGM-CSF (100 U/ml) and IL-4 (20 ng/ml) and FCS 10%. FACS analysis showed that most cells are mature DCs, CD11c$^+$, MHCII$^+$, B7-2$^+$, and that they also express the CD44 glycoprotein. In 5 days MLR, mature DCs strongly stimulated the proliferation of allogeneic Lewis lymph node cells (LNs).

To obtain immature DCs, BN bone marrow cells were grown as above with the exception that rat serum was used instead of FCS to avoid stimulation of DC maturation by xenogenic proteins. DCs had a lower MHCII and B7-2 expression intensity than mature DCs grown in the presence of FCS and a lower percentage of CD44$^+$ cells. By contrast CD11c was comparable in mature and immature DCs. In MLR, immature DCs stimulated the proliferation of allogeneic Lewis LN, although less efficiently than mature DCs.

2. Effect of pre-transplant infusion of donor DCs on kidney graft survival

Since FACS analysis showed that DCs express CD44 antigen, adenoviral transfection with CD44 was judged to be useless. Thus we evaluated the effect of pre-transplant infusion of donor DCs to prolong the survival of a kidney allograft from the same donor strain. Lewis rats were IV infused with either mature ($n=3$) or immature ($n=1$) BN DCs (6×10^6 cells each). The animals were given a 10-day course of cyclosporine A (CsA, 10 mg/kg) to

allow donor cell engraftment, then CsA was stopped and at day 11 the animals received a BN kidney transplant. Results (Figure 1) indicate that donor immature and mature DCs are both capable of slightly prolonging allograft survival, compared with rats receiving only CsA in the pre-transplant period.

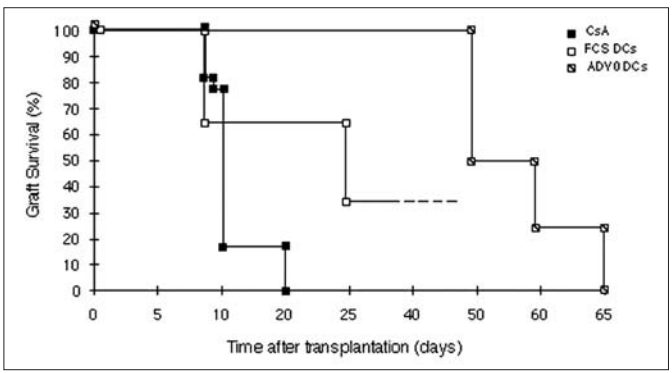

Figure 1.
Kidney graft survival.
Effect of BN FCS DCs or ADVO DCs preinfusion on kidney allograft survival in Lewis rats. 10 days before receiving a BN renal allograft, Lewis rats were treated either with BN FCS DCs or with BN ADV0 DCs together with a short-course cyclosporine. The dashed line indicats that a rat was still alive at the time of report writing.

3. Effect of ADV0 transfection on mature dendritic cell function

We then evaluated the effect of transfection with an adenoviral null vector (i.e., having no insert, ADV0) on function, *in vivo* migration properties and the tolerogenic potential of mature DCs, based on data that adenoviral transfection per se may induce DC maturation through the activation of NF-κB[1].

DCs were grown for 9 days in RPMI with GM-CSF and IL-4 and FCS 10%, then harvested and replated at 1×10^6 cells/ml in serum-free RPMI 1640. Cells were infected with replication-deficient adenoviruses at various MOI for 3 hours. Subsequently, serum, GM-CSF and IL-4 were restored without removing the adenoviruses and cell culture continued till day 11. The optimal transfection conditions were tested by using adenoviral vector encoding *E. coli* beta-galactosidase protein (Adβgal). DCs infected with Adβgal at various MOI were cytocentrifuged on glass slides and stained for XGal. We found that with 200 MOI, more than 80% of DCs were transfected (Fig. 2), thus this dose of adenovirus was used for all subsequent experiments. The viruses used are E1/E2a, E3 deleted and belong to the Ad5 serotype. FACS analysis of ADV0-transfected DCs showed that most cells are mature DCs, $CD11c^+$, $MHCII^+$, $B7\text{-}2^+$, and express CD44. CD44 levels in ADV0-transfected DCs were higher than in untransfected DCs, which may be the consequence of further maturation[2] induced by ADV0[1]. Since CD44 is an essential player in the migration of bone-marrow-derived cells into the thymus, we hypothesized that ADV0[1] DCs could have a better chance to colonize recipient thymus and to induce donor specific-tolerance after IV infusion than untransfected DCs. To test this possibility, four Lewis rats received 6×10^6 ADV0 DC IV together with a short course of CsA as above. Five hours after infusion peripheral blood and a cervical LN were obtained from each animal. One and 3 animals were sacrificed at day

5 and 11, respectively; thereafter peripheral blood and tissues (spleen, liver, lymph nodes, total thymocytes, MHCII$^+$ thymocytes, bone marrow) were collected. To evaluate distribution of DCs within recipient LNs, inguinal, mesenteric and cervical LNs were analyzed separately. To evaluate the presence of donor MHCII$^+$ cells in recipient thymus, the MHCII$^+$ cell fraction was purified using MACS magnetic microbeads after labelling thymocytes with OX6 monoclonal antibody. Genomic DNA was extracted and donor DNA was quantified by quantitative real-time PCR using primers and a fluorescent probe specific for the donor BN MHCII RT1.Bβ^n. The assay specifically detects BN DNA at a 1:10,000 dilution (corresponding to 3 DNA copies) in Lewis DNA. No signal is observed with Lewis DNA alone.

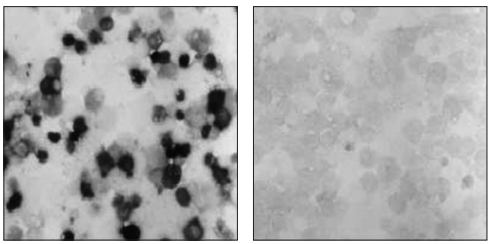

Figure 2. X-gal staining activity in Adβgal transfected DCs.
Left panel: Xgal staining on DCs transduced with Adβgal. Adβgal positive cells appear as dark blue stained.
Right panel: Xgal staining on non transduced DCs (negative control). Hematoxylin was employed to counterstain the cells.

At 5 hours after DC infusion, a faint (0.01%) donor microchimerism was detected in blood and cervical LNs. At 5 days high levels of donor DNA (1%) were found in inguinal LNs, intermediate levels (0.1%) in the blood and faint levels (0.01%) in the liver and the MHCII$^+$ fraction of thymocytes. The other tissues were negative. The high positivity found in inguinal LNs would indicate that donor DCs had migrated to the closest draining LNs after IV infusion (in the penile vein). At 11 days a faint but specific microchimerism was found in blood, in total thymocytes and in the MHCII$^+$ fraction of thymocytes; all the other tissues were negative. Thus, donor BN ADV0 mature DCs are capable of migrating and engraft into the Lewis rat thymus.

4. Effect of ADV0 transfection on tolerogenic properties of dendritic cells

Lewis rats ($n=4$) were given an intravenous injection of BN ADV0 DCs (6×10^6) together with a short course CsA, then the rats received a BN kidney transplant. As shown in Fig. 1, survival of the kidney allograft was prolonged in all rats given ADV0 DCs (median graft survival: 57 days), as compared with animals receiving CsA alone (median graft survival: 10 days), $p<0.01$. The tolerogenic properties of BN ADV0 DCs were comparable to those achieved with infusion of more than ten-fold the number of BN PBMC[3]. Interestingly, BN ADV0 DCs had better tolerogenic properties than untransfected DCs (median graft survival 24 days, $p<0.05$).

To evaluate whether prolonged graft survival in animals receiving donor ADV0 DCs was associated with T-cell hyporesponsiveness against donor antigens, LNs were collected from a recipient rat sacrificed 49 days after transplantation and cultured with irradiated splenocytes from donor BN or Lewis rats. LNs from this rat showed a much lower MLR proliferative response (SI=0.9) to donor stimulators than LNs from a naïve Lewis rat (SI=23). Addition of IL-2 (50 U/ml) only partially reversed anti-donor hyporesponsiveness

(+IL-2: SI=3.6), which suggests that some clonal deletion of donor-reactive T cells might have occurred. However, additional experiments are required to confirm the above hypothesis.

5. Cloning of rat CCR9

We then decided to over-express CCR9 in DCs to favour their migration into the thymus, and possibly increase their tolerogenic properties.

Since the rat CCR9 gene was not yet described, we cloned the corresponding cDNA. Detailed methods and results on rat CCR9 were reported in the first year progress report.

6. Analysis of CCR9 expression

CCR9 expression in several rat cell types was analyzed by real-time quantitative PCR. Detailed methods and results were reported in the first year progress report. The expression of CCR9 in total thymocytes is very high in comparison with that found in the other cell types.

7. Generation of E1/E2a/E3-deficient adenoviral vector encoding for rat CCR9 gene (ADV-CCR9)

Detailed methods and results on generation of ADV-CCR9 were reported in the first year progress report.

Fig. 3 shows PCR analysis from genomic DNA obtained from plaque 1, used to amplify the virus, using primers specific for the vector sequence flanking the CCR9 cDNA. The CCR9-specific amplified band is 1378 bp long.

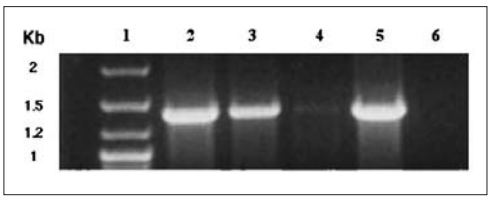

Figure 3. *Genomic DNA obtained from plaque of adenoviral recombination was amplified by PCR using primers specific for the vector sequence flanking the CCR9 cDNA. Lane 1: 2-log DNA ladder; lanes 2–4: different dilutions of genomic DNA obtained from plaque 1; lane 5: positive control; lane 6: without template.*

8. Effect of CCR9 transfection on dendritic cell function

BN DCs were grown from BN bone marrow for 9 days then transfected with ADV-CCR9 (200 MOI) as described above for ADV0. To evaluate the efficiency of transfection, CCR9 mRNA levels were evaluated by real-time quantitative PCR. ADV-CCR9 infection increased CCR9 expression in DCs by over 1000-fold compared with ADV0 DCs. Levels of CCR9 mRNA in transfected DCs almost reached those recorded in rat thymocytes.

To evaluate whether CCR9 was functionally expressed in ADV-CCR9 DCs, we needed to perform a chemotaxis response assay to the specific CCR9 ligand TECK. However rat TECK was not available, thus we first tested whether rat CCR9 could respond to mouse TECK. For this purpose, a migration assay was performed using 24-well Transwell plates with 5-µm pores and polycarbonate filters, using either rat thymocytes or mouse thymocytes as positive controls. Results showed that mouse but unfortunately not rat thymocytes migrate

in a dose-dependent manner to mouse TECK (Fig. 4), indicating that mouse TECK does not bind efficiently to rat CCR9.

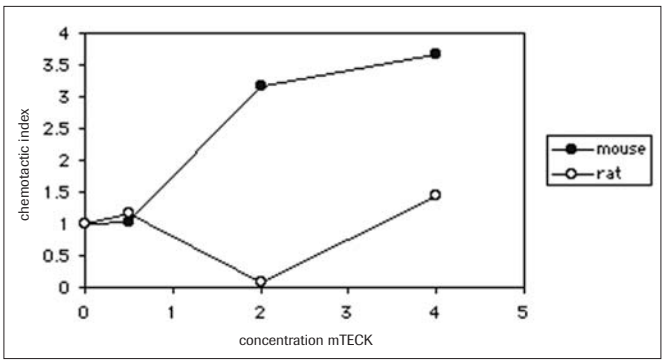

Figure 4.
Migration of mouse and rat thymocytes to recombinant mTECK.
Migration assay was performed in 24-well transwell plates with 5 µm pore polycarbonate filters using either rat thymocytes or mouse thymocytes as positive controls. 100 µl (10^6) of cells suspended in RPMI containing 0.5% BSA where placed in the upper chamber and 600 µl of medium alone or with different diluitions (0.5, 2, 4 µg/ml) of recombinant mouse TECK in the lower chamber. After incubation for 4h at 37°C the upper chamber was removed and the cells in the lower chamber were counted. The chemotactic index was calculated from the number of cells counted with the test sample divided by the number of cells counted with medium alone.

FACS analysis of ADV-CCR9 DCs showed lower percentages of $CD11c^+$ and $B7\text{-}2^+$ than ADV0 DCs. Most cells were $MHCII^+$ and $CD44^+$; however expression intensity of these antigens was lower than in ADV0 DCs (Table 1). ADV-CCR9 DCs were negative for B-cell, granulocyte, macrophage and T-cell antigens, thus excluding contamination of our preparation with other cell types. To evaluate whether transfection with ADV-CCR9 effectively downregulated CD11c and MHCII expression in DCs, FACS analysis was performed twice: at day 9 before transfection, and at day 11 after either ADV0 (control) or ADV-CCR9 transfection. While ADV0 had no effect, ADV-CCR9 markedly reduced the percentage of $CD11c^+$ cells and the intensity of MHCII expression as compared to the same cells before transfection (Table 2). We do not have an explanation for the mechanism by which CCR9 may have modified DC maturation since very little information is available in the literature on CCR9 intracellular signals. Consistent with the low expression of MHCII and of co-stimulatory molecules, ADV-CCR9 DCs failed to stimulate the proliferation of allogeneic Lewis LNs (Table 3).

Table 1. FACS analysis of DCs transfected with ADV0 or ADV-CCR9.

	ADV0 DCs		ADV-CCR9 DCs	
	% positive cells	MFI	% positive cells	MFI
CD11c	62 ± 4		29 ± 6	
MHCII	80 ± 4	187 ± 32	69 ± 7	75 ± 5 *
B7-2	73 ± 4	61 ± 13	45 ± 5	63 ± 22
CD44	87 ± 4	86 ± 20	78 ± 11	53 ± 5

Dendritic cells were labelled with primary antibodies (8A2, mouse anti rat CD11c, 1:20; OX6, mouse anti rat RT1.B, 1:20; 24F, mouse anti rat CD86 (B7-2), 1:20; OX50, mouse anti rat CD44, 1:20, washed and then labelled with secondary antibody (goat anti mouse IgG-FITC conjugated, 1:100). Cells labelled with antibodies anti B cells (OX33, mouse anti rat CD45RA, 1:50), anti macrophages (ED3, mouse anti rat ED3, 1:20), anti granulocytes (MOM/3F12/S2, 1:10), anti T cells (IF4, mouse anti rat CD3, 1:100) were $<8\%$ (data not shown).
*MFI = Mean Fluorescence Intensity; Data are mean ± SE (n=3); *$p<0.05$ vs ADV0 DCs*

Table 2. FACS analysis of DCs transfected with ADV0 or with ADV-CCR9 at day 9 (before transfection) and at day 11.

	FCS DCs day 9		ADV0 DCs day 11		ADV-CCR9 DCs day 11	
	% positive cells	MFI	% positive cells	MFI	% positive cells	MFI
CD11c	86		63		28	
MHCII	88	124	88	110	85	58

Dendritic cells were labelled with primary antibodies (8A2, mouse anti rat CD11c, 1:20; OX6, mouse anti rat RT1.B, 1:20), washed and then labelled with secondary antibody (goat anti mouse IgG-FITC conjugated, 1:100). MFI = Mean Fluorescence Intensity

Table 3. MLR allostimulatory activity of DCs transfected with ADV0 or ADV-CCR9.

		^{3}H-Thymidine uptake (CPM)	
	DCs: LNs	ADV0 DCs $n=1$	ADV-CCR9 DCs $n=1$
BN DCs + LW LYMPH NODES (LNs)	1:30	6549	690
	1:100	11935	930
	1:1000	60237	268

BN DCs were cocultured for 5 days with lymph nodes from LW rats at ratio DCs: LNs 1:30, 1:100 or 1:1000. During the last 18 hours ^{3}H-thymidine was added (1 µCi/well). The day after cells were harvested and the level of ^{3}H-thymidine incorporated was measured by a β-counter and expressed as CPM.

9. Effect of CCR9 transfection on migration and tolerogenic properties of dendritic cells

Four Lewis rats received 6×10^6 mature ADV-CCR9 DCs IV together with a short course of CsA. Tissue collection and microchimerism analysis were performed as above. At 5 hours after DC infusion, intermediate levels (0.1%) of donor microchimerism were detected in blood while cervical LNs were negative. At 5 days intermediate levels of donor DNA (0.1%) were found in inguinal LNs, and faint levels (0.01%) in blood and in the MHCII$^+$ fraction of thymocytes. The other tissues were negative. At 11 days intermediate levels of donor microchimerism were found in blood, while a faint but specific microchimerism was found in the MHCII$^+$ fraction of thymocytes; all other tissues were negative. Thus, BN ADV-CCR9 DCs are capable of migrating into the Lewis rat thymus; however their migration capability was not greater than that of ADV0 DCs.

To test the tolerogenic properties of donor ADV-CCR9 DCs, Lewis rats ($n=4$) were given an intravenous injection of BN ADV-CCR9 DCs (6×10^6) together with a short course of CsA, then a BN kidney was transplanted. As shown in Fig. 5, survival of the kidney allograft was prolonged in all rats given ADV-CCR9 DCs (median graft survival: 43 days), as compared

with animals receiving CsA alone ($p < 0.05$). The tolerogenic properties of BN ADV-CCR9 DCs were comparable to those achieved with infusion of the same number of BN ADV0 DCs.

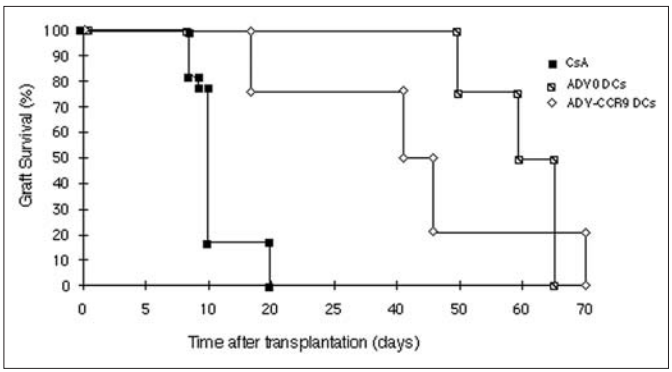

Figure 5.
Kidney graft survival.
Effect of BN ADVO DCs or ADV-CCR9 preinfusion on kidney allograft survival in Lewis rats. 10 days before receiving a BN renal allograft, Lewis rats were treated either with BNADV0 DCs or with BN ADV-CCR9 DCs together with a short-course cyclosporine.

LNs were collected from a recipient rat sacrificed 70 days after transplantation and cultured with irradiated splenocytes from BN rats or Lewis rats in MLR. LNs from this rat showed a much lower MLR proliferative response (SI = 1) to donor stimulators than LN from a naïve Lewis rat (SI = 46).

Conclusions

Results of this project document that mature allogenic ADV0 DCs expressing high levels of donor MHCII molecules and of CD44 glycoprotein, once infused under appropriate immunomodulating conditions, can migrate into the host thymus and activate tolerogenic mechanisms so that survival of a kidney transplant from the same donor strain is prolonged. Further studies are required to both optimize DC dose and mode of administration and to understand the mechanisms underlying the tolerogenic effect.

On the other hand, transfection with ADV-CCR9 greatly affects DC phenotype and function. The biochemical mechanisms responsible for the above phenomenon are under investigation.

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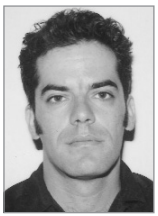
Dr. Miguel P. Soares, Principal Investigator
Prof. Fritz Bach, Co-Investigator



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Heme Oxygenase-1: An Anti-Inflammatory Molecule that Promotes Organ Graft Survival

Mouse-to-rat cardiac transplants survive long-term under brief inhibition of complement activation by cobra venom factor (CVF) and continuous T-cell immunosuppression by cyclosporine A (CsA)[1], a phenomenon we refer to as accommodation[2]. We have shown that expression of the protective gene heme oxygenase-1 (HO-1) in these grafts is essential to insure accommodation[3,4]. We have also shown that the protective effect of HO-1, in terms of sustaining xenograft survival, relies on its ability to catabolyze heme into the gas carbon monoxide (CO)[5].

In the absence of HO-1 expression, or under inhibition of HO-1 enzymatic activity by tin protoporphyrin (SnPPIX), mouse hearts transplanted in CVF- plus CsA-treated rats are rejected through a process that is characterized by widespread endothelial cell (EC) apoptosis[3,4,5]. HO-1-derived CO suppresses xenograft rejection by a mechanism that remains to be fully elucidated but that is associated with the inhibition of endothelial cell (EC) apoptosis[3,4].

In the past three years we have investigated the molecular mechanism underlying the protective effect of HO-1 in terms of suppressing EC apoptosis. In addition we have also addressed the mechanism by which the expression of HO-1 controls the pro-inflammatory phenotype associated with EC activation, another key event in the pathogenesis of xenograft rejection. We found that over-expression of HO-1 or induction of endogenous HO-1 expression by heme protects cultured EC from TNF-α-, etoposide- or serum starvation-mediated apoptosis[6]. Under inhibition of HO-1 activity, exogenous CO can fully substitute for HO-1 in terms of preventing EC apoptosis. The mechanism of action of CO is dependent on the activation of the p38 mitogen-activated protein kinase (MAPK) signaling transduction pathway[6]. How the activation of this specific signal transduction pathway contributes to suppress EC apoptosis remains to be established and is the focus of our present studies.

Given the key role of the transcription factor nuclear factor κB (NF-κB) in regulating the expression of anti-apoptotic genes in EC[7], we questioned whether CO interacted with this signal transduction pathway to suppress EC apoptosis. We found that when NF-κB activa-

tion is suppressed, CO no longer protects EC from undergoing TNF-α-mediated apoptosis. HO-1/CO does not seem to activate NF-κB in EC[8]. However, EC have basal levels of NF-κB activity, which sustain the expression of NF-κB-dependent anti-apoptotic genes, required to support the anti-apoptotic effect of HO-1/CO[8]. Expression of the NF-κB-dependent anti-apoptotic genes c-IAP2 and A1, but not A20 or MnSOD, restores the anti-apoptotic action of CO. This effect is abolished when p38 MAPK activation is blocked. These data suggest that HO-1/CO cooperates with a subset of NF-κB-dependent anti-apoptotic genes, i.e. c-IAP2 and A1, to protect EC from TNF-α-mediated apoptosis and that this effect is dependent on the ability of HO-1/CO to activate the p38 MAPK signal transduction pathway[8].

These data also suggest that HO-1-derived CO protects EC from undergoing apoptosis when these cells can activate the transcription factor NF-κB, such as when they are exposed to those pro-inflammatory stimuli that mediate the pathogenesis of xenograft rejection.

Given the above, specific inhibition of NF-κB activation in a xenograft may impair the protective action of HO-1 and thus prevent a xenograft from undergoing accommodation, a phenomenon we have shown to be dependent upon the expression of these genes[3,4].

The transcription factor NF-κB also plays a critical role in the expression of pro-inflammatory genes associated with EC activation that contribute in a critical manner to the pathogenesis of xenograft rejection. We questioned whether HO-1 would modulate the activity of the transcription factor NF-κB in a manner that would inhibit the expression of these proinflammatory genes, e.g. E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). We found that HO-1 inhibits TNF-α-driven up-regulation of E-selectin and VCAM-1 but not ICAM-1 expression, as tested at the RNA and protein levels. This effect is associated with the inhibition of NF-κB transcription activity. The data suggest that HO-1 inhibits the expression of a subset of NF-κB-dependent genes associated with endothelial cell activation, presumably via the inhibition of NF-κB activity (Soares et al., submitted for publication).

We have investigated whether these cytoprotective and anti-inflammatory functions of HO-1/CO could be used therapeutically to prevent the rejection of allografts. We have tested this hypothesis in three distinct experimental settings: a) cardiac ischemia reperfusion in rats, b) acute cardiac allograft rejection in mice, and c) chronic aorta allograft rejection in rats.

In the first experimental setting, inbred male Lewis rats were used as donors and recipients in a rat heart transplantation model. Hearts were preserved in University of Wisconsin solution (UW solution; 4oc) for 24 hours before transplantation. Under these conditions the ability of the transplanted hearts to regain function upon reperfusion was nonexistent. Up-regulation of HO-1 by CoPPIX administration to the donor suppressed ischemia reperfusion

injury and allowed grafts to regain function following reperfusion. The observations suggest that CO mediates the protective effects of HO-1, but does not exclude that other end products of HO-1 activity may act in a similar manner. The protective effect of HO-1 and/or CO in this experimental model was associated with the inhibition of endothelial and cardiomyocyte (Akamatsu et al., article in preparation).

In the second experimental setting we found that induction of HO-1 expression by CoPPIX prolonged the survival of MHC-incompatible DBA2/J (H-2d) cardiac grafts in B6AF1 (H-2k/d,b) mice, inducing long-term graft survival (>100 days) in 33% of the cases, as compared with untreated or ZnPPIX-treated controls where long-term graft survival was never observed. The data demonstrate that biliverdin inhibits alloreactive T-cell activation and proliferation, thereby prolonging allograft survival (Yamashita et al., submitted for publication).

In the third experimental setting we found that CO prevents arteriosclerotic lesions that occur following aorta transplantation in rats[9]. The protective effect of CO in this transplant model is associated with a profound inhibition of graft leukocyte infiltration/activation, as well as with inhibition of graft smooth muscle cell proliferation. The anti-proliferative effect of CO *in vitro* requires the activation of guanylate cyclase, generation of cGMP, activation of p38 mitogen-activated protein kinases and the expression of the cell cycle inhibitor p21Cip1. These findings demonstrate a protective role for CO in vascular injury and support its use as a therapeutic agent[9].

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Dr. Myron Cybulsky, Co-Applicant

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Role of DN Regulatory T cells in Long-Term Cardiac Graft Survival

Transplantation is an optimal treatment for patients with organ failure if graft rejection can be prevented. Non-specific immunosuppressive drugs currently in use for preventing rejection are associated with numerous problems, including opportunistic infection and malignancy. Developing novel therapies to produce specific immunosuppression is one of the major goals of transplantation research. Numerous studies have demonstrated that T regulatory (Tr) cells play an important role in down-regulating immune response to self- and allo-antigens. However, whether Tr cells also play a part in suppressing immune response to xenoantigens was not clear.

Mature $\alpha\beta$-TCR$^+$CD3$^+$CD4$^-$CD8$^-$ double-negative T regulatory (DN Tr) cells, originally identified in our laboratory, comprise 1–3% of peripheral lymphocytes in rodents, and 1–2% of human peripheral blood mononuclear cells. We have demonstrated that DN Tr cells express a unique set of cell surface markers and cytokine profiles, which differ from previously described lymphocytes. These DN Tr cells can suppress allogeneic immune responses mediated by CD4$^+$ and CD8$^+$ T cells *in vitro* in an antigen-specific and dose-dependent manner. They can significantly prolong skin allograft survival when adoptively transferred to naïve mice.

The goal of this ROTRF-supported project is to determine the role and mechanisms of DN Tr cells in the induction and maintenance of cardiac transplant tolerance. The specific objectives are as follows:

1. To develop a model in which long-term survival of cardiac grafts can be achieved.
2. To determine the role and mechanism of DN Tr cells in the prevention of cardiac allo- and xeno- graft rejection.
3. To study the migration of DN Tr cells *in vivo.*
4. To identify specific and selective cell surface markers for DN Tr cells.

During the last 21 months we have made the following progress on this project.

1. Development of a novel model in which permanent cardiac xenograft survival can be induced.

We previously demonstrated that pre-transplantation donor lymphocyte infusion (DLI) leads

to permanent acceptance of one MHC class I antigen-mismatched skin allograft. With ROTRF support, we have extended this study to a cardiac xenotransplantation model. We have successfully developed a model in which permanent (>200 days) Lewis-to-B6 concordant cardiac xenograft survival can be reliably achieved by combining a single dose of pre-transplant DLI with a short course of anti-CD4-depleting antibody treatment. This model allows us to further investigate the mechanisms leading to xenotransplantation tolerance.

2. Adoptive transfer of splenocytes from tolerant recipients prolonged xenograft survival in naïve mice.

To determine the role of Tr cells in down-regulating xenogeneic immune responses, spleen cells were harvested from B6 mice that permanently accepted Lewis heart grafts after DLI + anti-CD4 treatment, and adoptively transferred into B6 naïve mice, followed by transplantation of a Lewis heart graft 1 or 2 days later. We found that cardiac xenograft survival was significantly ($p<0.01$) prolonged in these recipients compared with untreated controls, indicating the existence of regulatory cells in the spleen of DLI + anti-CD4-treated recipients.

3. Increase of DN Tr cells in the spleen of tolerant recipients.

Various subsets of Tr cells have been identified in different animal models. In order to determine what type of Tr cells are involved in long-term rat-to-mouse cardiac xenograft survival, we examined whether there was a preferential activation of a T cell subpopulation in the recipients. Splenocytes were harvested from mice that received both DLI and anti-CD4 at various time points after transplantation, and the total numbers of $CD4^+$, $CD8^+$ and DN Tr cells were compared with those from mice that received anti-CD4 mAb alone. We found that both the total numbers and the kinetics of $CD4^+$ and $CD8^+$ T cells between the two treatment groups were similar. However, the DLI + anti-CD4-treated mice had significantly greater number of DN Tr cells in the spleen, compared with animals treated with anti-CD4 alone at all time points after transplantation. These results demonstrate that a combination of DLI and a short course of anti-CD4-depleting mAb leads to a significant increase in recipient-derived DN Tr cells. Because the only difference between the two treatments is the DLI, it suggests that pre-transplant DLI may facilitate activation of recipient DN Tr cells.

4. Splenic DN Tr cells are able to suppress proliferation of syngeneic $CD4^+$ and $CD8^+$ T cells.

To study whether recipient splenic DN Tr cells could suppress proliferation of naïve T cells to xenoantigens, we isolated DN Tr cells from the spleens of DLI + anti-CD4-treated B6 recipient mice and compared their ability to suppress proliferation of naïve CD4 or CD8 T cells. Our data indicate that DN Tr cells can suppress proliferation of xenogeneic CD4

and CD8 T cells, and suggest that DN Tr cells may contribute to permanent acceptance of xenografts.

5. Monitoring DN Tr cells *in vivo.*

To further understand the mechanism of how DN Tr cells induce donor-specific transplantation tolerance, we have developed a panel of DN Tr clones, as well as mutant clones that do not have regulatory function. We have demonstrated that infusion of DN Tr clones leads to long-term allogeneic heart graft survival. In order to study the migration of regulatory T cells *in vivo,* we are now injecting DN regulatory clones into mice and monitoring the fate of these DN Tr clones in transplant recipients.

6. Production of DN Tr-cell-specific monoclonal antibodies.

Accumulating evidence indicates that Tr cells play an important role in the down-regulation of immune responses to self- or allo-geneic antigens. However, studies of Tr cells have been restricted because of the lack of specific cell markers or antibodies to identify these cells. This is largely due to the difficulty of isolating and cloning Tr cells. My laboratory has produced a panel of DN regulatory T-cell clones and several natural mutant clones which have lost suppressive function. We have also produced a panel of monoclonal antibodies that are able to selectively bind to DN regulatory but not to mutant non-regulatory T cell clones. We are now attempting to produce mAbs that can directly recognize DN Tr cells in DLI-treated mice that have permanently accepted cardiac allo- or xeno-grafts.

7. Study of human DN Tr cells.

In collaboration with other investigators, we have started to investigate the regulatory function of human DN T cells. Our preliminary data suggest that human DN Tr cells can suppress allogeneic immune responses mediated by $CD4^+$ T cells *in vitro* through a similar mechanism to that in mice.

The data obtained from these studies supported by ROTRF resulted in publication of a research article, which reveals, for the first time, the role of DN Tr cells in long-term xenograft tolerance. Moreover, our success in developing long-term tolerance to cardiac xenograft allows us to further investigate the mechanisms involved in the induction and maintenance of cardiac xenograft survival.

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6. How to apply for an ROTRF grant

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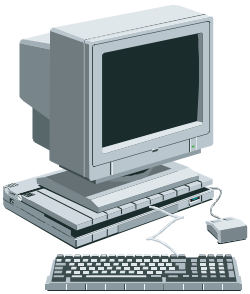

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