R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

April 2005

The Roche Organ Transplantation Research Foundation

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

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

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

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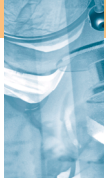


1. Preface

Funding Cycle XIII of the Roche Organ Transplantation Research Foundation (ROTRF) started with the submission of the Letters of Intent up to 1 October 2004. For the first time, applicants were given the opportunity to apply for conventional or clinical research grants. The response to the new clinical initiative was very positive with about 40% of the total applications submitted in Cycle XIII being applications for clinical research grants. In spring 2005, seven grants for conventional and three grants for clinical research applications were awarded by the ROTRF. The funding provided by the ROTRF continues to support the research of scientists working in the area of organ transplantation in many parts of the world.

The new clinical initiative aims to support research projects using human clinical material and patients, addressing new issues in organ preservation, human transplant pathology and other human transplant problems, and which promise to have a significant impact in the clinical setting in the near future.

For the first time, the ROTRF was present at this year's American Transplant Congress in Seattle with a Mini-Symposium as well as an information booth. Chaired by ROTRF Trustees Prof. Andrew Bradley and Prof. Allan Kirk, four young investigators gave high-quality presentations on the results achieved during their ROTRF-funded research.

Proof of the excellent work carried out by the ROTRF investigators is demonstrated by the quality of the presentations given at ATC 2005 and at previous European Congresses, the reports included in the Biannual Reports, and the papers published in peer-reviewed journals.

The ROTRF Board of Trustees wishes to thank the ROTRF Scientific Advisory Committee, and the ROTRF grantees for their competent and enthusiastic support contributing to the overall success of the Foundation.

The Foundation would like to express its gratitude to F. Hoffmann-La Roche Ltd for their generous support without which none of the research carried-out by investigators and the ROTRF activities would have been possible.

Finally, the ROTRF wishes the newly ROTRF-awarded investigators success in pursuing their research objectives.

On behalf of the Board of Trustees

Philip F. Halloran, MD, PhD, OC

2. Facts and Figures

Funding Cycle XIII – Letters of Intent Submission in October 2004

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that grants have been awarded to *conventional and clinical research applications* for the first time in funding cycle XIII.

Two million Swiss francs (CHF) were allocated to ten research projects, seven to conventional research and three to clinical research applications. The Board of Trustees and the Scientific Advisory Committee of the ROTRF were once again very pleased with the high quality and innovation demonstrated in the applications received.

The awarded grants will support research that aims to advance the science of solid organ transplantation, thereby improving the care of thousands of patients undergoing transplantation every year. The results of the funded research projects will contribute to the understanding of clinical and scientific aspects of transplantation, such as processes involved in chronic rejection, immune suppression and tolerance induction. The research supported by the ROTRF will also focus on the identification and validation of early molecular prognostic markers for the onset of chronic allograft nephropathy, aspects related to xenotransplantation, characterisation of islets with the final goal being to overcome organ shortage, and protection of islets from rejection.

The ROTRF received 108 letters of intent in funding cycle XIII up to the submission deadline (October 2004), from scientists around the world. Of the applications, 41.7% were received from Europe, the major countries being Germany (10.2%), UK (8.3%), and France (4.6%), and the same number of applications was received from North America, United States (38%) and Canada (3.7%). Australia/New Zealand (7.4%), South America (4.6%), Asia (3.7%) and Africa (<1%) accounted for the remaining 16.6% of the applications. Based on the reviews of the Scientific Advisory Committee, the Board of Trustees invited the 22 top-ranked applicants to prepare full paper applications. After thorough review of the full paper applications, grants were awarded to 10 projects.

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

Statistics on Applications to the ROTRF

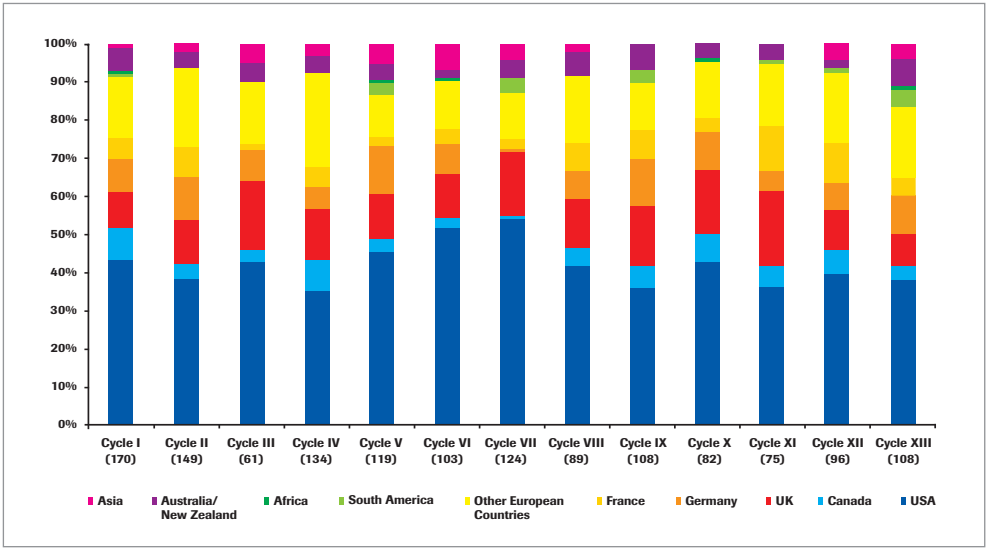


Figure 1. *Geographical distribution of the applicants who submitted Letters of Intent (LOI) during the first thirteen ROTRF funding cycles. The total number of LOIs submitted per cycle is shown in brackets.*

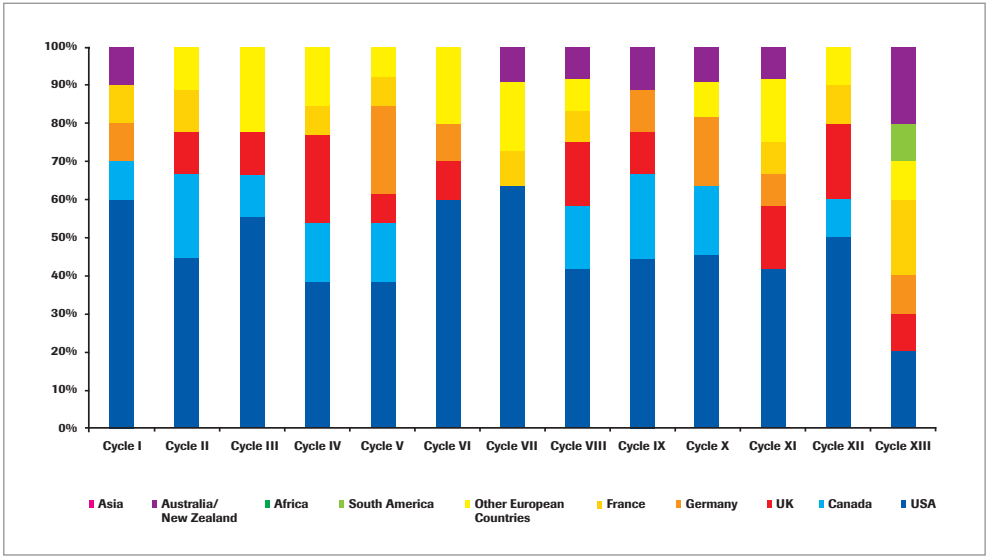
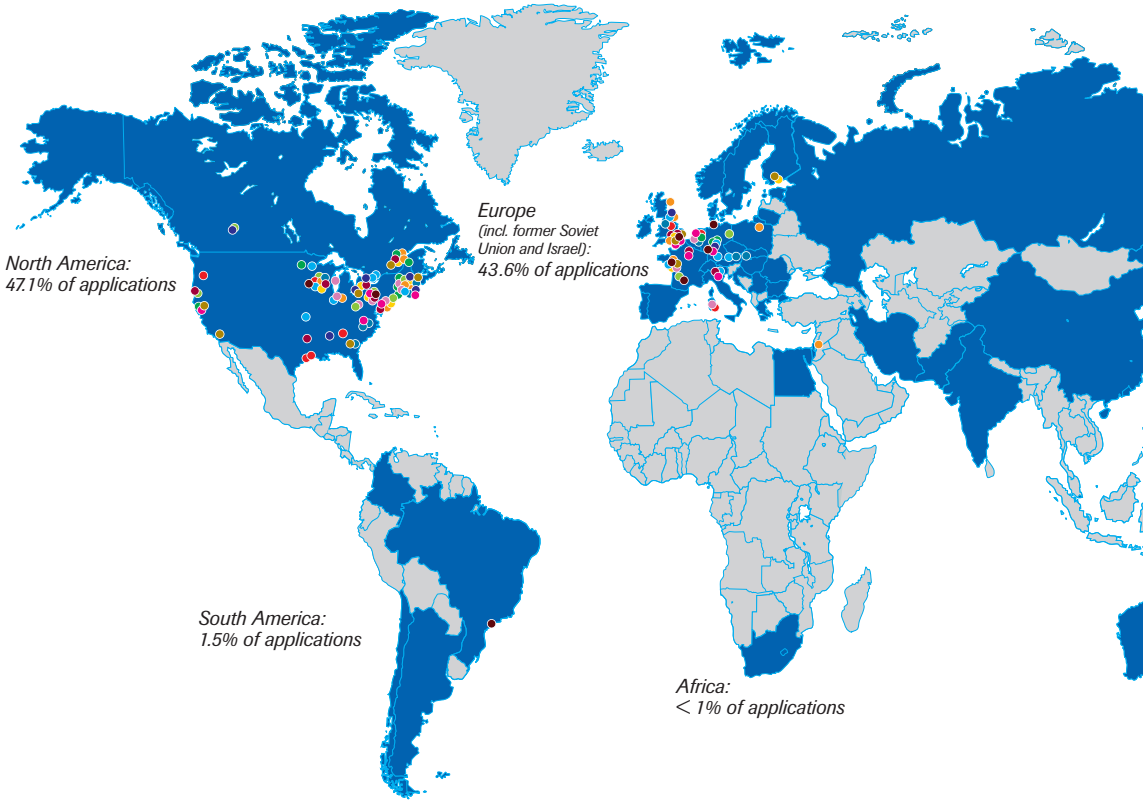
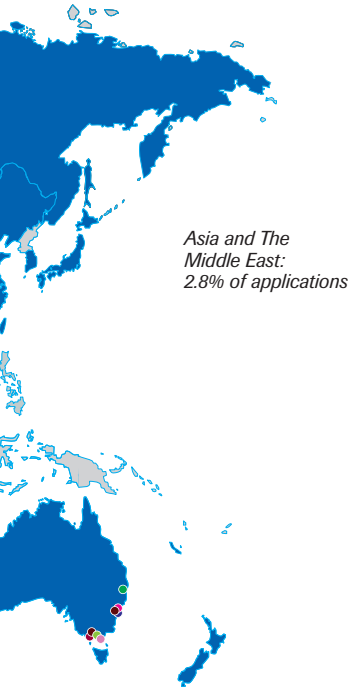


Figure 2. *Geographical distribution of the applicants who were awarded ROTRF grants during the first thirteen ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF Applications Worldwide





Asia and The Middle East: 2.8% of applications

Australia and New Zealand: 4.8% of applications

at least one application ever received

no application received

Cycle I Grantees

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

Cycle II Grantees

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

Cycle III Grantees

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

Cycle IV Grantees

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

Cycle V Grantees

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

Cycle VI Grantees

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

Cycle VII Grantees

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

Cycle VIII Grantees

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

Cycle IX Grantees

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

Cycle X Grantees

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

Cycle XI Grantees

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

Cycle XII Grantees

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

Cycle XIII Grantees

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

3. Grant Awards in Cycle XIII

3.1 Conventional Research Grant Awards

Dr. Eleanor Bolton, Principal Investigator
Mr. Gavin Pettigrew, Co-Investigator

Addenbrooke's Hospital, University of Cambridge, Cambridge, UK

The Role of B Lymphocytes in Chronic Transplant Rejection

This research project seeks a clearer understanding of the cellular and molecular processes resulting in rejection of transplanted tissues from genetically unrelated individuals. It is known that the different pathways (direct and indirect) through which foreign antigens expressed by donor tissues are processed and presented to recipient T cells influence the resulting pattern of graft rejection (acute and chronic). It is also generally accepted that the direct pathway of recognition is associated more with acute rejection which is easier to control with immunosuppressive drugs, while the indirect pathway contributes to chronic graft rejection for which there are currently no effective treatments.

Chronic rejection is now the major reason for transplant failure. Chronic allograft rejection is characterised by increasing obliteration of the lumen of blood vessels in the graft as a result of proliferation of intimal cells lining the vessels. This graft vasculopathy is thought to be mediated in part by antibodies recognising intact donor antigens expressed by the graft. It is unclear, however, how presentation of processed donor antigen via the indirect pathway during the recognition phase following transplantation can result in generation of antibodies that recognise intact, non-processed donor antigen as a target molecule in the effector phase resulting in graft destruction. We propose a novel hypothesis that B lymphocytes, which have receptors for intact donor antigen, may function as both antigen-presenting cells and antibody-secreting effector cells thereby contributing to chronic allograft vasculopathy.

We propose to use novel strains of transgenic mice in cardiac transplant experiments. The presence or absence of selected B cell antigens and transplantation (MHC) antigens in different strains of donor and recipient mice will influence the survival time of heart transplants. The relative effects on graft rejection will be analysed by cellular assays, including the Elispot assay, by antibody analysis and by studying the pathology and immunohistology of transplanted tissues. These studies will enable us to dissect the cellular and molecular interactions that may permit B lymphocytes to function as antigen-presenting cells. They may result in the rational design of strategies for inducing B cell tolerance, thereby diminishing the risk of chronic graft rejection.

Dr. Peter Cowan, Principal Investigator
Prof. Anthony d´Apice, Co-Investigator

St. Vincent's Hospital Melbourne, Fitzroy, Australia

Promoting the Growth of New Blood Vessels to Improve the Survival of Pancreatic Islet Grafts

Diabetes is a major health problem affecting millions of people worldwide. It is caused by the failure of the body's insulin-producing cells, the pancreatic islets. Although diabetes can be treated by regular insulin injections, long-term treatment can lead to many debilitating side effects including blindness, kidney failure and loss of limbs. A much better treatment option is to cure diabetes by transplanting islets from a human donor. However, there is a severe shortage of all donated human organs and cells, including islets. Transplantation of pig islets would eliminate the shortage overnight, because pig insulin works well in human patients, but there are several obstacles to overcome before pig-to-human islet transplantation becomes a medical possibility.

One problem is that islets need to 'grow' a new blood supply after they are transplanted into a new host. This is a slow process, and many of the transplanted islets die in the meantime because they are starved of oxygen and nutrients. Fortunately, it is possible to introduce genetic modifications into the donor pig to help overcome problems of this type. The aim of this project is to make the transplanted islets produce factors that accelerate growth of the new blood supply and therefore reduce the number of cells dying from starvation. The factors are vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1 (Ang-1). A potential additional benefit of expressing VEGF-A in transgenic pigs is that it may increase the yield of islets harvested from each pig.

If successful, this strategy will move pig-to-human islet transplantation one step closer to practice.

Dr. Lina Lu, Principal Investigator
Dr. Chandrashekhar Gandhi, Co-Investigator
Dr. Nogc Thai, Co-Investigator

University of Pittsburgh, Thomas E. Starzl Transplantation Institute, Pittsburgh, USA

Cell-Based Capsulation: A Novel Approach for Long-Term Islet Allograft Survival

Islet cell transplantation provides a hope for cure of diabetes. However, the results of islet allograft transplantation are plagued by the following problems: 1) early graft loss due to lack of rapid revascularization in newly transplanted islets; and 2) graft destruction by the immune system.

Scientists are exploring better ways to prevent islet allograft rejection. One of the approaches is capsulation of islets with semipermeable membrane that not only blocks out the immune attack, but also allows passage of nutrients and metabolites (glucose, oxygen and insulin). However, this artificial device has limitations, including poor biocompatibility of the capsule and lack of revascularization.

Recently, we have found that co-transplantation of self-hepatic stellate cells (HSC) can effectively protect islet transplants from rejection without the requirement of immunosuppressive therapy. HSC are known to be actively involved in liver repair and regeneration by synthesizing extracellular matrix protein and secrete cytokines and growth factors. We have demonstrated that HSC are powerful killers of activated T cells, which contribute to immune tolerance. Our preliminary data show that when co-transplanted with islet allograft, HSC form a multiple-layer capsule surrounding islet grafts and markedly prolong graft survival. Thus, we hypothesize that co-transplanted HSC may form a biological barrier which support islet engraftment by secretion of growth factor and prevents allograft rejection by eliminating activated T cells.

In this proposal, we will determine the impact of HSC on specific T cell responses towards islet allograft, and ascertain the efficacy of HSC on improving islet engraftment. We will also determine the effect of HSC on islet grafts to treat type 1 diabetes. The results of these studies will lead to development of novel strategies for improving the outcome of cell transplantation, such as, cure of diabetes by successful islet transplantation.

Prof. Frank Rösl, Principal Investigator
Prof. Edward Geissler, Co-Investigator
PD Dr. Ingo Nindl, Co-Investigator
Prof. Dr. Eggert Stockfleth, Clinical Collaborator

Deutsches Krebsforschungszentrum, Heidelberg, Germany

Reducing Skin Cancer in Organ-Transplanted Persons

The success of organ transplantation remains dependent on drugs that suppress the recipient's immune system from destroying the foreign transplanted tissue. Unfortunately, cancer cells are not as likely to be destroyed in transplant recipients when their immune system is inhibited. In addition, it is thought that the conventional immunosuppressant cyclosporine may directly act to promote cancer development. Skin cancer is particularly common in transplant recipients, to the extent that half or more have had cutaneous lesions by 10-20 years post-transplantation. Presently, the course of action is to remove the lesion, but lesions can be numerous, tend to recur, and have an increased risk to be aggressive. Therefore, it is not only troublesome and costly to continually treat the lesions; they eventually pose a serious risk for malignant disease.

The present study aims to better understand factors that could be altered in these patients to reduce the risk of skin cancer. We are particularly interested in studying the potential role of cutaneous papilloma virus (PV) infections in skin cancer development, and the influence of different immunosuppressive drugs on this process. For this purpose we have a unique animal available, referred to as *Mastomys natalensis* (a mouse-rat intermediate). Like humans, these animals are latently-infected with PV and spontaneously develop skin cancer. Using this model, we predict that a newer-age class of immunosuppressants, referred to as mTOR inhibitors, will reduce PV-induced skin carcinogenesis, in contrast to conventional cyclosporine immunosuppression. We aim to show that mTOR inhibitors interfere with molecular pathways critical for PV-cell latency/replication, and for PV-induced tumor angiogenesis, thus reducing the risk of skin cancer. To support our theory, PV-infected skin lesions will also be studied in transplant recipients on either mTOR inhibitors, or cyclosporine. This study will form a scientific basis for potentially reducing the problem of post-transplant skin cancer.

Dr. Alexandra F. Sharland, Principal Investigator
Dr. Dale Christiansen, Co-Investigator
Dr. Mark Gorrell, Co-Investigator
Dr. Peter MacDonald, Research Associate
Dr. Peter Tran, Research Associate

University of Sydney, Sydney, Australia

Pig Molecules which Activate Human Lymphocytes

Transplantation is a life-saving treatment for patients with organ failure. However, access to this treatment is steadily declining. Availability of donor organs is expected to worsen as the incidence of organ failure due to conditions such as diabetes and hepatitis C increases sharply over the next two decades. Pig to human xenotransplantation holds great promise as a means of alleviating this critical shortage, but its successful implementation poses many challenges. Hyperacute rejection results from binding of naturally occurring antibodies to carbohydrate molecules on the surface of pig cells. Initial results suggest that organs transplanted from genetically modified pigs lacking these carbohydrates are not subject to hyperacute rejection, and survive longer than any previous pig to human grafts. Whilst these reports are very encouraging, the genetic modification does not prevent the vigorous human anti-pig cellular immune response, and the gains in graft survival were achieved in the context of very significant immunosuppression, which remains a barrier to clinical applicability. Increasing our understanding of the function of NK cells and other components of the cellular response is now essential if progress towards ultimate clinical xenotransplantation is to continue.

NKG2D is an activating receptor, found on NK cells, T cells and macrophages (cells responsible for graft rejection). Activation through this receptor leads to target cell killing, and secretion of substances which can directly damage the graft. Some types of pig cells have surface molecules (ligands) which can bind to the human NKG2D receptor, and expression of these ligands can be induced in pig kidneys during transplantation. Interactions between pig ligands and the human NKG2D receptor are potentially of central importance in initiating and maintaining the human anti-pig cellular immune response, and are thus targets for therapeutic intervention. Understanding more about the ligands and their binding to human NKG2D is now imperative, and will allow us to determine the most appropriate strategy for preventing/blocking these interactions. In this project, we will examine expression of the ligands in normal and transplanted pig tissues, measure ligand-receptor binding and evaluate the ability of these ligands to activate human NK cells *in vitro*.

Prof. Joost van Meerwijk, Principal Investigator
Dr. Paola Romagnoli, Research Associate

INSERM U563, Purpan Hospital, Toulouse, France

Development of a Cell-Based Therapy against Short-Term and Long-Term Graft Rejection

Despite availability of very efficient immunosuppressive drugs, organ transplantation is still severely hindered by long-term graft rejection and serious toxicity as well as by the side effects of the drugs. Development of highly specific and durable therapies would therefore be of significant benefit to transplant recipients. We propose to develop a cell therapy for the induction of graft tolerance with these crucial properties.

We have recently published that rejection of bone-marrow grafts can efficiently be inhibited with a newly discovered population of regulatory T lymphocytes in mice. Our more recent data indicate that also transplanted hearts and skin can be protected using this regulatory T cell population in mice. Importantly, in contrast to immunosuppressive drugs, regulatory T cell-induced tolerance is specific (and therefore allows for immunological protection against infections) and durable (we have not observed so-called “chronic rejection”).

It will now be important to transpose the protocol allowing for the induction of transplantation tolerance with regulatory T cells to humans. First of all, it will be important to identify the immunosuppressive mechanisms responsible for the inhibition of graft rejection in our mouse model. Then, a logistic problem inherent to the human setting will need to be solved. To this end, we will study how immunosuppressive drugs can initially be used to inhibit rejection and later replaced with regulatory T lymphocytes. Finally, we will study the potential of human regulatory T lymphocytes to inhibit human graft rejection in mice harboring a “humanized” immune system. The described project will allow us to evaluate the feasibility of a regulatory T cell-based therapy for human organ transplantation. This therapy should lead to life-long graft acceptance in absence of generalized immunosuppression.

Dr. Bernard Vanhove, Principal Investigator
Dr. Gilles Blancho, Research Associate
Mr. David Minault, Research Associate
Mrs. Anne-Sophie Dugast, Research Associate
Mrs. Thomas Haudebourg, Research Associate
Mr. Bernard Martinet, Research Associate

ITERT, CHU Hotel Dieu, Nantes, France

Immunosuppression and Tolerance Induction by Selective Inhibition of CD28

Manipulation of immune responses in rodents can lead to the induction of regulatory mechanisms sustaining tolerance in transplantation, i.e., acceptance of allogenic grafts without pharmacological immunosuppression. Induction of tolerance in primates and in humans, however, is still the focus of intense investigations. T cell costimulation blockade is an immunosuppressive strategy that has been considered for more than a decade and now has several applications in the clinic. One of the most explored costimulation systems is that involving the CD28/B7/CTLA-4 receptors, where CD28 and CTLA-4 on T cells interact with B7 molecules on antigen-presenting cells.

While it has been known for a very long time that CD28/B7 interactions promote T cell activation, it is more recently that we understood that CTLA-4/B7 interactions are implicated in the extinction of immune responses and in the maintenance of immune tolerance. Thereafter, it became obvious that an agent that would selectively block CD28 and not CTLA-4 may be very adaptive to maneuvers aimed at inducing tolerance.

Inhibition of the CD28 receptor, however, is hard to achieve experimentally. Due to the dimeric nature of this receptor, most dimeric antibodies used to block CD28/B7 interactions actually stimulate rather than inhibit T cell activation. The use of monovalent antibody fragments or of a modulating antibody, however, demonstrated that CD28 inhibition could indeed induce transplant tolerance and reverse ongoing autoimmune diseases in rodents. In our laboratory, we have identified and are currently characterizing mechanisms sustaining anti-CD28 induced tolerance in kidney grafts in the rat, that are different from those previously described in mice. In parallel, we have developed a monovalent recombinant inhibitor of primate/human CD28 to investigate its action in the pre-clinical model of baboon kidney allotransplantation.

3.2 Clinical Research Grant Awards

Dr. Jan Anthoine Bruijn, Principal Investigator
Dr. Johan de Fijter, Co-Investigator
M.Sc. Marian Roos-van Groningen, Graduate Student
Dr. Ineke ten Berge, Collaborator
Dr. Sandrine Florquin, Collaborator

Leiden University Medical Center, Leiden, The Netherlands

Prognostic Markers in Protocolized Renal Allograft Biopsies

Chronic allograft nephropathy (CAN) is the major cause of renal graft loss. Interstitial fibrosis is generally seen in renal biopsies of patients with CAN. Myofibroblasts are the main effector cells responsible for the fibrosis and largely originate from tubular epithelial cells by epithelial-to-mesenchymal transition. Once CAN is histologically detectable in the biopsy, therapy is not effective. Due to the shortage of organs available for transplantation, it is essential to find and validate early molecular prognostic markers before the onset of overt fibrosis. Timely identification of high-risk patients enables more intense immunosuppressive medicative dosing and clinical follow-up. In addition, it will open the way to early, and thereby more efficient molecular intervention strategies.

We hypothesize that early gene expression levels in renal transplant biopsies are additive surrogate markers to histological and clinical parameters for outcome and may render novel targetable genes. This will be tested using protocolized transplant biopsies at 6 and 12 months from a prospective multicenter study. Outcome parameters are the extent of fibrosis in the 6-month biopsy, the change in the extent of fibrosis between 6 and 12 months, and the change in renal function between 6 months and 3 years. In the project we will focus 1.) on genes that have previously been shown in experimental studies to be involved either in promotion or inhibition of fibrogenesis and epithelial-to-mesenchymal transition, and 2.) on novel genes, identified by microarray analyses on kidney transplant tissue, which may exert anti- or pro-fibrogenic activities. Our goal is to determine the predictive value of mRNA levels and corresponding protein levels for graft outcome in comparison to that of histological and clinical variables.

mRNA and protein expression for multiple genes early after kidney transplantation are expected to be used as novel surrogate markers. Such markers may represent novel drug targets in the prevention or limitation of progressive allograft damage.

Dr. Luis A. Fernandez, Principal Investigator
Dr. Jon S. Odorico, Co-Investigator
Dr. Matthew S. Hanson, Research Associate
Mr. Eric Hatch, Research Associate

University of Wisconsin Medical School, Madison, USA

Characterization of Human Islet from Non-Heart Beating Donors

The transplantation of insulin-producing cell clusters known as islets of Langerhans, has emerged as the most promising option for achieving restoration of normal blood sugar control in people with type 1 diabetes mellitus. One limiting factor that has impaired the availability of this treatment is the shortage of donor organs. Donation after cardiac death (DCD; non-heart beating donors) is a viable alternative that will aid in reducing this shortage.

Our goal is to provide a comprehensive assessment of human islets from DCD donors and compare them to islets from donation after brain death (DBD) donors (heart beating donors). At present, islets for transplant are not typically isolated from DCD pancreata. Determination of the multiple mechanisms of adaptation that allows the cells to survive adverse metabolic conditions such as low oxygenation or limited nutrient supply will be the focus of our study. Also, characterization of the micro-environment in the liver after islet transplantation and its impact on islet function and insulin production will be studied in a rodent model. The knowledge acquired from this study will increase the utilization of pancreata that have the potential to cure type 1 diabetic patients. Understanding the biology of the islets and liver micro-environment after transplantation will open avenues for new therapeutic options that will not only impact the number of functional islets after transplantation, but also increase the number of patients that might be cured from this devastating disease.

Dr. Alvaro Pacheco-Silva, Principal Investigator
Dr. Aparecido B. Pereira, Co-Investigator
Dr. Niels O. Câmara, Co-Investigator
Dr. Kikumi S. Ozaki, Collaborator
Dr. Rogério Chinene, Research Associate
Dr. Georgia D. Marques, Research Associate
Dr. Marcos A. Cenedeze, Research Associate

Universidade Federal de São Paulo, São Paulo, Brazil

Detection and Prevention of Patients with Chronic Allograft Nephropathy

Renal transplantation is the best therapeutic option for patients with chronic renal disease. It contributes to improve life quality and decreases the relative risk of cardiovascular death when compared to patients kept on dialysis. One-year graft survival has improved a lot during the last 20 years, and currently is around 90% in recipients of kidneys from deceased donors. However, only 50% of kidney transplant patients will still have functioning organs after 10 years of transplantation. The main cause of graft loss after the first year is an entity denominated chronic allograft nephropathy (CAN).

Thus, CAN is currently the greatest barrier to long-term success of a renal transplantation. Many factors are known to be associated with CAN development and its progression but our understanding of these are still incomplete. CAN is characterized by a progressive diminution of renal function, and the histological analyses show evidence of progressive tubulointerstitial fibrosis and tubular atrophy. There is currently no clinical or invasive test that favors its early detection. Therefore, CAN can only be confirmed by graft biopsy when usually clinical alterations are already present. This late diagnosis makes it very difficult to establish any clinical maneuver to halt this process.

At early time points, CAN can be associated with tubulointerstitial injury, manifested by dysfunction of renal proximal tubular cells. We are proposing to use a new tool, the evaluation of proximal tubular function, for an early diagnosis and a better comprehension of CAN. Proximal tubular function can be assessed by the determination of urinary tubular proteins, especially retinol binding protein (RBP). We intend to reverse the tubular dysfunction by modifying immunosuppressive drugs, toward a less toxic schema. Genes and proteins involved in the initiation process of proximal tubular dysfunction and those related to CAN will also be investigated.

4. Progress Reports of ROTRF Grantees

Dr. Bert Binas, Principal Investigator
Prof. Fred Faendrich, Collaborator
Prof. Oliver Smithies, Collaborator
Dr. Thomas Coffman, Collaborator

Texas A & M University, College Station, USA

Mouse Blastocyst Stem Cell Immune Resistance

This report spans the time from June 2004 till March 2005.

Aim 1: Compare the identities of rat ES-like cells (RESC) and various mouse blastocyst-derived stem cells.

The aim mainly involves RT-PCR and plating assays. We are focusing at present on the RT-PCR. We designed primers for FAS ligand and genes characteristic of pre-implantation stem cell types. The primers were picked from regions where rat and mouse sequences are identical, ensuring identical amplification efficiency from mouse and rat samples. In contrast, the control HPRT primers were designed such that they are either rat-specific or mouse-specific, so that the origin of the sample (rat or mouse) can be verified. We have been analyzing RESC, mouse ES, and mouse endoderm cell lines with selected primer pairs. Note that we have not yet included mouse TS cells in this analysis, since the available TS cells are still too precious (see Aim 2). The preliminary results demonstrate the expected expression pattern (Fig. 1). That is, RESC (lane 2) but not mouse ES (lane 3) or mouse endoderm (lane 4) cells express FASL; mouse ES cells (lanes 6, 9) but not mouse endoderm (lanes 7, 10) or RESC (lanes 5, 8) express oct4 and nanog; and mouse endoderm (lane 14) but not mouse ES (lane 13) or RESC (lane 12) express HNF4a. The analysis will be expanded by further genes and by TS cells, and we will also use these cell lines for plating assays soon.

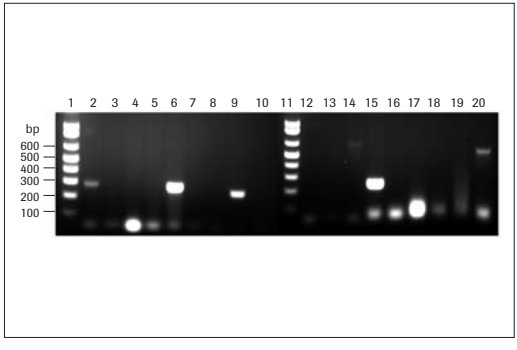


Figure.1. RT-PCR of selected genes in permanent mouse and rat pre-implantation stem cell lines. *RNA was extracted with TRIZOL and treated with DNAse before the RT-reaction. Genes: FAS ligand (lanes 2-4); oct4 (lanes 5-7); nanog (lanes 8-10); HNF4a (lanes 12-14); rat HPRT (lanes 15-17); mouse HPRT (lanes 18-20). Cell lines: RESC (lanes 2, 5, 8, 12, 15, 18); mouse ES (lanes 3, 6, 9, 13, 16, 19); mouse extraembryonic endoderm (lanes 4, 7, 10, 14, 17, 20). Molecular weight ladder: lanes 1 and 11. Note that bands <200bp are not true amplicons and that an HPRT negative ES cell strain was used (lane 19).*

Aim 2: Test the known mouse blastocyst stem cell lines for immune resistance.

The essence of this aim is the isolation and portal vein injection of fluorescently labeled mouse blastocyst stem cells from a defined genetic background. We have generated three TS cell lines (genetic background Balb/c X C57Bl6(GFP)) that are presently at passage five and in the process of expansion but not yet available in large amounts, plus one extraembryonic endoderm line. Figure 2 illustrates a characteristic TS cell colony of one line. An unexpected problem that hampered cell line derivations was the low percentage of productive matings. Twenty-five mating days (out of many more attempts) yielded vaginal plugs, but only on 12 mating days embryos resulted, and of these only 8 mating days yielded normal blastocyst numbers (6-10 blastocysts) and were suitable for cell line derivation. Of these eight productive matings, the most recent one yielded the lines described above. It turned out that the poor blastocyst yields were due to construction noise associated with renovation of the Texas A&M University animal facility over a considerable fraction of the last half year (evidence can be provided upon request). This problem affected especially the C57Bl6 strain, which is the background of our GFP-fluorescent mouse line and known to be sensitive to environmental stress, and it hampered also our backcrossing program designed to increase the genetic purity of our GFP-transgenic mice.

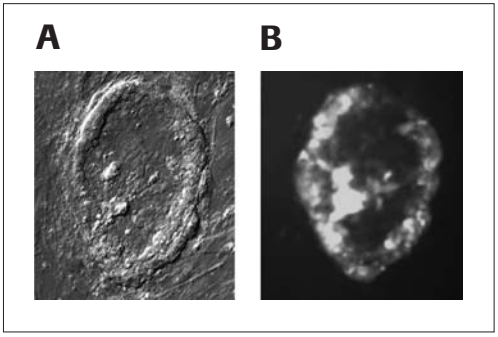


Figure 2. Representative colony of new mouse TS cell line derived from the Balb/c X C57Bl/6 (GFP) genetic background. A) *Bright field;* ***B)*** *Epifluorescence image.*

According to the director of our animal facility, construction is now completed. However, in light of the breeding difficulties we have taken action to make us less dependent on the GFP-C57Bl6 strain. Our laboratory has successfully adopted lentiviral technology to efficiently introduce GFP expression into wild-type cells (Fig. 3). With this technique, our cell line derivations no longer depend on the GFP-transgenic mice (that are mainly C57Bl6 background but may carry residual DNA from the original transgenic strain). To take further advantage of lentiviral technology, we also produced a red fluorescent protein (dsRed2)-expressing vector (Fig. 4) that will enable us to label the cells to be co-injected with the TS cells. This should save us experiments since now two cell types can be traced in one experiment.

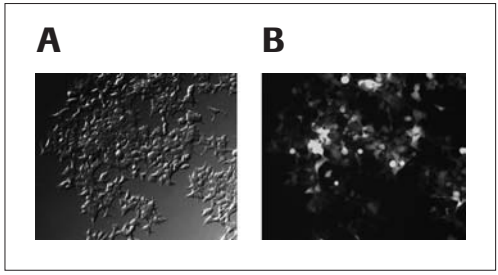


Figure 3. Transduction of 293 T cells with GFP-expressing lentiviral vector pFUGW. A) *Bright field;* ***B)*** *Epifluorescence image. Note that the transduction efficiency is ~100%.*

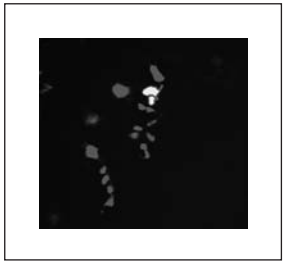


Figure. 4. Transfection of 293 T cells with a lentiviral dsRed2-expressing vector derived from pFUGW. *This figure demonstrates that the new dsRed2-expression vector expresses functional dsRed2. Note that this is a transfection and not a transduction experiment; therefore the number of dsRed2-expressing cells is <100%. Transductions will be performed soon.*

While I initially intended to hire a postdoc, I work now with two PhD students on the project. Mrs. Epple-Farmer (MS in Reproductive Physiology) started in June 2004 and has since successfully learned the techniques of stem cell isolation and intraportal vein injection. Mr. Debeb (DVM, and MS in Veterinary Science) started in January 2005 and has rapidly learned lentiviral techniques and RT-PCR and will also participate in the cell derivation/injection if needed.

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

Children's Hospital, Harvard Medical School, Boston, USA

Vascular Endothelial Growth Factor in Acute and Chronic Rejection

Specific Aims:

There have been no revisions or modifications to the specific aims. The overall objective of this proposal is to define the function of VEGF in allograft rejection. We hypothesize that VEGF is a pro-inflammatory cytokine via its ability to regulate intragraft mechanisms of rejection. To test this hypothesis, we have a research plan to investigate the mechanism of function of VEGF in ischemia reperfusion injury, acute and chronic rejection.

Progresses on Specific Aims:

1. VEGF, a well-known angiogenesis factor also functions as a pro-inflammatory cytokine, in part via its ability to regulate endothelial cell expression of adhesion molecules and chemokines. We have recently shown that VEGF mediates leukocyte trafficking to allografts in association with rejection. Since hypoxia is a major stimulus for the expression of VEGF, we speculate that VEGF acts as a mediator of graft injury during the ischemia-reperfusion (I/R) period. To test our hypothesis, we used an established C57BL/6 mouse kidney I/R model. The left renal artery was clamped for 45 minutes and serum creatinine and tissue were examined at 0, 2, 6, 24, 48 hours and at 4 and 6 days. Animals were untreated or treated with a blocking anti-VEGF serum (0.8 ml on day -1 and 0) or with a specific VEGF post-receptor kinase inhibitor PTK 787/ZK222584 (200 mg/kg/day on day -1 and 0). We found that untreated animals (n=11 at 24 hours) developed acute renal failure (ARF) manifested by an increase in serum creatinine peaking 24 hours following ischemia but returned to baseline between days 2 to 6. ARF was also associated with a transient local leukocytic infiltration of the kidney and a transient increase in the mRNA expression of the chemokines MIP-2, MIP-1 and MCP-1, TCA-3, IP-10 and RANTES (peaking at 6 hours following ischemia). Treatment with either anti-VEGF (n=4 at 24 hours) or the VEGFR inhibitor PTK 787 (n=4 at 24 hours) resulted in a marked inhibition of pro-inflammatory events. Treatment during the period of ischemia with either VEGF antagonist maintained early chemokine expression at baseline level, and creatinine level was normal at all times examined. Inhibition of T cell activation was also shown to limit I/R injury. Here, we treated a group of animals (n=12 at 24 hours) with the immunosuppressive agent mycophenolate mofetil (MMF) which we found also partially inhibits VEGF post receptor

responses in endothelial cells. We found that MMF alone attenuated I/R injury but MMF in combination with either VEGF antagonist was most effective to inhibit both chemokine expression and ARF in this model.

Together these findings demonstrate that VEGF is a mediator of kidney I/R injury. We suggest that that blockade of either VEGF or post VEGFR signals in combination with known immunosuppressives will be therapeutic to limit renal injury in the immediate post transplantation period.

2. We investigated the therapeutic efficacy of endostatin, an anti-angiogenesis agent in alloimmune responses *in vivo*. We first evaluated its effect in recipients of fully MHC mismatched allografts (C57BL/6 into BALB/c). Control recipients received a non-specific Fc fusion protein. We found that endostatin treatment only minimally prolonged survival at therapeutic anti-angiogenesis doses (20 mg/kg/d; MST = 10 days vs. MST 8 days in controls) but markedly prolonged allograft survival at higher doses, (40 mg/kg/d; MST= 36.7 days, $P=0.0002$ vs. controls). High dose endostatin significantly inhibited leukocytic infiltration of CD4 and CD8 T cells ($P<0.001$ and $P<0.05$ vs. control) into cardiac allografts. In addition, high dose treatment significantly downregulated the local mRNA expression of cytokines (IFN-γ and IL-4, $P<0.05$ and $P<0.005$ vs. control), chemokines (RANTES, $P<0.05$) and chemokine receptors (CXCR3, $P=0.007$). *In vitro*, we also found that high dose endostatin has direct effects on T cells to inhibit both mitogen and alloantigen-dependent T cell activation responses. These findings are suggestive that endostatin has biological effects independent of angiogenesis. Angiogenesis is a component of chronic inflammation and we also wished to evaluate the effect of endostatin/Fc on chronic rejection. We used the MHC class II mismatched B6.C-H2bm12 into C57BL/6 model of allograft vasculopathy in which chronic rejection occurs by 6 weeks post-transplant associated with increased expression of endothelial cell CD31 and the angiogenesis factor VEGF. C57BL/6 recipients were treated with anti-angiogenic doses of endostatin/Fc (20 mg/kg, three times a week), either in the initiation phase (0-3 weeks) or in the progression phase (4-6 weeks). While early treatment had no effect on outcome, we found that delayed treatment significantly inhibited the development of graft vascular disease ($n=8$, $P<0.05$ vs. controls). The effect of delayed treatment was also associated with downregulation of local cytokine, chemokine and adhesion molecule expression.

Thus, endostatin, a known angiogenesis inhibitor effects both angiogenesis-dependent and independent mechanisms of allograft rejection. Endostatin that binds endothelial cells at high affinity and T cells at low affinity has the unique ability to inhibit both T cell and endothelial cell responses and is therapeutically effective in acute and chronic allograft rejection.

3. Ischemia and reperfusion injury (IRI) are prime antigen-independent inflammatory factors in the dysfunction of liver transplants. The precise contribution of T cells in the mechanism of IRI remains to be elucidated. As CD154-CD40 costimulation pathway provides essential second signal in the initiation and maintenance of T cell dependent immune responses, this study was designed to assess the role of CD154 signaling in the pathophysiology of liver IRI. A mouse model of partial 90 minutes warm hepatic ischemia followed by 6 hours of reperfusion was used. Three animal groups were studied: i) WT mice treated with Ad-β-gal vs. Ad-CD40 Ig; ii) untreated WT vs. CD154 (MR1) mAb treated WT mice; and iii) untreated WT vs. CD154 KO mice. The disruption of CD154 signaling in all three animal groups ameliorated otherwise fulminant liver injury, as evidenced by depressed sGOT levels, as compared with controls. These beneficial effects were accompanied by local downregulation of VEGF expression, inhibition of TNF-α and Th1 cytokine production, and induction of anti-apoptotic (Bcl-2/Bcl-xl) but depression of pro-apoptotic (caspase-3) proteins. By utilizing gene therapy approach, pharmacological blockade, and genetically targeted mice in parallel, these findings document the benefits of disrupting the CD154 to selectively modulate inflammatory responses in liver IRI.

This study reinforces the key role of CD154-CD40 T cell costimulation in the pathophysiology of liver IRI.

4. In addition, we are developing a model of transplant-associated cancer to assess the effects of immunosuppressants on cancer development. We propose that the regulation of the expression and function of VEGF by immunosuppressants may impact angiogenesis and thus cancer growth. In a nude mouse model we have found that rapamycin inhibits VEGF-induced angiogenesis whereas cyclosporine increases the angiogenesis response. MMF has no notable effect. In addition, in an *in vivo* model of melanoma cancer growth, we have found that rapamycin inhibits cancer growth *in vivo*. The mechanism involved in these observations will be assessed in future studies.

Plans

We plan to continue the current studies as outlined in the research plan.

Publications

1. Haskova Z, Boulday D, Kiessling S, Flynn E, Chandraker A, Briscoe DM. Function of vascular endothelial growth factor in kidney ischemia-reperfusion injury. *Am J Transplant, In press.*
2. Sho M, Akashi S, Kashizuka H, Nomi T, Kuzumoto Y, Tsurui Y, Kanehiro H, Hisanaga, Ko S, Robertson SW, Nakajima Y, Briscoe DM. A potent anti-angiogenic factor, endostatin prevents acute and chronic allograft rejection. *Am J Transplant, In press.*
3. Ke B, Shen X-D, Gao F, Tsuchihashi S, Farmer DG, Briscoe DM, Busuttil RW, Kupiec-Weglinski JW. The CD154-CD40 T cell costimulation pathway in liver ischemia and reperfusion inflammatory responses. *Transplantation, In press.*

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

The Ohio State University, Columbus, USA

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

The purpose of this project is to determine the mechanisms by which a novel immunotherapeutic strategy targeting both LFA-1 and CD40/CD40L costimulation induces long-term suppression of alloreactive (CD4-independent) $CD8^+$ T cells, which are typically resistant to conventional immunotherapies. This therapeutic regimen has been previously reported to regulate alloreactive CD4-dependent immune responses through the induction of $CD4^+$ regulatory T cells. However, we have reported that this dual therapy regulates alloreactive (CD4-independent) $CD8^+$ T cells and promotes robust long-term acceptance of hepatocellular allografts. These results are of particular interest since $CD8^+$ T cells are normally resistant to immunosuppressive strategies that readily regulate $CD4^+$ T cell alloimmune responses. To our knowledge, this is the first therapeutic strategy, which indefinitely regulates CD8-dependent rejection responses across a complete MHC mismatch.

To date, the majority of our experiments have focused on Specific Aims I and III of our original research proposal. These aims have been approached using a functional model of hepatocellular transplantation in which allogeneic hepatocytes are transplanted to the liver by intrasplenic injection. The activity of (CD4-independent) $CD8^+$ T cells can be studied in CD4 KO or CD4-depleted C57BL/6 hepatocyte transplant recipients[1].

Specific Aim I: Determine the effect of immunotherapy targeting LFA-1 and CD40/CD40L costimulation on recruitment and activation of host inflammatory cells after hepatocyte transplant.

1. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation does not interfere with the total number of leukocytes trafficking to the liver after hepatocyte transplantation.

In these experiments, CD4 KO ($H\text{-}2^b$) mice were transplanted with $2x10^6$ allogeneic FVB/N ($H\text{-}2^q$) hepatocytes by intrasplenic injection and treated with anti-CD40L mAb (MR1, days 0, 2, 4, 7 post-transplant) and anti-LFA-1 mAb (days 0-6 post-transplant). Allograft recipients were sacrificed ten days following transplantation and liver infiltrating cells (LICs) were isolated and counted by trypan blue exclusion.

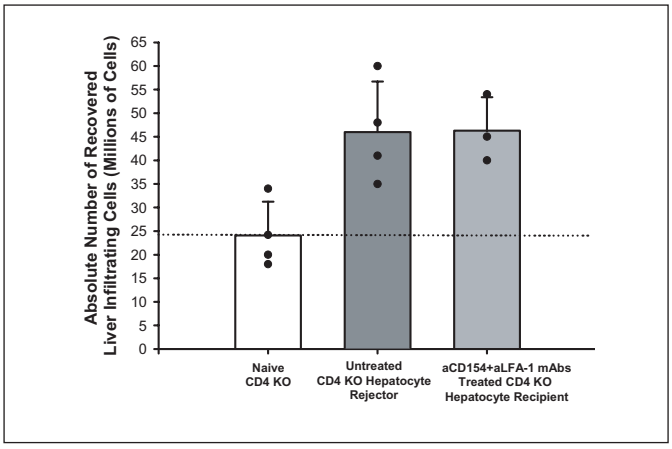


Figure 1. LICs in CD4 KO mice.

Results: CD4 KO recipients receiving dual therapy exhibited a significant increase in the total number of LICs in comparison to naïve CD4 KO mice ($P=0.009$); the number of LICs in treated mice did not significantly differ from that of CD4 KO rejector mice receiving no immunosuppression ($P=$ns, Fig. 1).

2. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation does not interfere with the trafficking of adaptive and innate leukocyte subsets to the liver after hepatocyte transplantation.

Targeting of LFA-1 could potentially influence the trafficking of both innate and adaptive immune effectors. For this reason, we also evaluated the accumulation of specific leukocyte subsets within the liver of CD4 KO allogeneic hepatocyte recipients. For these experiments, LICs isolated at day 10 post-transplant were evaluated for the presence of adaptive immune effectors ($CD4^+$ and $CD8^+$ T cells) and innate immune effectors (neutrophils [$GR\text{-}1^+$], macrophages [$F4/80^+$], and NK cells [$NK1.1^+$]).

Results: Among the adaptive immune effectors, $CD8^+$ T cell accumulation in the liver following hepatocyte transplantation was significantly increased in CD4 KO mice receiving dual therapy compared to both naïve CD4 KO mice and untreated CD4 KO hepatocyte rejectors ($P<0.001$ and 0.007, respectively). Among innate immune effectors, neutrophil accumulation was also significantly increased in treated CD4 KO hepatocyte acceptors versus both naïve CD4 KO mice and untreated CD4 KO hepatocyte rejectors ($P<0.03$ and $P=0.009$, respectively). NK cells were significantly increased in both treated hepatocyte acceptors ($P=0.003$) and untreated hepatocyte rejectors ($P=0.002$) in comparison to naïve CD4 KO mice. Macrophage accumulation did not differ significantly between groups (Fig. 2).

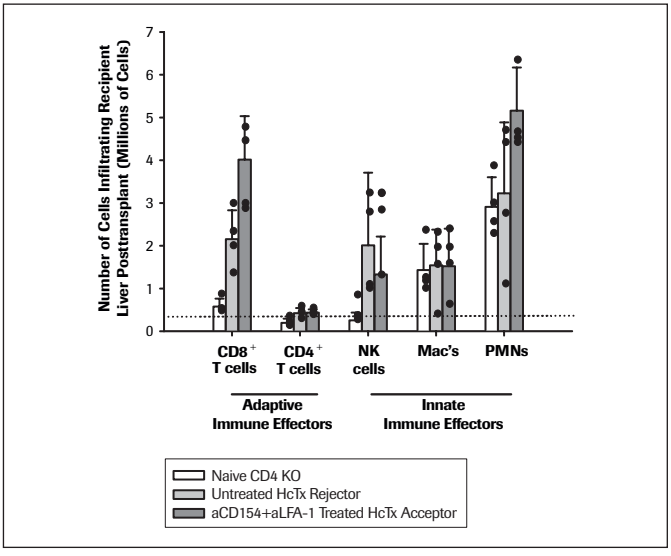


Figure 2. Accumulation of specific leukocyte subsets within the liver of CD4 KO allogeneic hepatocyte recipients.

Given these results, treatment of CD4 KO hepatocyte recipients with anti-LFA-1 and anti-CD40L mAbs did not significantly impair the accumulation of total leukocytes or specific innate or adaptive immune cell subsets in the liver, despite the known role of LFA-1/ICAM-1 interactions in leukocyte recruitment.

After determining the specific leukocyte subsets trafficking to the liver after hepatocyte transplantation, immunohistochemistry was performed to determine where these cells reside after accumulation in the liver post-transplant. For these experiments, CD4 KO hepatocyte recipients were either treated with anti-LFA-1 and anti-CD40L mAbs or were untreated, and the liver was harvested seven-ten days following transplant. Liver tissue was stained for 1) the transgenic reporter product, human α_1-anti-trypsin (hA1AT), which is expressed by the donor hepatocytes, 2) $CD3^+$ lymphocytes, and 3) standard H & E.

Results: In treated CD4 KO recipients, immunostaining for the presence of hA1AT demonstrated numerous allogeneic hepatocytes engrafted throughout the liver parenchyma and adjacent to both portal tracts and central veins (Fig. 3A,B). Few hepatocytes were detected in untreated recipients with decreasing serum hA1AT levels (Fig. 3C,D). Immunostaining for $CD3^+$ lymphocytes demonstrated $CD3^+$ T cells scattered throughout the liver parenchyma with occasional leukocyte clusters in both treated and untreated hepatocyte recipients (Fig. 3E-H); H & E stains also demonstrated occasional foci of leukocyte clusters in both treated and untreated hepatocyte recipients (Fig. 3I-L).

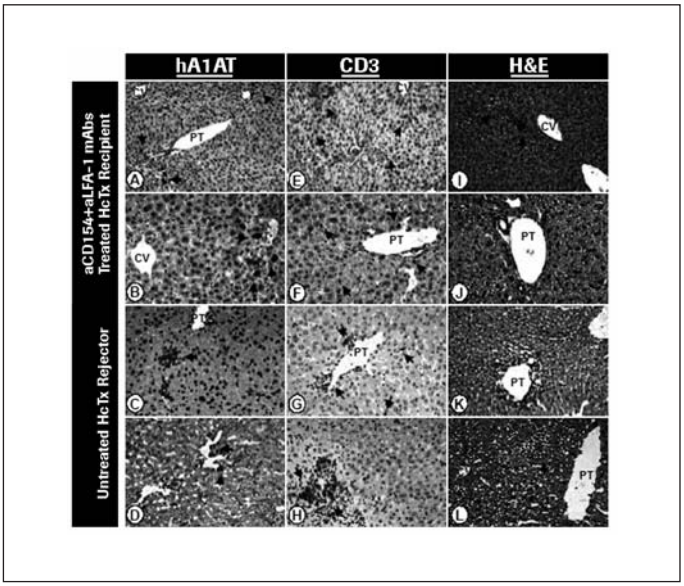


Figure 3. Immunostaining of liver post-transplant after treatment with anti-LFA-1 and anti-CD40L mAbs.

3. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation influences expression of the adhesion molecule CD103 and activation markers CD69 and CD25 on LICs after hepatocyte transplantation.

For these experiments, LICs seven-ten days post-transplant from treated and untreated CD4 KO hepatocyte recipients were analyzed by flow cytometry for expression of CD103, CD69, and CD25. Results were compared to control LICs from naïve CD4 KO mice.

Results: Approximately one third of $CD8^+$ LICs in untreated CD4 KO hepatocyte rejectors expressed CD103 by three and ten days post-transplant (data not shown). In contrast, $CD8^+$ T cells infiltrating the liver of treated hepatocyte recipients did not up-regulate CD103 expression in comparison to $CD8^+$ T cells in the liver of control naïve CD4 KO mice. Similarly, expression of the early T cell activation antigen, CD69, by $CD8^+$ LICs ten days post-transplant in treated CD4 KO hepatocyte recipients was suppressed in comparison to untreated CD4 KO hepatocyte rejectors ($P=0.003$; data not shown). Allogeneic hepatocyte transplantation induced expression of the activation marker $CD25^{lo}$ (IL-2 receptor) on day 10 $CD8^+$ LICs in untreated hepatocyte rejectors when compared to naïve mice ($P=0.002$). Day 10 $CD8^+$ LICs from hepatocyte recipients treated with dual therapy of anti-LFA-1 and anti-CD40L mAbs did not demonstrate expression of $CD25^{lo}$. Unexpectedly, a population of $CD8^+CD25^{hi}$ LICs was detected in treated recipients (data not shown).

Collectively, these data indicate that combined blockade of LFA-1 and CD40/CD40L mediated signaling does not affect the accumulation of $CD8^+$ T cells at the site of transplantation, but does influence the induction of the adhesion molecule CD103 on host $CD8^+$ T cells infiltrating the liver. Dual therapy also influences the expression of activation markers CD69 and CD25 on $CD8^+$ LICs.

4. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation influences the *in vivo* development of alloreactive cytotoxic $CD8^+$ T lymphocytes after hepatocyte transplantation.

For these experiments, flow cytometric analysis was performed in order to examine the expression of the CD43 activation-associated glycoform on day 10 $CD8^+$ LICs. CD43 expression is known to correlate with cytotoxic effector function.

Results: CD43 expression was significantly up-regulated on $CD8^+$ LICs from untreated CD4 KO hepatocyte rejectors in comparison to those of naïve CD4 KO mice ($P=0.02$). In contrast, CD43 was suppressed on day 10 $CD8^+$ LICs of CD4 KO hepatocyte recipients receiving dual therapy with anti-LFA-1 and anti-CD40L mAbs (data not shown). These results suggested that short-term dual therapy does suppress the development of $CD8^+$ cytotoxic T cells.

Next, the *in vivo* development of allospecific cytotoxic T lymphocytes (allo-CTLs) was examined using an *in vivo* allospecific cytotoxicity assay. For these experiments, syngeneic C57BL/6 ($H\text{-}2^b$) target splenocytes and allogeneic FVB/N ($H\text{-}2^q$) target splenocytes were isolated and stained with differing concentrations of CFSE (0.2 μM and 2.0 μM, respectively). Hepatocyte recipients and control mice received $20x10^6$ syngeneic and $20x10^6$ allogeneic target splenocytes by tail vein injection mixed in a 1:1 ratio. Splenocyte target CFSE staining and ratios were verified by flow cytometric analysis prior to injection. Spleens from hepatocyte transplant recipients were harvested 18 hours after CFSE-labeled target cell injection and were analyzed by flow cytometry. Percent cytotoxicity was calculated as:

$$\%\text{SpecificLysis} = \left[\frac{\left(\frac{\#\text{CFSE}^{high}\text{Naive}}{\#\text{CFSE}^{low}\text{Naive} + \#\text{CFSE}^{high}\text{Naive}} \right) - \left(\frac{\#\text{CFSE}^{high}\text{Experimental}}{\#\text{CFSE}^{low}\text{Experimental} + \#\text{CFSE}^{high}\text{Experimental}} \right)}{\left(\frac{\#\text{CFSE}^{high}\text{Naive}}{\#\text{CFSE}^{low}\text{Naive} + \#\text{CFSE}^{high}\text{Naive}} \right)} \right] x100$$

Results: The presence of allo-CTLs in untreated hepatocyte rejector mice was readily detectable with *in vivo* lysis of 52.96% ± 8.7% of allogeneic targets. Allospecific cytotoxicity was abolished by the depletion of host $CD8^+$ T cells with anti-CD8 mAb (2.43, 0.25 mg, i.p., day 12 and 14 relative to transplantation, data not shown). In contrast, allo-CTLs were not detected in CD4 KO hepatocyte recipients, which received dual therapy with anti-LFA-1 and anti-CD40L mAb (data not shown).

These data suggest that short-term targeting of LFA-1 and CD40/CD40L costimulation acts to suppress activation and *in vivo* development of alloreactive $CD8^+$ T cells, therefore resulting in long-term hepatocyte allograft survival.

Specific Aim II: Determine the role of host or donor cell expression of ICAM-1 on the efficacy of anti-LFA-1 mAb treatment for inhibition of (CD4-independent) $CD8^+$ T cell-mediated hepatocyte rejection.

Experiments addressing the role of host or donor cell expression of ICAM-1 on the efficacy of this immunosuppressive strategy for suppression of $CD8^+$ T cell-mediated hepatocyte allograft rejection have yet to be performed.

Specific Aim III: Determine whether targeting LFA-1 and CD40/CD40L costimulation-mediated signals induces regulation of alloreactive (CD4-independent) $CD8^+$ T cells.

5. Long-term hepatocellular allograft survival in CD4 KO recipients induced by immunotherapy with LFA-1 and anti-CD40L mAb is not abrogated by adoptive transfer of naïve $CD8^+$ T cells.

These experiments were designed to determine the robustness of long-term hepatocyte allograft survival induced by short-term therapy with anti-LFA-1 and anti-CD40L mAbs. For these experiments, treated hepatocellular allograft recipients with long-term hepatocyte allograft survival (LTS) >60 days were adoptively transferred with $2x10^6$ naïve $CD8^+$ T cells and monitored for allograft function and survival.

Results: Following $CD8^+$ T cell transfer, seven of eight CD4 KO recipients maintained hepatocyte allograft acceptance for at least an additional 60 days following adoptive transfer, though serum hA1AT levels decreased slightly from baseline levels (Fig. 4).

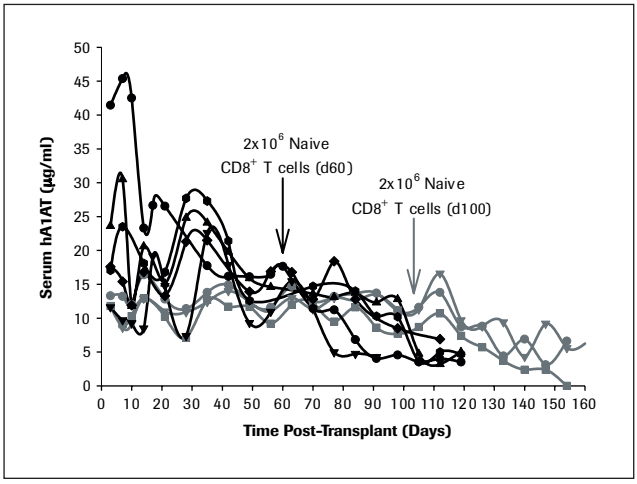


Figure 4. Long-term hepatocyte allograft survival induced by short-term therapy with anti-LFA-1 and anti-CD40L mAbs.

6. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation results in augmented donor-reactive delayed type hypersensitivity (DTH) responses *in vivo*.

For these experiments, anti-LFA-1- and anti-CD40L mAb-treated CD4 KO (H-2^b) mice which had accepted allogeneic FVB/N (H-2^q) hepatocytes > 60 days were assessed for alloreactive DTH responses. DTH was measured as footpad swelling in response to allogeneic (FVB/N) or

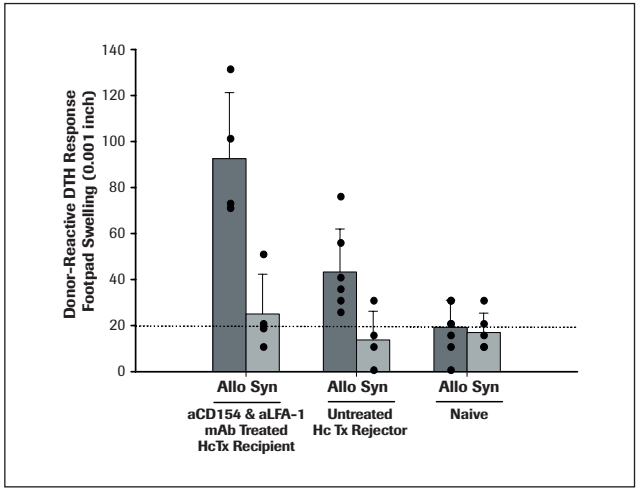


Figure 5. Alloreactive DTH response in anti-LFA-1- and anti-CD40L mAb-treated CD4 KO (H-2^b) mice.

syngeneic (C57BL/6) cellular antigen. Antigenic cells were injected directly into the footpad and swelling was measured 18 hours after injection.

Results: Unexpectedly, alloreactive DTH responses were significantly increased in CD4 KO hepatocyte recipients receiving dual therapy in comparison to untreated hepatocyte rejector mice ($P=0.01$, Fig. 5). DTH responses to control syngeneic antigen were similar between the two groups ($P=$ns, Fig. 5). However, this augmented DTH response in treated hepatocyte recipients did not interfere with long-term hepatocellular allograft survival. These results also demonstrated that long-term hepatocellular allograft survival induced by anti-LFA-1 and anti-CD40L mAbs was not due to clonal deletion or ignorance.

7. Immunotherapy targeting LFA-1 and CD40/CD40L costimulation induces allograft acceptance associated with linked antigen recognition.

For these experiments, an F1 generation of hepatocyte donors was created by crossing transgenic FVB/N (H-2^q) hA1AT positive mice with non-transgenic B10.BR (H-2^k) mice, to produce an F1 (H-2^{qxk}) generation. Some progeny expressed the reporter transgene product, hA1AT and were used as F1 hepatocyte donor mice. Some preliminary studies were required to first establish acute rejection responses of C57BL/6 recipients transplanted with hepatocytes from F1 donor mice. These were performed by transplanting C57BL/6 mice with F1-hA1AT hepatocytes to determine the time course of rejection without immunosuppression. Similarly rejection responses in untreated CD4 KO hepatocyte recipients were determined, though in these studies hepatocytes were transplanted by injection under the kidney capsule. Some recipients received treatment with anti-LFA-1 and anti-CD40L mAbs and were monitored for production of the reporter product, hA1AT. At 60 days post-transplant, the hepatocyte transplant-bearing kidney is removed, the animal is monitored until serum hA1AT levels return to zero, and then is re-transplanted with F1-hA1AT hepatocytes by injection under the kidney capsule of the remaining kidney. Acceptance of the F1 hepatocyte transplant in the absence of additional immunosuppression would indicate operational tolerance through linked antigen recognition.

Results: Preliminarily, we have determined that F1 (H-2^{qxk}) hepatocytes are rejected in a similar fashion to FVB/N (H-2^q) hepatocytes when transplanted into C57BL/6 or CD4 KO mice under the kidney capsule with median survival times of 10 and 17 days, respectively (data not shown). Studies addressing the induction of operational tolerance by dual therapy targeting LFA-1 and CD40/CD40L costimulation are currently ongoing. To date, the sequential hepatocyte transplant studies have only been performed in CD4 KO hepatocyte recipients. However, preliminary results are quite exciting since 2 of 4 recipients demonstrated acceptance of the F1 hepatocyte transplant for >30 days in the absence of immunosuppression (data not shown).

Publications

1. Lunsford KE, Koester MA, Eiring AM, Gao D, Horne PH, Bumgardner GL. Targeting LFA-1 and CD154 suppresses the *in vivo* activation and cytolytic development of (CD4-independent) $CD8^+$ T cells. *Submitted for publication.*
2. Lunsford KE, Eiring AM, Koester MA, Gao D, Bumgardner GL. Long-term regulation of $CD8^+$ T cells by short-term costimulation. *Am J Transplant* 2004; 4(632):330; (Abstract).
3. Lunsford KE, Horne PH, Wang Y, Gao D, Eiring AM, Bumgardner GL. Role of alloantibody in $CD4^+$ T cell dependent immune damage of hepatocytes. *Am J Transplant* 2004; 4(1093):457; (Abstract).
4. Lunsford KE, Koester MA, Eiring AM, Bumgardner GL. Mechanisms of immune regulation of CD8-dependent immune responses by short-term immunotherapy targeting LFA-1 and CD40/CD154 costimulation. Oral Presentation, *The Society of University Surgeons 66th Annual Meeting* 2005; (Abstract).
5. Lunsford KE, Koester MA, Eiring AM, Horne PH, Bumgardner GL. Mechanisms of immune regulation for (CD4-independent) CD8-dependent immune responses by short-term immunotherapy targeting LFA-1 and CD40/CD154 costimulation. *Am J Transplant* 2005; 5(11):268; (Abstract).
6. Lunsford KE, Koester MA, Horne PH, Eiring AM, Qureshi Z, Bumgardner GL. Mechanisms of activation of (CD4-independent) CD8-dependent immune responses to parenchymal cell allografts. *Am J Transplant* 2005; 5(11):364; (Abstract).
7. Horne PH, Lunsford KE, Eiring AM, Wang Y, Gao D, Bumgardner GL. $CD4^+$ T cell-dependent immune damage of liver parenchymal cells is mediated by alloantibody. *Transplantation, In press.*
8. Bumgardner GL. Evidence for multiple allograft rejection mechanisms within the same experimental system. *Curr Op Organ Transpl, In press.*
9. Walker J, Bumgardner GL. Hepatocyte transplantation: current status and future potential. *Curr Op Organ Transpl, In press.*

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

Specific Aim 1: To assess if enhanced revascularization of syngeneic islet grafts correlates with better functional islet mass after transplantation.

Previous studies from our laboratory described a useful mice model to evaluate the role of angioblast in islet revascularization[1]. Briefly, FEV/N mice underwent bone marrow (BM) transplant from transgenic mice constitutively expressing β-galactosidase (β-gal) encoded by LacZ under the transcriptional regulation of an endothelial cell-specific promoter, TIE-2[2]. Reconstitution of the transplanted BM yielded FEV/N-TIE-2-LacZ mice in which expression of LacZ is restricted to BM-derived cells expressing TIE-2-LacZ and is not observed in other somatic cells[1]. Detection of TIE-2-LacZ fusion transcripts by RT-PCR in peripheral blood, BM and spleen of the BM Tx recipients confirmed reconstitution of the BM and the origin of the incorporated cells in FEV/N-TIE-2-LacZ mice. Minimal detection of TIE-2-LacZ was demonstrated in the liver. Using this model, reconstituted animals underwent intrahepatic islet auto-transplantation (FEV/N donors). As a control, some recipients received, instead of islets, an intrahepatic infusion of donor lymphocytes. An islet dose-dependent increase in liver TIE-2-LacZ detection was demonstrated after transplantation. The endothelial cell-specific promoter was detected as early as three days post-transplantation. In contrast, no detection of TIE-2-LacZ was evident in reconstituted recipients given non-LacZ BM (FEV/N donors), or animals that received lymphocytes instead of islets. These results are in accordance with observations that demonstrated the extensive capacity of angioblasts to migrate before *in situ* differentiation and plexus formation. The localization and *in situ* differentiation of angioblasts into foci of islet revascularization was confirmed by immunohistochemistry[1]. Having demonstrated the role of angioblast in the islet revascularization process, we examined the effect of peripheral angioblast mobilization from the BM in order to enhance islet revascularization and thus islet engraftment. As previously reported[3-5], treatment with GM-CSF (250 μg/m²/subcutaneous/7 days), increased the number of cells with endothelial cell-specific antigens, CD34 (↑315 ±34% by day 4), Flk-1 (↑260 ±21%) and VE cadherin (↑158 ±34%). Circulating cells mobilized with GM-CSF consisted principally of cells taking up acetylated low density lipoprotein (acLDL). In order to asses the impact of GM-CSF treatment in islet revascularization following transplantation, FEV/N-TIE-2-LacZ reconstituted animals received a seven day

course of GM-CSF starting four days before the transplant. Diabetes was induced in syngeneic recipients 4 days before the transplant by a single intraperitoneal injection of streptozotocin, 250 mg/kg and confirmed in all recipients by serum non-fasting glucose levels > 350 mg/dl for 3 consecutive days after induction. Islets were isolated following standard techniques and hand-picked to maximize islet purity. Because islet culture probably have significant effect in the survival of intraislet endothelial cells[6] and thus islet revascularization, islet were kept in culture for 48 hours, protocol currently in place in most of the clinical islet transplant centers. As an initial step, we infuse an optimal dose of syngeneic islets into the portal vein (450 islet equivalents (IEQ) per animal). Four weeks after the transplant, we demonstrated a significant and dose-dependent increase in islet vascular density in syngeneic islets after intraportal transplantation (Fig. 1). Next, we will evaluate if the increase in islet vascular density will translate better islet graft performance, which is the long-term goal of this proposal. To this end, we indirectly evaluated functional islet mass in recipients described above by metabolic testing. Arginine-stimulated acute insulin release ($AIR_{arginine}$) was performed in overnight-fast recipients after intravenous injection of arginine, 0.3 g/kg body weight, infused in 30 seconds. Thirty μl of whole blood were collected in 3 μl heparin (5000 U/ml) at -5, 0, 2, 5, and 7 minutes after stimulus injection.

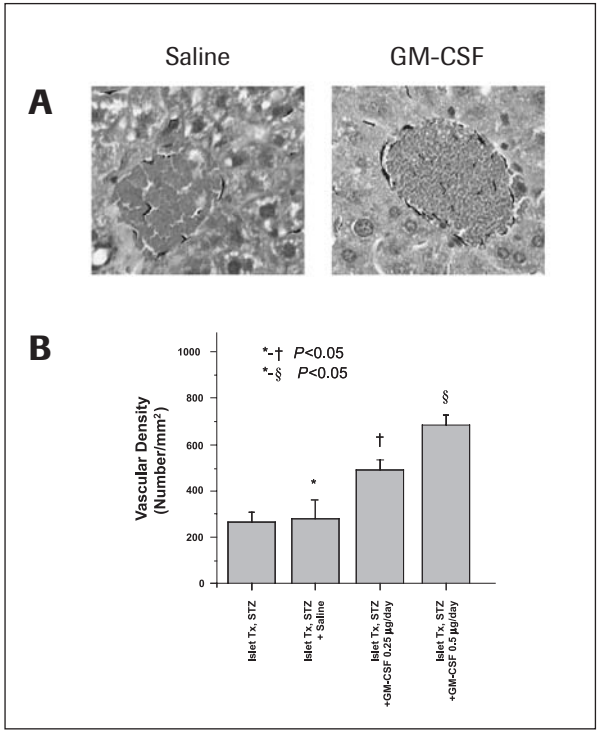

Figure 1. GM-CSF increases angioblast localization and in situ differentiation into foci of syngeneic islet revascularization. *Diabetic FEV/N-TIE-2-LacZ reconstituted mice or FEV/NJ (not reconstituted) recipients received 450 syngeneic (FEV/N) IEQ/mouse into the portal vein as we described[1]. Recipients received GM-CSF (0.25 or 0.5 μg/day/7 days starting 4 days before the transplant) or saline as indicated. On day 30 post-transplant, the whole liver was excised and analyzed for islet vascular density, as described[1,7,8]. **A)** Microvascular endothelium (dark) in transplanted islets with or without GM-CSF treatment. **B)** Semi-quantitative evaluation of islet vasculature performed as described[1]. Results are expressed as mean ± SD (n=8 animals per group).*

Samples were immediately centrifuged, supernatants were collected, and stored at -20° C. Mouse insulin was determined by ELISA (Kamiyama Biomedical Company, Seattle, WA). Higher $AIR_{arginine}$ was demonstrated in recipients mobilized with GM-CSF and treated with syngeneic islet transplant compared with similar, control recipients given vehicle (Fig. 2). In addition, 30 days after the transplant, the liver of some recipients (n=4) was homogenized in acid/ethanol (0.1 N HCl in 100% ethanol) and stored overnight at -80° C. The homogenates were centrifuged at 14,000g for 7 minutes and the amount of insulin was measured in the supernatants by ELISA. In correlation with the metabolic studies, higher total insulin content was demonstrated in livers harvested from animals with enhanced islet revascularization (GM-CSF group, insulin=377 ±88 ng) compared with animals given vehicle (172 ±32 ng, $P<0.05$). Overall, these results demonstrated that promoting islet revascularization after intraportal islet transplantation by mobilizing endothelial progenitor cells from the BM promotes islet engraftment and thus functional islet mass after transplantation.

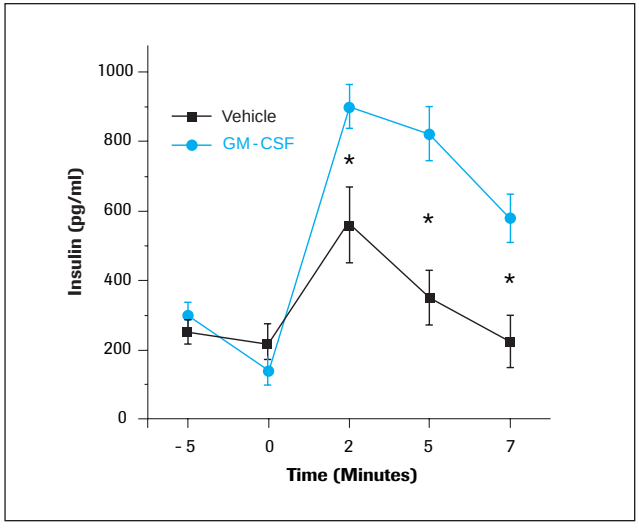

Figure 2. Arginine-stimulated insulin release after PIT in syngeneic recipients treated with and without GM-CSF. *On day 30 post-transplant, after overnight fast, recipients (treated with vehicle or GM-CSF, and intraportal infusion of syngeneic, 450 IEQ, as described in Fig. 1) received arginine, 0.3 grams/kg body weight/i.v. in 30 seconds. Blood samples were collected in heparin tubes at -5, 0, 2, 5, and 7 minutes after stimulus injection. Mouse insulin was determined by ELISA. Results are expressed as mean ± SD (n=4). * P< 0.05.*

Specific Aim 2: To enhance survival and functional islet mass in islet allograft recipients by angioblast mobilization with GM-CSF.

To evaluate the effects of angioblast mobilization with GM-CSF in islet allograft recipients receiving an immunosuppressive protocol currently in use in clinical islet transplantation, FEV/N-TIE-2-LacZ reconstituted animals received streptozotocin four days before the transplant, as described above. The initial control group received allogeneic islets (donor C57Bl6,

600 IEQ/animal infused into the portal vein) with no immunosuppression and no treatment with GM-CSF. The means survival time was 8.4 ±4.2 days (n=5). Experiments are currently underway in groups of animals given immunosuppression with and without GM-CSF and animals given GM-CSF alone without immunosuppressive therapy.

Publications

1. Contreras JL, Smyth CA, Eckstein C, Bilbao G, Thompson JA, Young CJ, Eckhoff DE. Peripheral mobilization of recipient bone marrow-derived endothelial progenitor cells enhances pancreatic islet revascularization and engraftment after intraportal transplantation. *Surgery* 2003; 134(2):390-8.
2. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 1995; 376(6535):70-4.
3. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999; 85(3):221-8.
4. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 1999; 18(14):3964-72.
5. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; 5(4):434-8.
6. Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A, Lin PC, Gannon M, Powers AC. Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* 2004; 53(5):1318-25.
7. Mattsson G, Carlsson PO, Olausson K, Jansson L. Histological markers for endothelial cells in endogenous and transplanted rodent pancreatic islets. *Pancreatology* 2002; 2(2):155-62.
8. Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* 2002; 51(5):1362-6.

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Mechanisms of Renal Allograft Rejection

Aim: To define the molecular basis of the lesions that characterizes clinical graft rejection.

To be applied to human clinical transplantation, the expanding knowledge of molecular immunology must be linked to the unmet needs in the clinic. Traditional methods of exploring mechanisms have failed to establish the mechanism by which the alloimmune response injures the tissue. We believe that this is due to failure of the experimental systems to model the human pathology. The key link to making animal models relevant is that the animal pathology must simulate the human lesions, which permits investigations to move from the clinic to the laboratory and back. In the early 1990s clinical transplantation changed its focus from graft survival to graft function and pathology (biopsy-proven rejection, based largely on tubulitis). Basic immunology must now do the same, focusing on pathologic lesion development rather than graft failure. In this project, we focused on mechanisms of development of pathologic lesions typical of clinical rejection. Our first goal is to understand tubulitis. We will study the mechanisms of tubulitis, the role of CD103 and E-Cadherin, and microarray patterns. We plan similar studies of cardiac myocyte necrosis, bile ductulitis, and endothelial arteritis. This will facilitate studies of immune regulation: we will know what is being regulated. Establishing the mechanisms of allograft injury will also facilitate the development of novel diagnostic tests and interventions. We will thus investigate mechanisms of tubulitis in our mouse kidney transplant model adapted to permit the observation of progressive tubulitis.

Proposal

We proposed to study tubulitis mechanisms, including changes in cadherin expression and the potential changes in epithelial cell biology, e.g. altered transcription; evolution of tubulitis in CD103 deficient hosts; and correlation of tubulitis to the evolution of microarray patterns.

Progress

Our goal was ultimately to understand how effector T cells and other inflammatory cells produce the histologic lesions that characterize human kidney transplant rejection. This would permit the first complete mechanistic understanding between basic immunology/inflammation and human-disease phenotypes in transplantation. Until now, there has been no mechanistic understanding of how the alloimmune response damages tissue. Many of the

existing concepts in the immunologic literature are in fact either naïve or completely wrong when applied to the lesions that characterize human rejection. We started by establishing a mouse kidney transplant model which develops the Banff lesions (namely tubulitis). We then used knock-out mouse hosts, two different types of B cell/immunoglobulin knock-outs and perforin knock-outs, and granzyme A/granzyme B double knockouts. This established that the tubulitis lesions were largely independent of B cells and immunoglobulin, and that they were also independent of perforin and of granzyme A/B. We have established the time course of the lesions over the period from day 3, 4, 5, 7, 14, 21, 42. The model is unique in that the contralateral kidney of the host is left in place in order to preserve kidney function and allow us to study the evolution of the lesions. By day 42, there is about 30% patchy ischemic necrosis but the arteries and veins are still patent. The technical failure rate (anastomotic failure/ureteric obstruction) is an infection rate, which is about 15%. Most rejecting kidneys remain largely viable through these six weeks of observation. We have also performed a large number of isografts, at days 3, 4, 5, 7, 21 and have duplicate arrays at each of these points with three kidneys per array. Many normal kidney controls – in wild-type and various knockout mice - are also in progress. These materials allow us to interrogate the pathology using Affymetrix microarrays. We have now performed 100 Affymetrix microarrays on various mouse kidneys, all with detailed characterization of their histopathology to allow correlations. Each microarray containing the RNA pool from histologically verified kidneys. The results form a remarkable database which provided unique new insights into the pathogenesis of graft rejection. Included are knockout mice with disruption of the genes for immunoglobulin production, perforin, granzyme A/B, CD103, interferon-γ, interferon-γ receptors, transcription factor IRF1, donor TAP1, donor β2-microglobulin, and a number of other genes. Our strategy is to perform the microarrays in duplicate, with each microarray containing the RNA extracted from three different kidneys. The results of the duplicate microarrays are highly correlated (R values between 0.9 – 0.96). We also have some replicate microarrays on the same RNA pools showing R values of 0.96 to 0.98. These results indicate the extraordinary reproducibility of our experimental system and of the Affymetrix microarrays. In addition, we have performed validation of the Affymetrix results using RT-PCR for approximately 50 genes. The results are summarized in a series of five papers[1-5].

Major Attraction of Funds

This ROTRF grant had the remarkable effect of permitting us to establish microarray technology and apply it to mouse kidney transplants, and then attract a large Genome Canada grant. This three-year project allows us to expand from the ROTRF project in the mouse to explore mouse and human kidney transplant pathology using Affymetrix microarrays. This is an extraordinary achievement: without the ROTRF grant, we would not have been able to secure this major investment, which now establishes us as the leading center in exploring the transcriptome of kidney graft rejection, using both knockout mice and human material. This will be accomplished within the Banff consensus process to ensure that all results are in the public

domain. We are hosting the first workshop to define the transcriptome criteria for graft rejection (http://transplants.med.ualberta.ca/Nephlab/workshop/index.html). This will be held in Edmonton as a workshop before the 8th Banff Congress on Allograft Pathology.

Summary

The ROTRF grant entitled "Mechanisms of Renal Allograft Rejection" has permitted us to establish a new system for microarray analysis of the mouse kidney transplant rejection, and to explore the relationship between pathology and immunologic events. This is now the basis of a major new initiative to extend these findings to human organ transplantation.

Publications

1. Jabs WJ, Sedlmeyer A, Ramassar V, Hidalgo LG, Urmson J, Afrouzian M, Zhu LF, Halloran PF. Heterogeneity in the evolution and mechanisms of the lesions of kidney allograft rejection in mice. *Am J Transplant* 2003; 3:1501–9.
2. Halloran PF, Urmson J, Ramassar V, Melk A, Zhu LF, Halloran BP, Bleackley RC. Lesions of T-cell-mediated kidney allograft rejection in mice do not require perforin or granzymes A and B. *Am J Transplant* 2004; 4:705–12.
3. Hidalgo LG, Urmson J, Halloran PF. IFN-γ decreases CTL generation by limiting IL-2 production: a feedback loop controlling effector cell production. *Am J Transplant* 2005; 5:651–61.
4. Vongwiwatana A, Tasanarong A, Rayner DC, Melk A, Halloran PF. Epithelial to mesenchymal transition during late deterioration of human kidney transplants: the role of tubular cells in fibrogenesis. *Am J Transplant* 2005; 5:1367–74.
5. Melk A, Schmidt BMW, Vongwiwatana A, Rayner DC, Halloran PF. Increased expression of senescence-associated cell cycle inhibitor p16 in deteriorating renal transplants and diseased native kidney. *Am J Transplant* 2005; 5:1375–82.

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Modulation of TRAF-Dependent Signaling in EBV$^+$ B Cell Lymphomas

The original specific aims of the grant were:

1. **Determine the role of EBV in modulating the signaling pathways used by TNFR family members to activate responses in B cell lymphomas and**
2. **Determine the role of LMP1 and TRAF signaling pathways in the modulation of B cell activation.**

We have made important progress in both aims and our findings are summarized below.

1.1 Relative expression of TNFR family members on paired EBV-infected and uninfected B cell lines

With the goal of assessing the effect of EBV on signaling through TNFR family members, the initial experiments in Aim 1 were intended to identify paired uninfected and EBV-infected B cell lines with similar levels of expression of the TNFR family members. In preliminary data submitted with the proposal we had demonstrated that the paired BL41 lines and the paired Ramos lines express comparable levels of CD27, CD40 and TNFR1. We have now examined expression of these molecules, along with CD30 and TNFR2, on the paired BL30, BL41, and BJAB lines (Fig. 1). These data indicate that the BL30 and BL41 lines express similar levels of CD27, CD40, and TNFR1 irrespective of EBV infection. In contrast, EBV infection markedly affects the levels of expression of CD27 and CD40 in the BJAB lines. CD30 was not detected on any of the cell lines, though we could readily detect CD30 expression on Jurkat cells (data not shown). TNFR2 was absent or expressed at low levels on all cell lines.

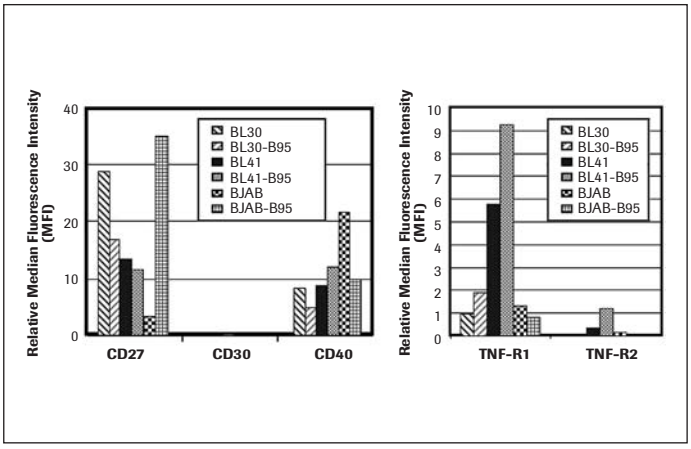

Figure 1. Expression of TNFR family members on paired B cell lines. *Cells were labeled with fluorochome conjugated mAb to CD27, CD30, CD40, TNFR1, TNFR2 or isotype controls and analyzed by FACS. Data are expressed as the relative median fluorescence intensity (MFI) as calculated by subtracting the MFI of isotype control from the MFI obtained by staining with the specific TNFR Abs.*

1.2 Relative expression of TRAF proteins in paired EBV-infected and uninfected B cell lines

We also compared the relative levels of TRAFS 1, 2, 5, and 6 in the BL30, BL41, Akata, and BJAB parental cell lines and their EBV (B.95 strain) infected counterparts. Figure 2 shows Western blots for TRAF1 and TRAF2 in the uninfected and EBV-infected versions of these cell lines.

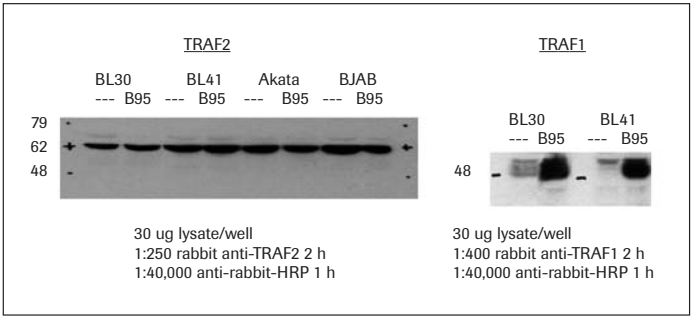

Figure 2. Western blot for TRAF1 (right) and TRAF2 (left). *Whole cell lysates were prepared and separated by SDS-PAGE, transferred to membranes and blotted for TRAF1 or TRAF2 with specific antibodies.*

TRAF2 (Fig. 2), as well as TRAF5 and TRAF6 levels (data not shown) were similar in the cell lines irrespective of EBV infection. Density analysis using β-actin as a control showed there was no significant difference in the levels of TRAF2, TRAF5, and TRAF6 between infected and uninfected paired lines (data not shown). LMP1, a latent viral gene expressed in our EBV-infected cell lines, is known to up-regulate TRAF1 expression. Thus, as anticipated, TRAF1 levels were significantly increased in EBV-infected BL30, BL41 (Fig. 2) and BJAB cells (data not shown).

In summary, the levels of expression of CD30 and TNFR2 were absent or low on all the cell lines tested, thus, these molecules were not considered for further study. We have not pursued CD27 at this time since we have found it difficult to identify reagents that reliably signal through this molecule. We have also eliminated the BJAB cells from this study since the levels of cellular receptors are quite different in the infected and uninfected counterparts. Therefore, on the basis of the data shown in Result 1.1 and Result 1.2 we have initially focused our signaling studies on the CD40 and TNFR1 molecules expressed on the BL30 and BL41 lines.

1.3 Effect of EBV infection on expression of cell surface molecules in response to TNF-α or CD40 stimulation

We first analyzed the effect of TNF-α on expression of CD86 and ICAM on the paired BL30 and BL41 cell lines. Our results indicated that baseline expression of these molecules tends to be higher on the EBV-infected counterpart than on the parental cell line. Nevertheless, expression of CD86 and ICAM was relatively unchanged by TNF in both infected and uninfected BL30 and BL41 cell lines (data not shown). Next we analyzed the effect of CD40 stimulation on CD80, CD86, ICAM, and CD95 expression in the paired cell lines (Fig. 3). CD40 signaling induced by agonist anti-CD40 mAbs had minimal effect on CD80, CD86, and CD95 in the uninfected BL30 lines and minimal effect on CD80 and CD86 in the uninfected BL41 line. In contrast, CD40 signaling increased expression of ICAM on both BL30 and BL41, and CD95 on BL41. Interestingly, signaling through CD40 in EBV-infected BL30 and BL41 uniformly inhibited expression of CD80, ICAM, CD86 and CD95. These data indicate that EBV infection alters signaling through CD40 and subsequent cellular function. Future experiments will analyze the effect of EBV infection on cytokine production, cell growth and apoptosis in response to TNF and CD40 signaling and on recruitment of TRAF proteins to CD40.

2.1 Development of an inducible system for LMP1 signaling using nerve growth factor receptor-LMP1 chimeric molecules

To more specifically examine the role of the viral protein LMP1 on cellular signaling pathways we developed nerve growth factor receptor (NGFR)-LMP1 chimeric molecules from constructs obtained from Wolfgang Hammerschmidt. These molecules contain the extracellular and transmembrane regions of NGFR and the cytoplasmic domains of LMP1, and thereby, have the advantage of allowing inducible LMP1 signaling when NGFR is crosslinked with anti-NGFR antibodies. Since LMP1 activates NFκB we utilized NFκB luciferase reporter assays in 293 cells to test the function of these molecules. As shown in Fig. 4 (top panel) crosslinked NGFR.LMP1 (+), but not uncrosslinking NGFR-LMP1 (-) or empty vector (pcDNA3), induced NFκB-dependent reporter activity comparable to wild-type LMP1 (SG5.LMP1). Western blots (Fig. 4, bottom panel) for LMP1 showed the NGFR-LMP1 molecules were of higher molecule weight than wild-type LMP1, as expected, and were expressed at similar levels.

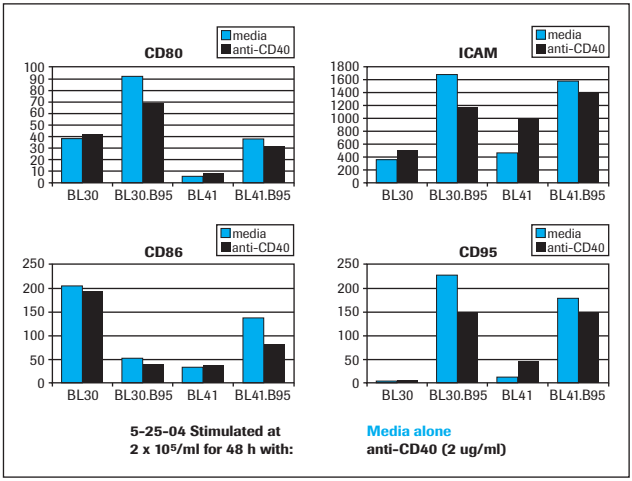

Figure 3. Effect of EBV infection on CD40-induced expression of CD80, CD86, ICAM and CD95. *Cells were cultured in media alone (blue) or with agonist anti-CD40 mAbs (black) for 48 hours and analyzed for expression of cell surface markers by immunofluorescent staining and flow cytometry. Data are expressed as the relative MFI as described in Figure 1.*

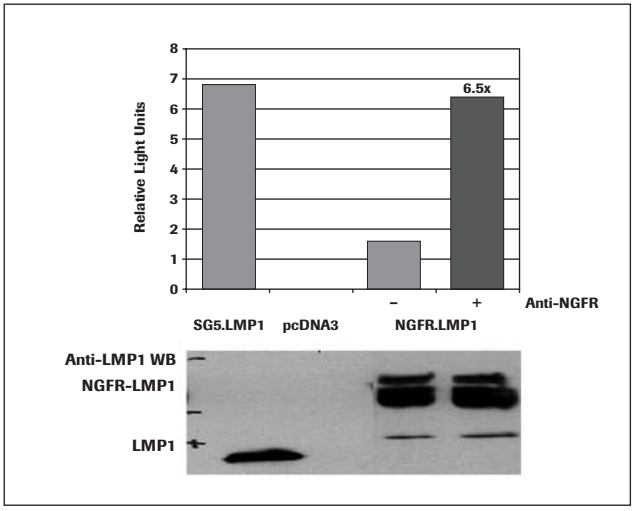

Figure 4. NFκB luciferase reporter assay (top) and Western blot for NGFR-LMP1 constructs. *293 cells were transfected with constructs for wild-type LMP1 (SG5.LMP1), NGFR.LMP1 or empty vector (pcDNA3), along with renilla as an internal control, and the NFκB luciferase reporter. After 18 hours NGFR was crosslinked with anti-NGFR and anti-mouse Ig for 6 hours before luciferase activity was measured. For Western blots, lysates were made of transfected 293 cells and blotted for LMP1.*

We have generated similar NGFR-LMP1 chimeric molecules in which the two key signaling domains of LMP1, CTAR1 and CTAR2, have been mutated to prevent signal transduction. CTAR1 mutants show modest NFκB activation after NGFR crosslinking whereas CTAR2 mutants show markedly diminished NFκB activation in the luciferase reporter assay (data not shown). These findings are in agreement with previous reports showing that CTAR1 contributes about 20% and CTAR2 contributes 80% of the NFκB activation activity of LMP1.

2.2 Expression of NGRF-LMP1 chimeric molecules in BL30, BL41, and BJAB

We have used electroporation, single-cell cloning, and G418 selection to generate stable clones of BL30 AND BL41 expressing the NGFR-LMP1 chimeric molecules (data not shown).

Clones were selected in G418 and expanded for two-three weeks before FACs analysis for surface NGFR expression. Lysates of the cell lines were analyzed by immunoprecipitation and Western blot for LMP1 to detect expression of the chimeric molecule. We have generated and are currently characterizing a panel of NGFR-LMP1 transfectants in BL30 and BL41 to obtain low, medium, and high expressers of the chimeric molecule.

2.3 Function of NGRF-LMP1 chimeric molecules and CTAR mutants in BL30 and BL41

Since LMP1 is known to up-regulate ICAM expression, we tested whether the NGFR-LMP1 constructs expressed in BL30 and BL41 were functional by FACs analysis of ICAM expression before and after NGFR crosslinking.

Whereas crosslinking of NGFR in BL30-NGFRLMP1 and BL41-NGFRLMP1 transfectants induced ICAM up-regulation, anti-NGFR labeling without crosslinking had no effect on ICAM expression (data not shown). These results indicate that NGFR-LMP1 is inducible and functional in stable BL30 and BL41 transfectants. These reagents, along with BL30 and BL41 lines expressing the CTAR mutant forms of the chimeric NGFR-LMP1 molecules, will be used to test the effects of LMP1 signaling on TNF-α and CD40-mediated cellular growth, apoptosis, cytokine production, receptor expression, and TRAF recruitment in future studies.

2.4 Effect of EBV and LMP1 on recruitment of TRAF proteins to CD40

In our original hypothesis we proposed that LMP1 may compete with CD40 for TRAF proteins and that this would lead to alteration in CD40 signaling outcome. One prediction is that less TRAF proteins would be recruited to CD40 in the presence of LMP1 signaling. To test this we utilized paired BL30 and BL41 cell lines as well as clones of BL30 and BL41 that express the inducible NGFR-LMP1 molecule. Figure 5 shows that EBV infection or LMP1 signaling results in reduced recruitment of TRAF3 to CD40 when CD40 is crosslinked and activated. Similar data were obtained for TRAF2 (data not shown). We will continue to assess the effect of LMP1 on the recruitment of other TRAF proteins to CD40 and will determine if there is enhanced binding of TRAF proteins to LMP1.

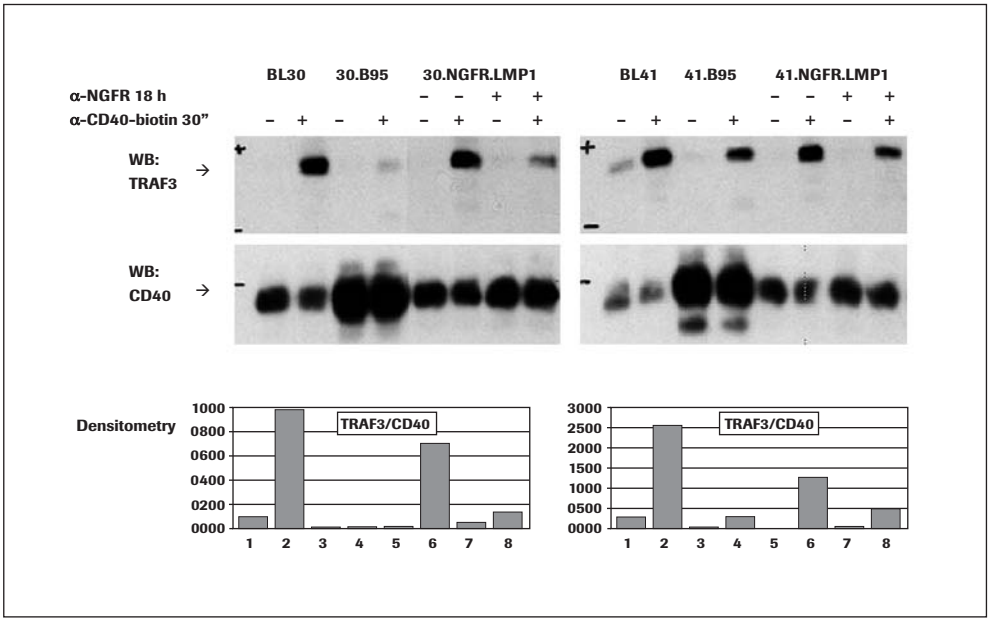

Figure 5. EBV infection and NGFR.LMP1 signaling inhibits TRAF3 association with crosslinked CD40. *As shown in the 4 right lanes of each panel, LMP1 was activated in BL30.NGFR.LMP1 and BL41.NGFR.LMP1 clones for 18 hours (+) or cells were left untreated (-). Then the paired cell lines (BL30 and BL41) (first four lanes in each panel) and the transfectants were either untreated (-) or CD40 was crosslinked with anti-CD40-biotin+streptavidin (+) for 30 minutes. CD40 was immunoprecipitated and proteins were separated by SDS-PAGE, transferred to nylon membranes and blotted for TRAF3 or CD40. Densitometry is shown below. EBV-infected cells, and cells in which NGFR.LMP1 was crosslinked, show reduced association of TRAF3 with CD40.*

Publications

Lambert SL, Martinez OM. LMP1 protein of Epstein-Barr virus effects CD30 signaling in human B cells. *American Association of Immunologists* 2005; (Abstract).

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Defining the Molecular Basis of T Cell Allorecognition

Original Aims

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

Summary of work to date and achievements

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

We next determined the crystal structure of the anti-HLA-B*/FLR–specific "public" TcR to 1.5Å, representing a significant advance on previously determined TcR structures. This crystal structure reveals that five of the six hypervariable loops adopt novel conformations providing a unique combining site that contains a deep pocket predicted to overlay the HLA B8-peptide complex. The findings hinted at a structural basis for the immunodominance of this clonotype in the immune response to EBV. This was further cemented when we solved the structure of this immunodominant or "public" TcR complexed with the HLA-B*/FLR binary ligand. Residues encoded by each of the highly selected genetic elements of an immunodominant clonotype recognising EBV were critical to the antigen specificity of the receptor. Upon recognising antigen, the immunodominant TcR undergoes extensive conformational changes in the complementarity-determining regions (CDRs), including the disruption of the canonical structures of the germline-encoded CDR1α and CDR2α loops to produce an enhanced fit with the HLA-peptide complex. TcR ligation induces conformational changes in the TcRα constant domain thought to form part of the docking site for CD3ε. These findings indicate that TcR immunodominance is associated with structural properties conferring receptor specificity and suggest a novel structural link between TcR ligation and intracellular signalling.

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

The next set of experiments examined the potential of antagonist ligands to inhibit T cell alloreactivity. Alloreactive T lymphocytes are central mediators of graft-versus-host disease and allograft rejection. The public LC13 CTL clonotype has allospecificity for the alloantigens HLA3 B*4402 and B*4405 despite being, driven by cross-reactive stimulation with the common, persistent herpesvirus EBV. Since such alloreactive memory CTL expansions have the potential to influence transplantation outcome, altered peptide ligands (APLs) of the target HLAB* 0801-binding EBV peptide, FLRGRAYGL, were screened as specific antagonists for this immunodominant clonotype. One APL, FLRGRFYGL, exerted powerful antagonism of a prototypic T cell clone expressing this immunodominant TcR when co-stimulated with target

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

In other experiments we explored the impact of single-residue polymorphisms on the T cell alloreactivity between members of the HLA-B44 group of related allotypes. HLA-B*4402 and B*4403 are naturally occurring MHC class I alleles that are both found at a high frequency in all human populations and yet they only differ by one amino acid on the α2 helix (B*4402 Asp156$\rightarrow$B*4403 Leu). $CD8^+$ T lymphocytes discriminate between HLA-B*4402 and B*4403 and these allotypes stimulate strong allogeneic responses reflecting their known barrier to haemopoietic stem-cell transplantation. While HLA-B*4402 and B*4403 share $>$95% of their peptide repertoire, B*4403 presents many more unique peptides than B*4402, consistent with the stronger T cell alloreactivity observed towards B*4403 compared with B*4402. Crystal structures of B*4402 and B*4403 show how the polymorphism at position 156 is completely buried and yet alters both the peptide and the heavy chain conformation, relaxing ligand selection by B*4403 compared with B*4402. Thus, polymorphism of the naturally selected single residue between HLA-B*4402 and B*4403 modifies both peptide repertoire and T cell recognition, and is reflected in the paradoxically strong alloreactivity that occurs across this single residue class I mismatch. The findings also suggest that the maintenance of this dimorphism in diverse human populations is related to the differential selection of developing and mature T cells by these two allotypes. It is well known that HLA class I polymorphism creates diversity in epitope specificity and T cell repertoire. We also showed that HLA polymorphism also controls the choice of Ag presentation pathway. A single amino acid polymorphism that distinguishes HLA-B*4402 (Asp116) from B*4405 (Tyr116) permits B*4405 to constitutively acquire peptides without any detectable incorporation into the TAP-associated peptide-loading complex (PLC) even under conditions of extreme peptide starvation. This mode of peptide capture is less susceptible to viral interference than the conventional loading pathway used by HLA-B*4402 that involves assembly of class I molecules within the PLC. Thus, B*4402 and B*4405 are at opposite extremes of a natural spectrum in HLA class I dependence upon the PLC for Ag presentation. These findings unveil a new layer of MHC polymorphism that affects the generic pathway of Ag loading revealing an unsuspected evolutionary trade-off in selection for optimal HLA class I loading versus effective pathogen evasion.

Future Goals

We have two current aims. The first is to identify the allopeptide responsible for the T cell alloreactivity of the LC13 clonotype with HLA-B*4402/05. This information would be used to determine the structure of the LC13 TcR associated with its alloligand. This will provide the first glimpse of a naturally alloreactive T cell receptor interacting with allogeneic MHCp enabling a comparison to its cognate self-+viral ligand (HLA-B8/FLR). Our second aim is to examine the thermodynamics of LC13 TcR binding to its cognate ligand by isothermal calorimetry.

Publications

1. Kjer-Nielsen L, Clements CS, Purcell AW, Brooks AG, Whisstock JC, Burrows SR, McCluskey J, Rossjohn J. A structural basis for the selection of dominant αβ T cell receptors in antiviral immunity. *Immunity* 2003; 18(1):53-64.
2. Macdonald WA, Purcell AW, Mifsud N, Ely LK, Williams DS, Gorman JJ, Clements CS, Kjer-Nielsen L, Koelle DM, Burrows SR, Tait BD, Holdsworth R, Brooks AG, Lovrecz GO, Lu GO, Rossjohn J, McCluskey J. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire and T cell recognition. *J Exp Med* 2003; 198 (5):679-91.
3. Webb AI, Dunstone MA, Chen W, Aguilar MI, Chen Q, Chang L, Kjer-Nielsen L, Beddoe T, McCluskey J, Rossjohn J, Purcell AW. Functional and structural characteristics of NY-ESO-1 related HLA-A2 restricted epitopes and the design of a novel immunogenic analogue. *J Biol Chem* 2004; 279(22):23438-46.
4. Zernich D, Purcell AW, Macdonald WA, Kjer-Nielsen L, Ely LK, Laham N, Crockford T, Mifsud NA, Tait BD, Holdsworth R, Brooks AG, Bottomley SP, Beddoe T, Peh CA, Rossjohn J, McCluskey J. Natural HLA class I polymorphism controls the pathway of antigen presentation and susceptibility to viral evasion. *J Exp Med* 2004; 200:13-24.
5. Webb AI, Borg NA, Dunstone MA, Kjer-Nielsen L, Beddoe T, McCluskey J, Carbone FR, Bottomley SP, Aguilar MI, Purcell AW, Rossjohn J. The structure of H-2K(b) and K(bm8) complexed to a herpes simplex virus determinant: evidence for a conformational switch that governs T cell repertoire selection and viral resistance. *J Immunol* 2004; 173(1):402-9.
6. Borg NA, Ely LK, Beddoe T, Macdonald WA, Reid HH, Clements CS, Purcell AW, Kjer-Nielsen L, Miles JJ, Burrows SR, McCluskey J; Rossjohn J. The CDR3 regions of an immunodominant T cell receptor dictate the 'energetic landscape' of peptide-MHC recognition. *Nat Immunol* 2005; 6:171-80.
7. Kranz DM. T cell receptor CDRs: starring versus supporting roles. *Nat Immunol* 2005; 6:130-1.
8. Turner SJ, Kedzierska K, Komodromou H, La Gruta NL, Dunstone MA, Webb AI, Webby R, Walden H, Xie W, McCluskey J, Purcell AW, Rossjohn J, Doherty PC. Lack of prominent peptide/MHC features limits T cell receptor repertoire diversity in virus-specific $CD8^+$ T populations. *Nat Immunol* 2005; 6(4):382-9.
9. Jones YE. Favorite flavors of surfaces. *Nat Immunol* 2005; 6(4):365-6.
10. Ely LK, Green KJ, Beddoe T, Clements CS, Miles JJ, Bottomley SP, Zernich D, Kjer-Nielsen L, Purcell AW, McCluskey J, Rossjohn J, Burrows SR. Antagonism of antiviral and allogeneic activity of a human public CTL clonotype by a single altered peptide ligand: implications for allograft rejection. *J Immunol, In press.*

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Vascularization of Engineered Human Skin Equivalents

Synthetic skin grafts have been in clinical use for several years with limited success. These constructs enhance wound healing but generally fail to become stably engrafted. Graft failure does not seem associated with immunological rejection since recipients do not appear to become immunized. It is our hypothesis that delayed development of graft perfusion by host angiogenesis/vasculogenesis is a major limitation on the success of synthetic graft survival. The overall goal of this project is to develop a synthetic skin construct that is rapidly perfused following orthotopic engraftment. Our strategy for accomplishing this involves incorporating vascular endothelial cells (ECs) within the synthetic graft, allowing these cells to catalyze rapid blood vessel formation *in vivo*. Our specific aims are (1) to optimize a method for incorporation of human ECs into a synthetic skin construct; (2) to determine if inclusion of allogeneic (the host) ECs increases the immunogenicity of the graft; and (3) to determine if grafts containing ECs become perfused more rapidly than grafts which do not and if such grafts survive better than grafts lacking EC. Substantial progress has been made in the first aim and experiments are underway in pusuit of the second and third aims.

Incorporation of endothelial cells into synthetic skin grafts

Our initial approach was to use serially passaged human umbilical vein ECs (HUVECs) as a source of ECs. These cells are readily available in large quantities and are well characterized. We prepared decellularized human dermis from dermatome-harvested human cadaver skin, seeded and then differentiated human keratinocytes on the upper surface of the dermis, and then seeded HUVECs on the basal surface. Twenty-four hours later, the construct was orthotopically engrafted on to the backs of immunodeficient C.B-17 SCID/bg mice. As reported in late 2003[1], this approach sometimes resulted in invasion by the HUVECs into dermis in a manner suggestive of re-endothelialization of pre-existing vascular channels and that these re-endothelialized channels established anastomoses with host vessels present at the base of the graft. Many of these human endothelial-lined tubes acquired a coating of smooth muscle alpha-actin expressing cells derived from the host. However, success was variable in that HUVECs frequently (more than half the time) failed to become incorporated into the graft. Although HUVECs are readily obtained and easily cultured, they are relatively fragile, undergoing rapid apoptosis when suspended or deprived of serum and/or growth factor. Thus HUVEC apoptosis is a possible explanation of our frequent failures. We had previously shown that

transduction of a Bcl-2 gene with retrovirus rendered HUVECs resistant to apoptosis under many conditions that led to death of untransduced or control gene transduced cells, including survival in three-dimensional culture. Moreover, Bcl-2-transduced HUVECs appeared more adept at forming vessels when implanted into immunodeficient mice within gels formed from rat-tail type I collagen and human plasma fibronectin. A more recent study[2] showed that vessels formed from Bcl-2 transduced HUVECs are mature by a variety of criteria, including morphology, cytokine responses and permselectivity. Therefore, we also analyzed synthetic skin formed from Bcl-2-transduced HUVEC and found that this modification consistently enhanced the frequency of success in creating vascularized skin. Our current success rate approaches 100%. We also have studied a number of additional human EC types. EC isolated from dermis (human dermal microvascular endothelial cells or HDMECs) did not appear to perform better than HUVECs. Most recently, we have used magnetic beads to isolate $CD34^+$ cells from umbilical cord blood or adult peripheral blood (collected by leukapheresis from young adult volunteer donors) and differentiated these cells into endothelium in culture by addition of vascular endothelial growth factor (VEGF). EC colonies routinely grow out and, after a lag of 14-21 days, rapidly outgrow other cell populations within the initial isolation resulting in nearly homogenous EC populations. We have characterized these cells and find they uniformly express characteristic EC markers including VE-cadherin, PECAM-1, endoglin and TNF-inducible E-selectin. In other words, they are unequivocally EC. Endothelial progenitor cell-derived EC (EPC-EC) will routinely invade acellular dermis and form microvessels when incorporated into synthetic skin grafts. These findings are being prepared for publication. We have recently established conditions for retroviral transduction of EPC-EC, and we plan to determine if and how introduction of Bcl-2 affects the behaviors of such cells, specifically looking at resistance to apoptosis, formation of mature vessels *in vivo*, and efficiency of vessel formation in acellular dermis and synthetic skin.

Effects of incorporating ECs within synthetic skin constructs on graft immunogenicity

Cultured human ECs are capable of stimulating cytokine production and proliferation of allogeneic human T cells. Under similar conditions, keratinocytes, fibroblasts or smooth-muscle cells do not. EC-responsive T cells belong to the memory ($CD45RO^+$) subsets of both $CD4^+$ and $CD8^+$ T cells. It is not simple to determine if human ECs contribute to allogeneic T cell responses *in vivo* because natural tissues often contain a variety of other cell types, such as dendritic cells or macrophages, that may also serve to activate T cell responses. However, synthetic skin grafts of the sort we have developed do not contain such cell types, and we will be able to compare grafts constructed with or without human ECs. Since we have now developed methods for orthotopic transplantation of such synthetic grafts, we can use the model of allogeneic orthotopic skin-graft rejection we had previously developed involving sequential skin transplantation followed by adoptive transfer of human peripheral blood mononuclear cells or isolated peripheral blood T cells. We have just begun these experiments. Parallel experiments (supported by other grants) have shown that adoptively transferred

T cells will attack and destroy allogeneic HUVECs implanted into immunodeficient mice in collagen/fibronectin gels and that transduction of the HUVECs with Bcl-2 prevents EC death but not infiltration of the gel by adoptively transferred T cells. Thus, we will also test the effect of Bcl-2 transduction of HUVECs upon rejection of synthetic skin grafts.

Effects of incorporating ECs within synthetic skin constructs on graft perfusion and survival

Our initial report of HUVEC-containing synthetic skin grafts showed that this modification led to earlier perfusion of the grafts, especially within the central regions, assessed by injection of fluorescent dextran prior to harvest and fixation. (We also used laser Doppler measurements on grafts in the living animal but are uncertain to what extent these measurements reflect flow through the graft as opposed to flow through the underlying mouse tissues.) We interpret these findings to imply that host (mouse) vessels primarily grow into the grafts from the lateral borders and that the presence of human EC induces a parallel process of revascularization by forming conduits within the graft that connect to host vessels in the wound bed along the basal surfaces of the graft. Many potential recipients of skin grafts, namely the elderly and diabetics, show impaired angiogenesis and vasculogenesis. This may account for why synthetic skins are poorly vascularized in patients despite apparently adequate vascularization in immunodeficient mouse hosts. Ultimately, it may be advantageous to test grafts with and without ECs using diabetic or elderly mice. In the interim, we have established that rapamycin inhibits angiogenesis/vasculogenesis in immunodeficient mice, probably by blocking EC replication via mTOR inhibition. Grafts lacking human ECs do not become vascularized in rapamycin-treated mouse hosts and often ulcerate secondary to ischemia. Preliminary experiments support the conclusion that grafts containing human ECs still become vascularized in rapamycin-treated hosts. Experiments are in progress to determine if this produces a statistically significant enhancement of graft take and survival.

Plans for the final year

In the final year of the grant from the ROTRF, we plan to complete our analysis of skin grafts populated by EPC-ECs and compare these to grafts containing HUVECs; determine if Bcl-2 transduction improves the performance of EPC-ECs as it does of HUVECs; fully characterize the responses of adoptively transferred human T cells to synthetic skin grafts with and without allogeneic (to the T cells) ECs and determine whether Bcl-2 expression by the ECs has any effect on this process. Finally we will use rapamycin-treated mouse recipients to determine whether incorporation of ECs into synthetic skin constructs improves early perfusion and graft survival.

Publications

1. Schechner JS, Crane SK, Wang F, Szeglin AM, Tellides G, Lorber MI, Bothwell ALM, Pober JS. Engraftment of a vascularized human skin equivalent. *FASEB J* 2003; 17:2250-6.
2. Enis DR, Shepherd BR, Wang Y, Qasim A, Shanahan CM, Weissberg PL, Kashgarian M, Pober JS, Schechner JS. Induction, differentiation and remodeling of blood vessels following transplantation of Bcl-2-transduced endothelial cells. *Proc Natl Acad Sci USA* 2005; 102:425-30.

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Prevention of Allograft Rejection by Specific Tolerance Induction Using $CD4^+CD25^+$ Regulatory T Cells

To prevent rejection of allogeneic grafts by the immune system, immunosuppressive drugs are given chronically to transplanted patients. Although very effective, these treatments are toxic and only partially block chronic rejection. Alternative treatments are thus explored, such as the use of the $CD4^+CD25^+$ regulatory T cells (Treg). We and others have shown that Treg have the capacity to efficiently block alloreactive responses and can be used to prevent graft-versus-host disease[1-4], for which clinical protocols are being proposed. In rodent models, rejection of allogeneic skin or heart transplants can be prevented by administration of Treg[5-7]. However, these studies were performed in lymphopenic recipients and the Treg were obtained from other manipulated animals, which were previously rendered tolerant to donor allo-antigens (allo-Ags). The goal of our project is to explore the possibility of using Treg to prevent graft rejection in a context that could be applicable in a clinical situation. Recent data have shown that administration of Ag-specific Treg induces specific tolerance to this same Ag[8,9]. A rational explanation is that these specific Treg are activated locally by Ag-presenting cells presenting cognate Ag, which turns on suppressive activity of Treg since this activity is dependent on engagement of their T cell receptor[10,11]. We thus expect that prevention of allograft rejection using Treg would be effective only if the injected Treg are specifically re-activated by donor allo-Ags. A therapeutic effect may thus be achieved by injecting Treg previously selected *in vitro* to recognize specifically donor-type major histocompatibility complex (MHC) allo-Ags through the direct and/or indirect pathways. Along this line, we showed that Treg specific for recipient-type allo-Ag are superior to non-specific Treg in their capacity to prevent graft-versus-host disease[12]. In this project, we are studying the possibility to prevent allograft rejection by injecting Treg specific for donor-type allo-Ag.

Progress made on the specific aims and achieved results.

1. Capacity of Treg specific for allo-Ag presented by the direct pathway to prevent graft rejection (part of specific aim 3 of the research proposal).

BALB/c Treg specific for C3H allo-Ag presented by the direct pathway were obtained *in vitro* as previously described[2]. During the culture, Treg exhibited dramatic expansion (Fig. 1A). After four weeks of culture, Treg were highly suppressive and were indeed specific for C3H allo-Ag (Fig. 1B). We then tested the capacity of these allo-specific Treg to prevent rejection of allogeneic skin. BALB/c control mice rejected allogeneic C3H skins between 9 to 15 days after transplantation whereas syngeneic skins were not rejected. Unfortunately, the administration of 10 million BALB/c Treg specific for C3H allo-Ag had no effect since allogeneic skin was rejected at the same kinetics as in the controls (Fig. 1C). Similar findings were obtained in another genetic combination. BALB/c Treg specific for B6 allo-Ag did not prevent rejection of B6 skin grafted in BALB/c mice (not shown).

BALB/c Treg specific for C3H allo-Ag presented by the direct pathway were also tested in islet transplantation. Preliminary data suggest that injection of these Treg did not prevent rejection of allogeneic C3H pancreatic islets, grafted in BALB/c mice rendered diabetic by administration of streptozotocin (data not shown). We will perform additional experiments to confirm these findings. However, in order to obtain a therapeutic effect of allo-specific Treg in transplantation, we are now exploring other approaches described below.

2. Generation of Treg specific for allo-Ag presented by the indirect pathway (part of the specific aim 1 of the research proposal).

After transplantation, donor allo-Ag are presented to CD4 T cells by the direct and the indirect pathways, this latter pathway being predominant, if not exclusive, when organs poor in passenger leukocytes expressing MHC class II molecules such as pancreatic islets are grafted. Since Treg turn on their suppressive activity only after being activated[10,11], Treg specific for allo-Ag presented by the indirect pathway are likely better than Treg specific for allo-Ag presented by the direct pathway to prevent graft rejection. Although we did not encounter substantial difficulties in selecting these latter alloreactive Treg, probably because they represent a significant proportion of the Treg polyclonal repertoire[2,13], selecting the rare Treg specific for allo-Ag presented by the indirect pathway appeared a much more difficult task. We thus had to modify culture conditions, described below, suitable for selecting rare Ag-specific Treg. In another project aimed at generating Treg specific for pancreatic islet Ag that have a therapeutic potential in type 1 diabetes[14,15], we have set up culture conditions allowing selective expansion, among the diverse and autoreactive repertoire of polyclonal Treg, of rare Treg specific for a nominal Ag. We investigated the potential of different dendritic cell subsets to expand antigen-specific Treg in mice. The successful selective *in vitro* expansion of rare islet-specific Treg from polyclonal Treg could only be achieved by stimulation with $CD8^+$ splenic dendritic cells presenting islet antigens. This dendritic cell

subset had the unique capacity to strongly activate specific Treg in the presence of the cognate Ag while low background proliferation was obtained in the absence of exogenous Ag. We thus obtained expanded islet-specific Treg that exerted potent bystander suppression on diabetogenic T cells and prevented type 1 diabetes[16]. Using a similar culture protocol using $CD8^+$ dendritic cell as Ag presenting cells, we expect to be able to generate Treg specific for allo-Ag presented by the indirect pathway. After testing their suppressive activity and their specificity *in vitro*, we will analyse their capacity to prevent rejection of allogeneic skin and islet grafts. Co-administration of Treg specific for allo-Ag presented by the direct and the indirect pathways could further increase the chance to reach our goal.

3. *In vivo* survival and activation of Treg specific for allo-Ag (part of specific aim 2 of the research proposal).

We have shown that Treg specific for an islet Ag are specifically reactivated and expanded in lymph nodes draining the pancreas, exerting potent and local bystander suppression[16,17]. This suggests that alloreactive Treg would be strongly activated and have increased survival in the presence of their cognate allo-Ag. To test this hypothesis, BALB/c Treg specific for C3H allo-Ag, and control BALB/c Treg specific for third party C57BL/6 allo-Ag, were transferred in non-irradiated (BALB/c x C3H)F1 mice. Compared to the latter Treg, the former specific Treg had increased activation and expansion over 30 days after adoptive transfer. We are now analysing whether allo-specific Treg would be specifically activated in lymph node draining skin or islet allografts or within the transplants. It will be important to analyse if there is any correlation between the duration of a putative expansion of specific Treg in these tissues and their possible therapeutic effects. We are also testing other culture conditions, such as low IL-2 concentration or addition of IL-15 or TGF-β, to increase survival of Treg *in vivo* without success so far (data not shown).

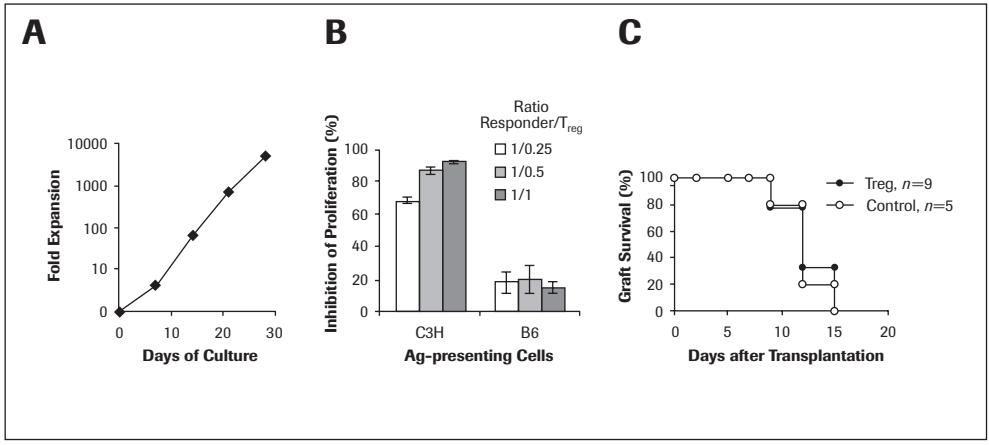

Figure 1. *Treg specific for allo-Ag presented by the direct pathway were obtained after stimulation of highly purified BALB/c Treg by allogeneic splenocytes from C3H mice, as previously described[2]. **(A)** Living cells, counted every week, exhibited dramatic expansion in the culture. After four weeks of culture, expanded Treg were harvested seven days after the last restimulation for in vitro and in vivo analyses **(B, C). (B)** The suppressive activity and specificity of Treg were tested in vitro. Expanded Treg, co-cultured at the indicated ratio with BALB/c CD25-depleted responder T cells, were stimulated by irradiated C3H or third party C57BL/6 splenocytes. Since suppression by Treg requires activation via their T cell receptor[10,11], the fact that significant inhibition of T cell proliferation was obtained in the presence of C3H Ag-presenting cells but not C57BL/6 Ag-presenting cells indicated that expanded Treg were indeed specific for C3H allo-Ag. **(C)** Expanded C3H-specific Treg were analyzed for their capacity to prevent allogeneic skin rejection. BALB/c or C3H tail skins were grafted on the back of anesthetized BALB/c mice. The same day, mice were not injected (control) or injected intravenously with 10 million C3H-specific Treg. Mice were then examined every two-three days for determination of the kinetics of graft rejection. The administration of Treg specific for C3H allo-Ag presented by the direct pathway did not delay rejection of C3H skin grafts. Data were pooled from two independent experiments. All syngeneic BALB/c skin grafts were accepted (not shown).*

Publications

1. Taylor PA, Lees CJ, Blazar BR. The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 2002; 99:3493-9.
2. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) Immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med* 2002; 196:401-6.
3. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donortype CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 2002; 196:389-99.
4. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of allopeptide-specific human $CD4^+CD25^+$ regulatory T cells *ex vivo*. *Blood* 2003; 102:2180-6.
5. Kingsley CI, Karim M, Bushell AR, Wood KJ. $CD25^+CD4^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol* 2002; 168:1080-6.
6. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance. *J Immunol* 2002; 168:5558-65.
7. Chiffoleau E, Beriou G, Dutartre P, Usal C, Soulillou JP, Cuturi MC. Role for thymic and splenic regulatory $CD4^+$ T cells induced by donor dendritic cells in allograft tolerance by LF15-0195 treatment. *J Immunol* 2002; 168:5058-69.

8. Klein L, Khazaie K, von Boehmer H. *In vivo* dynamics of antigen-specific regulatory T cells not predicted from behavior *in vitro*. *Proc Nat Acad Sci USA* 2003; 100:8886-91.
9. Lee MK 4th, Moore DJ, Jarrett BP, Lian MM, Deng S, Huang X, Markmann JW, Chiaccio M, Barker CF, Caton AJ, Markmann JF. Promotion of allograft survival by $CD4^+CD25^+$ regulatory T cells: evidence for *in vivo* inhibition of effector cell proliferation. *J Immunol* 2004; 172:6539-44.
10. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998; 10:1969-80.
11. Thornton AM, Shevach EM. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J Immunol* 2000; 164:183-90.
12. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, Cohen JL. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest* 2003; 112:1688-96.
13. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193:1285-94.
14. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA. *In vitro*-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 2004; 199:1455-65.
15. Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. $CD25^+CD4^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* 2004; 199:1467-77.
16. Fisson S, Djelti F, Trenado A, Billiard F, Liblau R, Klatzmann D, Cohen JL, Salomon BL. Therapeutic potential of self-antigen-specific $CD4^+CD25^+$ regulatory T cells selected *in vitro* from a polyclonal repertoire. *Submitted for publication.*
17. Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL. Continuous activation of autoreactive $CD4^+CD25^+$ regulatory T cells in the steady state. *J Exp Med* 2003; 198:737-46.

Prof. Ann M. Simpson, Principal Investigator
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Dr. Bronwyn O'Brien, Co-Investigator

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Correction of Diabetes Using Primary Liver Cells

Background

Type I diabetes mellitus is caused by the autoimmune destruction of pancreatic β cells. Current treatment of the disease requires daily insulin injections to control blood glucose levels. Despite rigid maintenance and monitoring of blood glucose levels, the chronic complications of diabetes still develop. Transplantation of pancreatic tissue is restricted by the scarcity of donors and requires patients to be immunosuppressed. This problem could be overcome by genetically engineering an "artificial β cell" to mimic the function of pancreatic cells. The target cells in this proposal are liver cells which express glucokinase and the glucose transporter GLUT 2, as do pancreatic β cells. Studies by our group have shown that the introduction of the insulin gene into two liver cell lines (HEP G2ins/g and Huh7ins)[1,2] has resulted in synthesis, storage and regulated secretion to glucose and other metabolic substances, and correction of diabetes in a mouse model. This ability to store insulin is linked to the expression of a β cell transcription factor. Using an efficient viral transduction system we have established that delivery of a modified insulin and a β cell transcription factor directly into the liver of streptozotocin (STZ)-diabetic rats, resulted in storage of insulin, normal glucose response curves and reversal of diabetes for eight months.

Aims

Using our efficient viral transduction system we have three aims:

1. To determine whether the expression of insulin alone and together with the β cell transcription factor, induces storage of insulin and permanent reversal of diabetes in non-obese diabetic (NOD) mice (0-36 months);
2. To determine whether we can achieve better physiological control of insulin production from the livers of NOD mice, if we transfer insulin under the control of a glucose- and insulin-sensitive promoter (13-36 months);
3. To determine whether the insulin-secreting cells in NOD mice are subject to autoimmune attack (10-36 months).

Research Outcomes

The following report is for the first year of a 3-year grant (approximately 9 months since obtaining actual funding and starting work) thus we have been exclusively working on aim 1.

1. Reversal of diabetes in NOD mice:

a) Determination of correct vector concentration:

As mentioned above our initial studies were in a STZ-induced diabetic rat model. Therefore, we firstly needed to determine the optimum concentration of vector for transduction of NOD mice. At the commencement of the project Dr. Ren (Research Fellow) spent some time adapting the unique surgical procedure that she has developed in the Wistar rat model to the much smaller NOD mouse. Once she had accomplished this, we transduced the mice with 5 concentrations of vector (based on earlier experiments), which expresses enhanced green fluorescent protein (EGFP). We then determined by flow cytometry and confocal microscopy the most appropriate concentration of vector to obtain 70% transduction of the liver tissue, which is comparable to the percentage of transduced cells following transduction with the vector containing EGFP in the rat model (data not shown). Figure 1 illustrates NOD mouse liver tissue 1 month after transduction with EGFP. A large number of cells (approximately 70%) express EGFP. This concentration of vector has been used in later studies.

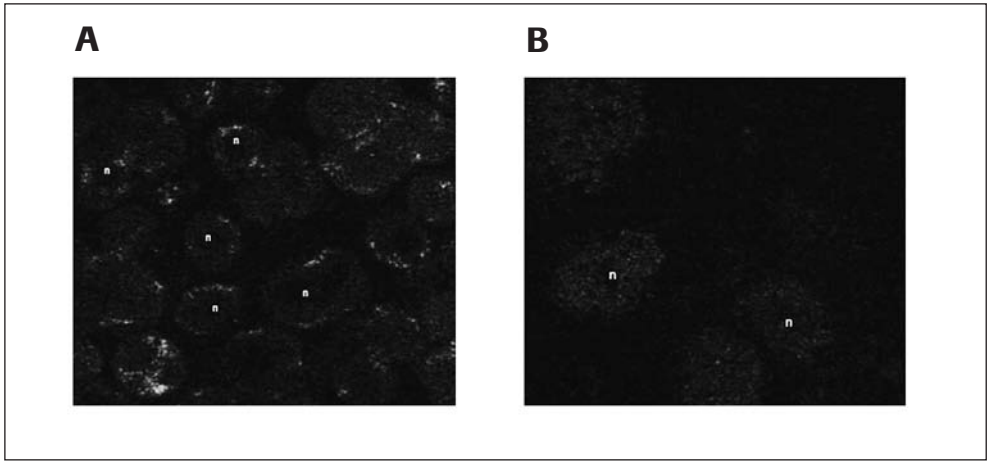

Figure 1. NOD mouse liver tissue one month after transduction with EGFP. *Confocal analysis of EGFP expression recorded from NOD mouse hepatocytes isolated by collagenase perfusion from the liver of an animal transduced by* ***(A)*** *the viral vector containing the EGFP gene and* ***(B)*** *saline alone, one month after initial transduction. It is obvious from this figure that stable expression of EGFP is visible in a large proportion of cells, with no staining of the nucleus (n). There is a small amount of autofluorescence present in the control.*

b) Delivery of the insulin cassette to diabetic NOD mice:

Female NOD mice obtained from the Australian Research Council facility were used in the study. These mice are characterised by insulitis and a marked decrease in pancreatic content, which occurs between 12-18 weeks of age in females. The onset of diabetes is marked by glucosuria and non-fasting blood glucose levels exceeding 11 mM on two subsequent occasions. The mice for these preliminary studies were divided into three groups. One group were untreated controls, one group received empty vector and one group received the insulin cassette (data not shown), $n=3$ per group. Blood glucose levels were determined by tail-vein pricks and glucometer measurement. The mice were followed for a period of two months.

Figure 2A illustrates the pancreas of a NOD mouse before the onset of diabetes at approximately 6 weeks of age. There is a significant number of insulin-containing cells and limited lymphocytic infiltration. By contrast, Fig. 2B is a section of a pancreas from a NOD mouse with blood glucose >11 mM. No appreciable positive staining can be seen for insulin and significant lymphocytic infiltration into the islets. This is typical of all pancreatic tissue of animals used for experimentation. No indication of pancreatic regeneration was seen in animals treated with the insulin cassette after two months.

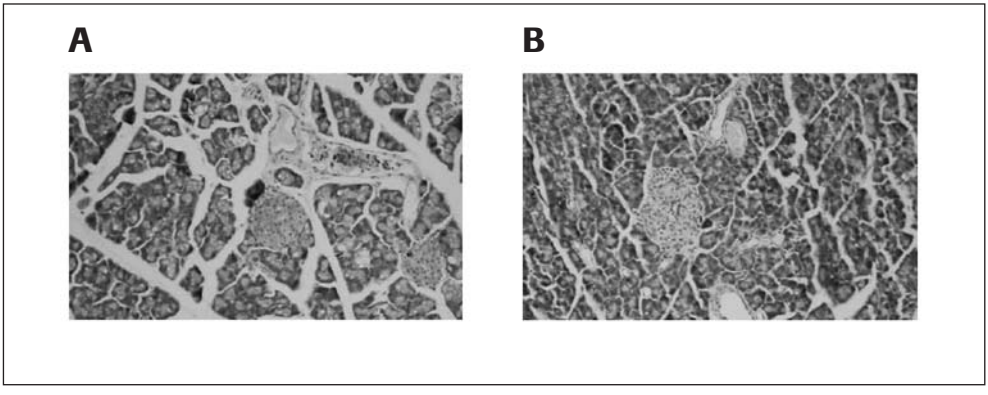

Figure 2. The pancreas of a NOD mouse before the onset of diabetes. *Photomicrographs of* ***(A)*** *a section of the pancreas of a NOD mouse before the onset of diabetes as judged by blood glucose level and* ***(B)*** *a section of the pancreas from a diabetic NOD mouse. After immunohistochemistry for insulin using immunoperoxidase, positive cells appeared brown. Original magnification = 200 X.*

Figure 3A represents a section of liver from a NOD mouse that has been transfected with the empty vector after immunohistochemistry for insulin. There is no evidence of insulin storage in the animals. By comparison it can be seen in Fig. 3B, which is a section of a liver from an animal transduced with the insulin cassette, that a large number of the cells in the liver have been converted into cells that stain positively for insulin. Unlike other studies that have engineered β cell neogenesis from liver cells[3,4], the insulin-containing cells are not isolated to the area surrounding the portal circulation, but are scattered throughout the liver tissue. Tissue has been collected and fixed for electron and immuno-electron microscopy studies, to establish whether or not the insulin is stored in granules as it is in pancreatic β cells, but the results have not been processed as yet. Samples have also been taken to measure insulin storage by acid/ethanol extraction.

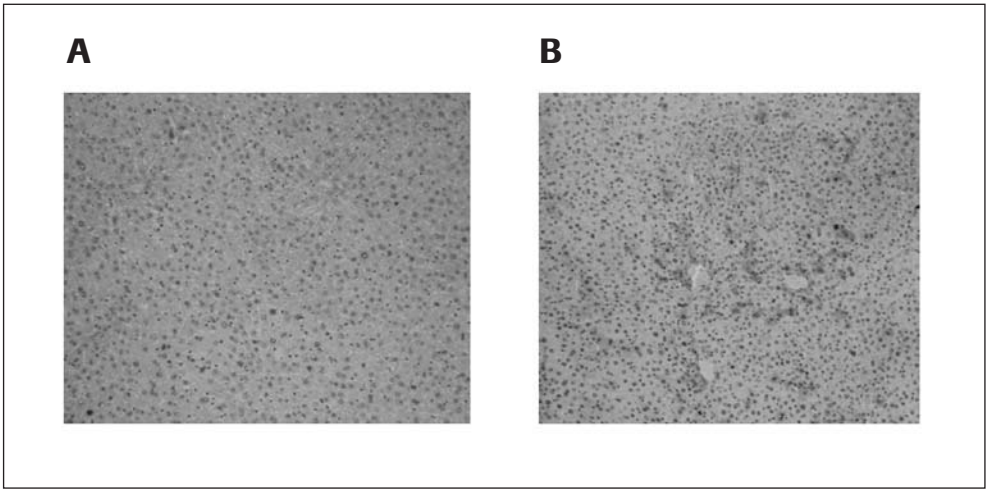

Figure 3. Section of liver from a NOD mouse. *Photomicrographs of* ***(A)*** *a section of the liver of a NOD mouse transduced with the viral vector alone; sacrificed 2 weeks after transduction due to high blood glucose levels;* ***(B)*** *A section of the liver of a NOD mouse transduced with the viral vector containing the insulin cassette for a period of 2 months. After immunohistochemistry for insulin using immunoperoxidase, positive cells appeared brown. Original magnification = 200 X.*

The reversal of diabetes is seen almost immediately in the animals transduced with the insulin cassette and normal blood glucose was maintained for two months, when the animals were sacrificed (Fig. 4). By comparison, the blood glucose levels of the untransduced diabetic control animals continued to increase and these animals were sacrificed at much earlier time points. Likewise, the blood glucose of animals transduced with the empty vector continued to increase similarly to the diabetic control animals (data not shown).

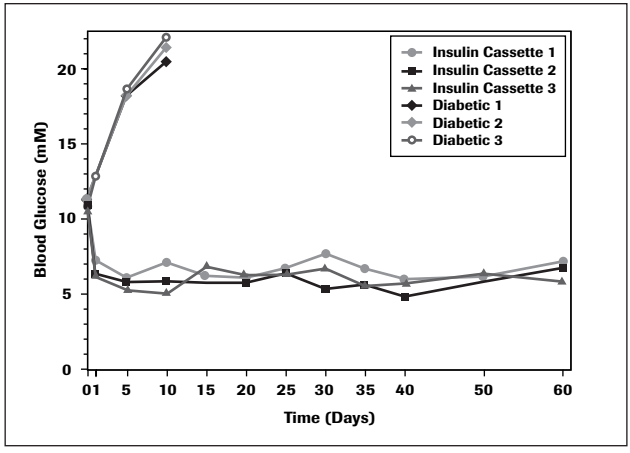

Figure 4. Serum glucose of NOD mice treated with the vector containing the insulin cassette or untreated (diabetic). *Blood glucose levels fell rapidly to normal levels on addition of the insulin cassette and normoglycaemia was maintained for two months when the animals were sacrificed. Mice treated with the vector alone (results not shown) or untreated remained diabetic until sacrifice.*

Figure 5 illustrates the results of an intravenous glucose tolerance test following a 16 hour (960 min) fast. It can be seen that the blood glucose levels of animals transduced with the insulin cassette returned to normal within 30 minutes, whilst the blood glucose of diabetic control animals remained at diabetic levels throughout. By comparison it took non-diabetic control animals with original blood glucose levels between 5-6 mM, 50 minutes for blood glucose levels to normalise. This was most likely due to the fact that whilst these animals exhibited normal blood glucose levels, the autoimmune destruction of the β cells had commenced, thereby reducing the β cell population. In the NOD mouse model, animals develop insulitis from 5 weeks of age with the onset of diabetes occurring asynchronously in female mice from 12 weeks of age. Therefore, the ability that such pre-diabetic mice have to normalise blood glucose levels after glucose challenge may be reduced. Therefore, in subsequent experiments we plan to also use age-matched non-obese resistant (NOR) mice as control animals for intravenous glucose tolerance tests. The NOR mouse is a NOD-related syngenic recombinant strain that possesses approximately 12% of C57Bl/KsJ-derived genes resulting in resistance to invasive insulitis, β cell destruction and diabetes.

Samples have been collected for further immunohistochemistry looking for evidence of general pancreatic transdifferentiation such as glucagon and somatostatin expression in the liver of transduced animals. Samples have also been taken from a range of body tissues for PCR analysis to determine if insulin expression is solely limited to the liver as was the case in the rat model. The expression of β cell transcription factors in the liver will also be examined. Blood samples have been collected to measure insulin, c-peptide and general liver function tests as outlined in the proposal. Histological samples of liver have also been prepared to look for the beginning of autoimmune attack in the liver, but thus far there is no evidence of this from the preliminary work that has been completed.

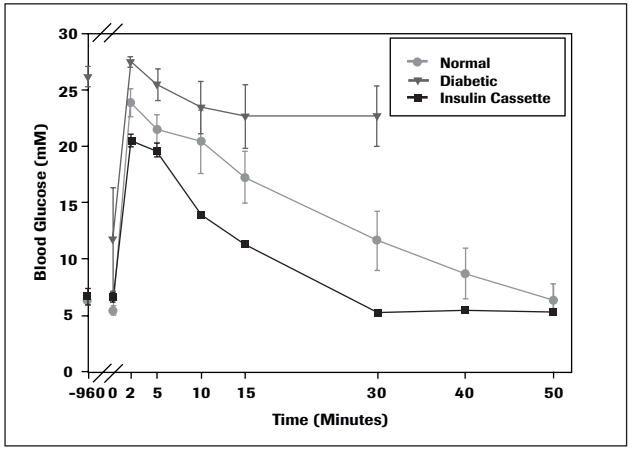

Figure 5. Intravenous glucose tolerance test. *After an overnight fast on non-diabetic control mice, diabetic mice and diabetic mice treated with insulin cassette two months previously. The animals were given an intravenous dose of glucose (0.5 g/kg body weight) at time zero, blood glucose levels were measured over 30-50 minutes (n=3).*

Whilst we need many replicates, which are underway, early indications are that we have accomplished the stable reversal of autoimmune diabetes by the transduction of only the insulin cassette and have maintained expression for two months. In the following year we will continue to work on aim 1 and commence aims 2 and 3 as detailed in the original proposal.

Publications

1. Simpson AM, Marshall GM, Tuch BE, Maxwell L, Szymanska B, Tu J, Beynon S, Swan MA, Camacho M. Gene therapy of diabetes: glucose-stimulated insulin secretion in a human hepatoma cell line (HEP G2ins/g). *Gene Ther* 1997; 4:1202-15.
2. Tuch BE, Szymanska B, Yao M, Tabiin MT, Gross DJ, Holman S, Swan MA, Humphrey RK, Marshall GM, Simpson AM. Function of a genetically modified human liver cell line that stores, processes and secretes insulin. *Gene Ther* 2003; 10:490-503.
3. Ber I, Shternhall K, Perl S, Ohanuna Z, Goldberg I, Barshack I, Benvenisiti-Zarum L, Meivar-Levy I, Ferber S. Functional, persistent, and extended liver to pancreas transdifferentiation. *J Biol Chem* 2003; 278:31950-7.
4. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat Med* 2003; 9:596-603.

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Activation of Macrophages and Endothelium by Non-Complement Fixing Antibodies in Allograft Rejection

Antibody-mediated rejection is clinically critical because this form of rejection is usually unresponsive to conventional anti-rejection therapy. Therefore, it has recently been recognized as a major cause of allograft loss. We developed a mouse model, immunoglobulin knockout (IgKO), that demonstrates a critical role of alloantibodies in acute allograft rejection. Cardiac allografts survive significantly longer in IgKO than in wild-type (WT) recipients.

Passive transfer experiments documented that different subclasses of IgG monoclonal antibodies against donor MHC class I antigens have different capacities to reconstitute allograft rejection in IgKO recipients. Mouse IgG1 antibodies that do not activate complement were found to interact with complement-activating antibodies to cause rejection of cardiac allografts. Parallel *in vitro* experiments revealed that IgG1 up-regulates chemokine production by endothelial cells and proinflammatory cytokine production by macrophages. These findings led us to the hypothesis that non-complement-activating IgG1 augments graft injury by stimulating endothelial cells through cross-linking antigens and by stimulating macrophages through their Fc receptors (FcR). On the other hand, complement-activating antibodies have the additional capacity to activate macrophages and T cells through their complement receptors (CR).

During the first year we made progress on Specific Aim 1:

Macrophage activation via engagement of FcγRI and FcγRIII by alloantibodies will be tested *in vitro* with macrophages from FcR KO mice.

Studies and results

This experimental aim is based on two of our preliminary findings: 1) IgG1 antibodies that bind to MHC class I stimulate endothelial cells to produce the chemokines MCP-1, IL-6 and RANTES; and 2) addition of macrophages to these cultures of IgG1 antibodies and endothelial cells results in the production of large quantities of IL-1-α, IL-6 and small amounts of TNF-α.

We developed two parallel models using mouse and human transformed endothelial cell populations in order to investigate mechanisms of antibody-mediated endothelial cell injury.

We performed preliminary experiments on mouse endothelial cells sensitized with 50 μg/ml of IgG1+IgG2b anti-MHC mAbs and co-cultured for 48 hours with C57BL/6 splenocytes or lymph node cells primed *in vitro* by a seven-day MLR with B10.A splenocytes or *in vivo* by six days exposure to a B10.A cardiac allograft. The initial screen of culture supernatants in protein macroarray assays revealed that endothelial cells sensitized with IgG1+IgG2b mAbs co-cultured with alloantigen primed splenocytes or lymph node cells expressed high levels of MCP-1, KC, IL-1, IL-6 and lower levels of RANTES. MCP-1 and IL-6 were further quantified in ELISA. In cultures of endothelial cells with splenocytes primed by B10.A alloantigen *in vitro* and *in vivo*, the presence of IgG1+IgG2b mAbs caused a 1.5-7 fold (*in vitro* vs. *in vivo* stimulation) increase of MCP-1 (Figs 1A, 2A) and 5-17 fold (*in vitro* vs. *in vivo* stimulation) increase of IL-6 (Figs 1B, 2B).

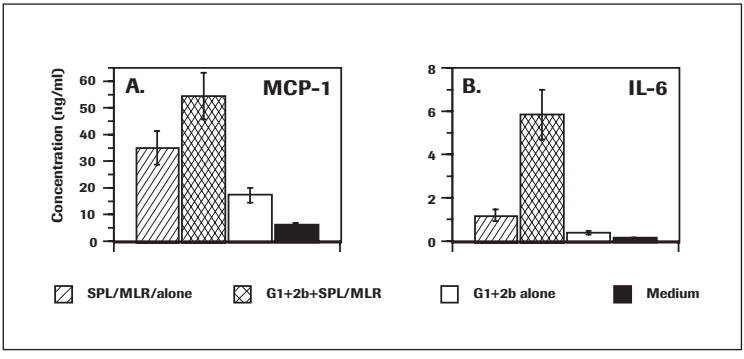

Figure 1. The effect of IgG1 and IgG2b mAbs on MCP-1 (A) and IL-6 (B) production in 48 hour co-cultures of SVEC4-10 endothelial cells and C57BL/6 splenocytes primed in vitro by B10.A splenocytes in 7-day MLR.

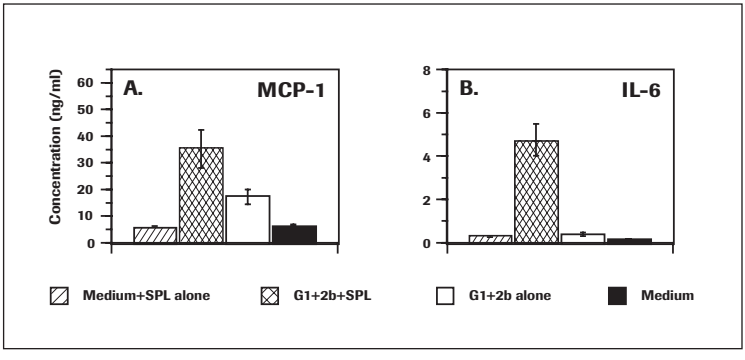

Figure 2. The effect of IgG1 and IgG2b mAbs on MCP-1 (A) and IL-6 (B) production in 48 hour co-cultures of SVEC4-10 endothelial cells and C57BL/6 splenocytes primed in vivo by 6 days exposure to a B10.A cardiac allograft.

The pattern of MCP-1 and IL-6 production in cultures of endothelial cells sensitized with anti-MHC IgG1+IgG2b mAbs and co-cultured with lymph node cells primed by B10.A cardiac allograft *in vivo* was very similar to cultures with primed splenocytes (Fig. 3).

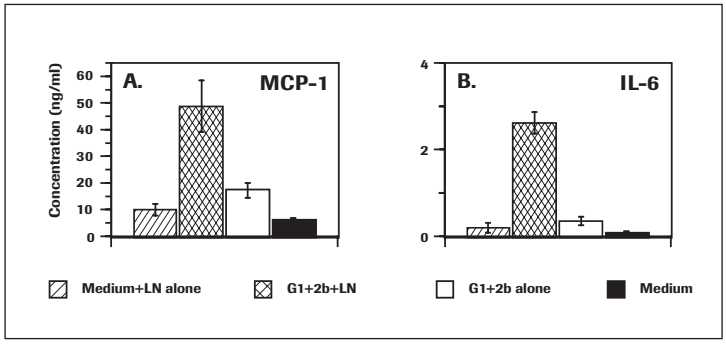

Figure 3. The effect of IgG1 and IgG2b mAbs on MCP-1 (A) and IL-6 (B) production in 48 hour co-cultures of SVEC4-10 endothelial cells and C57BL/6 lymph node cells primed in vivo by 6 days exposure to a B10.A cardiac allograft.

These data suggest that both allospecific mAbs and alloantigen-primed splenocytes or lymph node cells played a role of triggering factors in cultures of endothelial cells that result in the stimulation of MCP-1 and IL-6.

Mechanistically these findings can result from several possible interactions among macrophages, IgG1 and endothelial cells. One possibility is that the IgG1 antibodies are only necessary to stimulate endothelial cells to produce MCP-1 and this chemokine activates the macrophages to produce IL-6. Another possibility is that macrophage activation requires engagement of FcR by IgG1 alloantibodies in addition to stimulation by MCP-1. The role of Fc-FcR interactions was tested in mouse and human models with macrophages from FcγRIII KO mice and human macrophage cell line U 937 expressing FcγRI. All mouse IgG subclasses can bind to FcγRI and FcγRIII receptors. The affinity of binding different IgG subclasses to FcgRIII is shown in Table 1.

Receptor	The affinity of binding
FcγRI	10^{7}-10^{8} M^{-1} for IgG2a>>IgG1>IgG2b
FcγRIII	<10^{7} M^{-1} for IgG1>Ig2a>IgG2b

Table 1. The affinity of binding different IgG subclasses to FcγRIII.

Mice with a genetic mutation of the γ chain have impaired expression of FcγRIII and exhibit impaired antibody-mediated responses, including loss of macrophage-mediated antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis. We tested cultures of endothelial cells with macrophages from C57BL/6 WT and FcγRIII KO mice.

Based on the assumption that FcγRIII binds IgG1 very strongly (see the table above), we ask the question whether FcγRIII expressed by lymphoid cells is involved in IgG1-mediated endothelial cell injury. To this end we performed experiments on mouse endothelial cells sensitized with 50 μg/ml of IgG1 anti-MHC mAbs and co-cultured for 48 hours with C57BL/6 WT and FcRγIII KO splenocytes or lymph node cells primed *in vivo* by a 10-day exposure to B10.A skin transplants. The initial screen of culture supernatants in protein macroarray assays revealed that endothelial cells sensitized with IgG1 mAbs co-cultured with alloantigen primed splenocytes or lymph node cells expressed high levels of MCP-1, KC, and IL-6. MCP-1 and IL-6 were further quantified in ELISA. In cultures of endothelial cells sensitized with 50 μg/ml of IgG1 anti-MHC mAbs and co-cultured for 48 hours with lymph node lymphocytes or splenocytes from FcR KO mice primed by the exposure to B10.A skin allografts, the levels of both MCP-1 and IL-6 were significantly decreased in FcR KO mice compared to cultures of endothelial cells in the presence of lymphoid cells from WT skin recipients. (Figs 4,5).

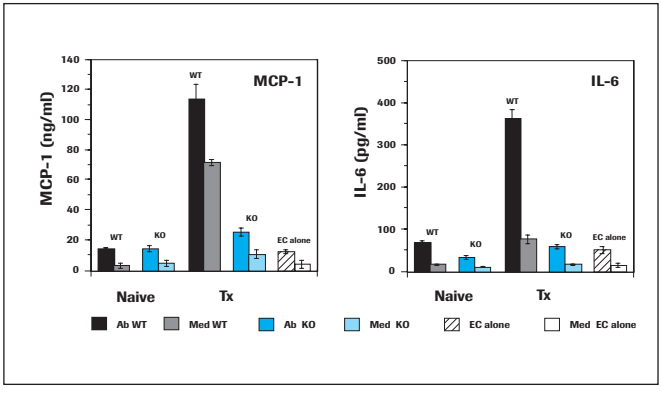

Figure 4. The effect of IgG1 mAbs on MCP-1 and IL-6 production in 48 hour co-cultures of SVEC4-10 endothelial cells with WT or FcRIII KO C57BL/6 lymph node cells primed in vivo by 10 days exposure to a B10.A skin allograft.

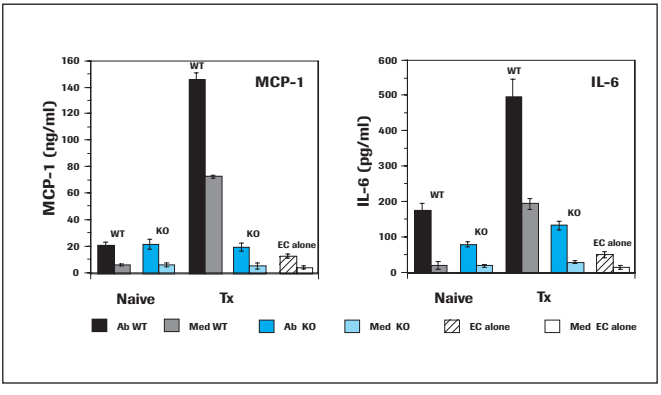

Figure 5. The effect of IgG1 mAbs on MCP-1 and IL-6 production in 48 hour co-cultures of SVEC4-10 endothelial cells with WT or FcγRIII KO C57BL/6 splenocytes primed in vivo by 10 days exposure to a B10.A skin allograft.

We also performed experiments on human brain microvascular cells (BMEC) sensitized with mouse anti-HLA mAb W6/32 (IgG2a) and co-cultured for 48 hours with human macrophages U937 stimulated for 24 hours with IFN-γ. IgG2a binds strongly to high affinity receptor FcγRI. Resting U937 macrophages do not express FcγRI. In contrast, U937 macrophages stimulated for 24 hours with IFN-γ demonstrate 10-log channel increase in intensity of staining for FcγRI. The presence of U937 macrophages expressing FcγRI in cultures with BMEC cells sensitized with mouse anti-HLA Ab caused significant downregulation of MCP-1 and IL-6 production compared to cultures with resting macrophages.

The experiments described above and performed in mouse and human endothelial cell models represent two lines of evidence suggesting that FcγRIII and FcγRI are involved in IgG1 and IgG2a-mediated endothelial cell injury, respectively.

We also performed some preliminary experiments relative to Specific Aim 2 regarding the expression of Cr1/2 receptor on alloantigen stimulated cells. Our findings indicate that non-complement-activating anti-MHC mAbs can augment injury to allografts by complement-activating anti-MHC mAbs. Based on the hypothesis that activated complement can interact with macrophages and T cells through complement receptors, we measured the expression of CR1/2+ receptor on alloantigen-stimulated T cells.

CR1/2 receptor was identified in $CD8^+$ and $CD4^+$ splenocytes stimulated in seven-day MLR, and in parathymic lymph nodes and spleens of C57BL/6 recipient exposed for six days to B10.A cardiac transplant. An additional 24-48 hours co-culture of these cells with endothelial cells sensitized with IgG1 and IgG2b anti-MHC mAbs increased the percentage of $CD8^+/CR1/2^+$ cells in MLR splenocytes (36% vs. 56%) and in parathymic lymph nodes (11% vs. 45%). There was no further expansion of $CD4^+$ cells expressing CR1/2 in cultures with endothelial cells. The expression of this receptor on alloantigen-primed cells would allow the interaction with split products of C3 (C3b, iC3b, C3dg and C3d) that covalently attached to the membrane and lead to further activation of lymphoid cells.

Publications

1. Qian Z, Loghmani M, Bieler J, Baldwin III WM, Wasowska BA. Antibody-mediated pro-inflammatory functions of endothelial cells, macrophages and T cells. *Transpl Proc* 2005; 37:32-4.
2. Qian Z, Loghmani M, Bieler J, Baldwin III WM, Wasowska BA. Antibody-mediated pro-inflammatory functions of endothelial cells, macrophages and T cells. *Transplantation* 2004; 78(2):561; (Abstract).
3. Wasowska BA, Qian Z, Bieler J, Baldwin III WM. Anti-MHC antibodies stimulate pro-inflammatory functions of endothelial and lymphoid cells. *25th Meeting of The International Society for Heart and Lung Transplantation* 2005; (Abstract).

5. Final Reports of ROTRF Grantees

Dr. William Burlingham, Principal Investigator
Prof. Hans Sollinger, Co-Investigator

University of Wisconsin Medical School, Madison, USA

Delayed-Type Hypersensitivity Status and Mycophenolate Mofetil Monotherapy

Hypothesis and Specific Aims

Hypothesis:

Successful withdrawal of immunosuppression in renal transplant patients depends on development of stable regulation to donor antigens, whereas patients who fail (i.e. reject after withdrawal) are likely to be those with evidence of sensitization to donor antigens by DTH assay.

Specific Aims:

One specific aim was proposed, namely to retrospectively monitor patients >55 years old undergoing steroid withdrawal for regulated delayed-type hypersensitivity (DTH) responses to their donor as part of a staged transition to mycophenolate mofetil (MMF) monotherapy from triple [steroid/cyclosporine (CyA)/MMF] therapy.

Results to date:

The trial has enrolled 30 patients to date, 20 in the steroid withdrawal arm and 10 in the control arm, out of a target enrollment of 75 (50 withdrawals, 25 controls). There have been three rejection events, all in the withdrawal group, and all in the living unrelated donor (LURD) patient subset, those receiving kidney transplants from spouse, friend or other unrelated living donor.

DTH findings were consistent with the development of anti-donor sensitization in all three cases of rejection in the withdrawal arm:

- In one case, DTH sensitization developed after the 6-month steroid withdrawal internally in a LURD patient, who subsequently went on to reject 2 months later and was returned to steroid therapy.
- In another case, the patient underwent steroid withdrawal, showing no change in DTH status (non-regulator) but experienced CyA nephrotoxicity and was put back on steroids and withdrawn from CyA for clinical reasons. One month later the patient experienced rejection and was found at that time to have developed anti-donor DTH sensitization.

- The third case was found to be a non-regulator at the time of enrollment; partially through the steroid withdrawal process (2 months), the patient developed a mixed acute cellular and antibody-mediated rejection. At this time the DTH was positive for anti-donor sensitization.

An interesting aspect of the ROTRF-sponsored research is that as a group LURD RTx recipients are poorly regulated toward their donors, as compared with either LRD haplotype-mismatched, or with deceased donor (DD), HLA-mismatched transplants[1]. This appears to be due to the high incidence of DR matching in our DD RTx program, and the lack of any such matching attempt in the LURD patients. The long-term consequences of low regulation in this latter patient group may be that maintenance levels need to remain higher in LURD than in LRD and/or DR-matched DD RTx recipients.

Publications

1. Jankowska-Gan E, Sollinger HW, Pirsch JD, Radke N, Lillesand H, Janus H, Schmidt M, Burlingham WJ. Recipients of kidney transplants from living unrelated donors (LURD) have a low incidence of regulated anti-donor DTH response. *Am J Transpl* 2005; 5(11):452; (Abstract).

Other publications resulting from this ROTRF grant support

2. Rodriguez DS, Jankowska-Gan E, Haynes LD, Leverson G, Munoz A, Heisey D, Sollinger HW, Burlingham WJ. Immune regulation and graft survival in kidney transplant recipients are both enhanced by human leukocyte antigen matching. *Am J Transpl* 2004; 4:537-43.

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Generation of Foxp3-Transduced CD4$^+$ Suppressive T Cells for Induction of Tolerance in Transplantation

Introduction

One of the major aims of research in the field of transplantation is to control the reaction of the immune system after grafting, to avoid graft reject or graft-versus-host disease (GVHD). To date, this can be achieved by the use of immunosuppressive drugs such as cyclosporine, methotrexate and /or corticosteroid. However, in spite of a significant increase of survival, these treatments are only partially effective and graft rejection or GVHD finally occur.

Thus, the ability to induce a state of immune tolerance that would alleviate the need for immunosuppression and that would decrease the occurrence of late allograft failure represents a major goal in the field of transplantation.

Recently, it has been demonstrated that a sub-population of T cell, the CD4$^+$CD25$^+$ regulatory T cell (Treg) can be used in mice to induce specific tolerance in the case of solid organ transplants or in GVHD. This approach needs high number of Treg to obtain a therapeutic effect.

The major goal of our project is to generate, entirely *ex vivo*, alloantigen (allo-Ag)-specific suppressive T cells (Tsup) that could be used to confer specific tolerance to allogeneic transplants. The present project relies on the association of cell and gene therapies. We first want to select *ex vivo* allo-Ag-specific cells from a starting population of CD4$^+$CD25$^-$ purified T cells by culturing them in the presence of allo-Ags. Combined with a phase of transduction with the Foxp3 gene described to confer suppressive activity in T cells, our goal is to generate allo-specific Tsup that will share with Treg the ability to efficiently and specifically suppress conventional alloreactive T cells without inducing general immunosuppression.

Achieved goals

Constructions

The first step of our work was to achieve the construction and the production of the lentiviral vector. We constructed a bicistronic lentiviral vector carrying the Foxp3 gene and the Thy1.1 reporter gene on both sides of the internal ribosome entry site (IRES), under the control of the regulatory sequences of the human cytomegalovirus (hCMV) promoter. At the same time, we constructed a monocistronic lentivirus carrying only the reporter gene Th-1.1 as control.

Functionality of the transgenes

Viral supernatants were generated by transient transduction of the human embryonic kidney epithelial cell line 293T with these lentivirus constructs. Lentiviral supernatant were collected and concentrated 100 times. These supernatants (titer $>10^8$) were tested on 293T cell line. The expression of the transgene was followed during 15 days by flow cytometry upon the membrane expression of the Thy-1.1 reporter gene. We observed a high level expression of the reporter gene on infected 293T cells with the Fox-Thy-1.1 construct. At day 15, Thy-1.1 expression was maintained but to a lower level. Similar observations were made using the monocistronic construction.

PCR on transduced 293T cells were performed at days 2 and 15 to verify the virus integration. We found a high expression of the transgene even after 15 days of culture.

Allogeneic activation of T cells

We tested different conditions of activation of T cells and different kinetics after allogeneic stimulation. We observed that at day 5 T cells highly divided and most divided cells expressed the CD25 activation marker, while only half of them were $CD69^+$ (data not shown). Thus, CD25 is a good candidate to select activated T cells, and, consequently cells that respond to the allogeneic stimulation.

Transduction of allogeneic T cell: preliminary results

In order to select $CD4^+$ conventional T cells specific for allo-Ags, highly purified $CD4^+CD25^-$ T cells isolated from C57Bl/6 mice were cultured in the presence of the same number of irradiated allogeneic splenocytes (allo-sp) from BALB/c mice. At day 5, allo-specific T cells were isolated by immunomagnetic selection based on the membrane expression of the CD25 activation marker. After 4 days of culture in the presence of IL-2, cells were infected by lentiviral supernatant with a MOI of 20. Twenty-four hours after the transduction, 97% of T cells expressed the Thy-1.1 reporter gene (Fig. 1).

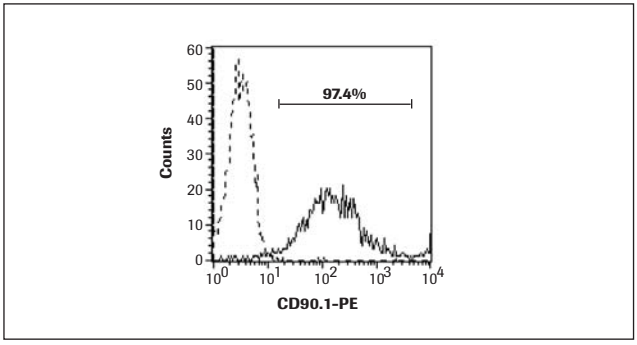

Figure 1: Expression of the reporter gene by the murine T cells transduced with the bicistronic lentivirus (black line) in comparison with non-transduced T cells (dashed black line).

The transduced cells expressed high levels of CD25 (78%). To increase the number of transduced T cells, we performed an additional round of stimulation with allogeneic irradiated splenocytes (ratio 1 T cells/20 splenocytes). After 8 days culture, transduced cells highly proliferated (from 1 x 10^6 to 25 x 10^6; Fig. 2). The percentage of CD25-expressing cells was maintained along the culture.

Non-transduced cells stimulated with the same protocol proliferate less than the transduced ones.

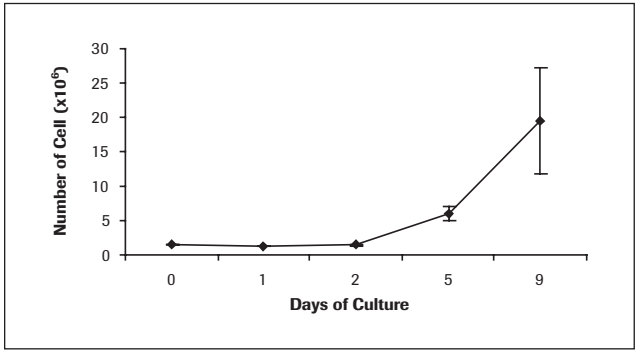

Figure 2: Proliferation of transduced T cell with Foxp3-ires-thy1.1.

To test the suppressive properties of transduced T cells, the proliferation of C57BL/6 conventional T cells stimulated by BALB/C irradiated splenocytes was compared to the proliferation observed when increasing amounts of transduced T cells were added to the co-culture. The transduced T cells population inhibited the proliferation of conventional T cells in a dose-dependent manner (Fig. 3).

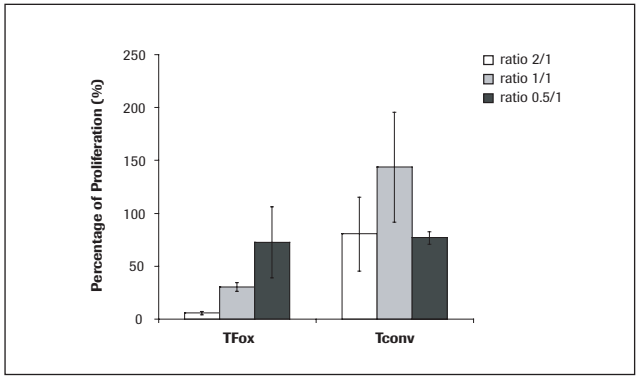

Figure 3: Test of the suppressive properties of the Tfox.
The proliferation of C57bl/6 conventional T cells stimulated by BALB/C irradiated splenocytes is compared to the proliferation observed when different ratios of Tfox are added.

Conclusions

- We demonstrated our capacity to produce lentiviral particules expressing Foxp3.
- Transgene expression was verified on 293 T.
- We developed culture procedure allowing the selection of activated T cells.
- We verified that at time of transduction, T cells still divided. This renders possible their transduction.
- In preliminary experiments, we observed that activated T cells could be transduced.
- Transduced T cells display suppressive properties *in vitro*.

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Regulatory Antigen-Presenting Cells for the Induction of Transplantation Tolerance

Introduction

Donor-specific tolerance induced by stem cell transplantation (SCT) offers the prospect of solid organ transplantation without the requirement for long-term immunosuppressive therapy. Tolerance in this setting is the result of mixed chimerism of donor and host haematopoetic cells and is predominantly due to central deletion of both donor and host reactive T cells by relevant thymic antigen-presenting cells (APC)[1]. To date, stable mixed chimerism across MHC barriers has only been achieved by the use of recipient conditioning with total body radiation (TBI) doses >600 cGy[2] or prolonged administration of cyclophosphamide and fludarabine to recipients that would be unacceptably toxic to potential solid-organ transplant recipients[3]. The only study demonstrating engraftment following low dose TBI (≤200 cGy) has been dependent on the administration of high dose cyclophosphamide after transplantation[4,5]. Although engraftment has been demonstrated across MHC barriers with co-stimulatory blockade alone, like the majority of these regimens, success is dependent on the transplantation of mega-doses of stem cells that are not feasible to achieve in clinical practice[2,6,7]. These studies have also utilised donor bone marrow devoid of T cells to prevent graft-versus-host disease (GVHD).

We have recently shown that stem cell mobilization with progenipoietin-1 (ProGP-1), a potent G-CSF and Flt-3L analogue results in the generation of large numbers of regulatory APC and T cells that induce tolerance after myeloablative stem cell transplant via IL-10 secreting regulatory T cells[8,9]. We hypothesized that the properties inherent in these "regulatory grafts" may permit transplantation across MHC barriers without toxic or myelosuppressive host conditioning and eliminate the requirement for T cell depletion to prevent GVHD.

The aim of this project is therefore to induce tolerance via haemopoietic chimerism prior to solid organ transplantation without immunosuppression. We have utilised an allogeneic H-$2^{b/k}$ → H-$2^{b/d}$ model of stem cell transplantation to mimic the haplo-type mismatched setting inherent in directed family transplantation. Donor and recipient mice in this strain combination have been bred such that they are disparate at the CD45 locus to enable tracking of donor cells. Recipient mice were conditioned with reducing doses of total body irradiation with or without T cell depleting antibodies, fludarabine or a combination of fludarabine and

cyclophosphamide. They were then transplanted with splenocytes (10^8) from donors that were pre-treated with ProGP-1 or control diluent that are representative of cell doses achievable in clinical peripheral blood stem cell aphaeresis products. Donor engraftment was monitored at various time-points after SCT by phenotyping of peripheral blood myeloid and T cells using flow cytometric analysis of CD45 disparity.

Results

We first determined the lowest radiation dose, that, in isolation allowed stable engraftment of T cell replete grafts in the absence of significant GVHD. A series of transplants were undertaken with dose-adjusted irradiation regimens. Conditioning with TBI at 500 cGy and above enabled 33-100% chimerism in peripheral blood of recipient mice transplanted with grafts from donors pretreated with ProGP-1 (Table 1). As previously shown, the treatment of donors with ProGP-1 prevented the induction of severe lethal GVHD following SCT after myeloablative doses of TBI that was universally the case following the transplantation of grafts from control donors[8]. However, at TBI doses below 500 cGy all grafts were consistently rejected, despite the treatment of donors with ProGP-1.

Radiation Dose cGy	Engraftment >5% donor cells		GVHD Mortality		GVHD Clinical Score	
	Control	ProGP-1	Control	ProGP-1	Control	ProGP-1
1100	NA*	100%	100%	0%	NA*	1.4 ± 0.4
700	100%	100%	0%	0%	2.6 ± 0.5	0.3 ± 0.3
600	100%	100%	0%	0%	2.0 ± 1.0	3.0 ± 0.0
500	33%	50%	0%	0%	0.0 ± 0.0	1.9 ± 0.9
400	0%	0%	0%	0%	0.8 ± 0.4	0.9 ± 0.4
300	0%	0%	0%	0%	0.3 ± 0.3	0.0 ± 0.0
200	0%	0%	0%	0%	0.0 ± 0.0	0.0 ± 0.0

Table 1. Comparison of donor engraftment, GVHD mortality and clinical score at 14 days post-SCT. *Mice were conditioned with varying doses of TBI at day 1; percentage of mice that had greater than 5% of donor cells at day +14 is recorded above in either control or ProGP-1 pre-treated mice. NA = data not available due to death prior to day +14 caused by GVHD. Clinical scores expressed as mean ± SE.*

We could therefore not achieve mixed chimerism with radiation doses in isolation that could realistically be delivered to potential solid organ transplant recipients. This is consistent with previous work demonstrating the inability to establish mixed chimerism following low dose TBI in isolation, even following the transplantation of very high doses of bone marrow[10]. Indeed, previous studies have required TBI doses of at least 300 cGy in conjunction with thymic irradiation and *in vivo* T cell depletion.

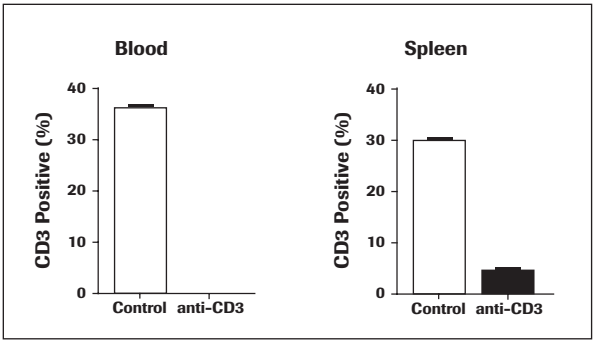

Figure 1. Anti-CD3 monoclonal antibody is effective in depleting CD3⁺ T cells from peripheral blood and spleen. *Either control diluent (□) or 500 µg anti-CD3 monoclonal antibody (■) was injected into B6D2F1 mice at day 5. The mice were then conditioned with 200 cGy at day 1. At day 0, flow cytometric analysis was performed to assess the percentage of CD3⁺ cells in diluent and anti-CD3 treated animals.*

In order to further reduce graft rejection by recipient T cells we added fludarabine (100 mg/kg IP on days -6 to -2) to low dose TBI (200 cGy on day 1) or combined fludarabine (100 mg/kg IP on days 8 to 4) and cyclophosphamide (100 mg/kg IP days 3 and 2). Disappointingly, all grafts were again rejected regardless of donor pretreatment with ProGP-1 (data not shown). We next added the anti-CD3 depleting antibody (145-2C11)[11] to low dose TBI which was administered to recipients at day 5 in a single dose of 500 µg per mouse. The administration of this antibody reduces recipient $CD3^+$ T cells in both peripheral blood and spleen by >90% (Fig. 1).

Next, we tested whether we could obtain mixed chimerism by conditioning recipients with anti-CD3 depleting antibody and 200 cGy of TBI. The transplantation of grafts from ProGP-1 treated donors resulted in stable mixed chimerism after transplant (Fig. 2, closed bars). In contrast, grafts from non-cytokine treated donors containing equivalent numbers of donor T cells were promptly rejected (Fig. 2, open bars). Surprisingly, clinical scores in SCT recipients were <2, consistent with the absence of GVHD, despite the robust engraftment in the cohort transplanted with ProGP-1 grafts.

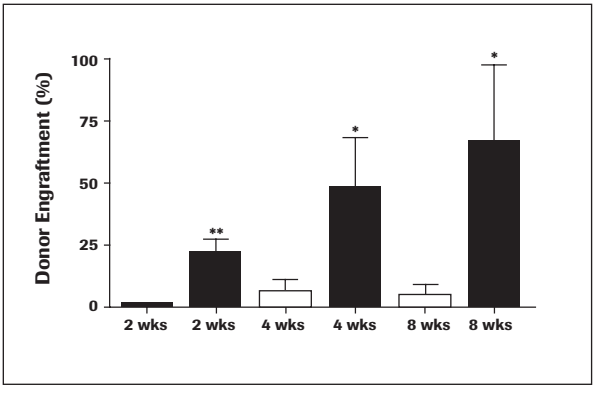

Figure 2. Donor pre-treatment with ProGP-1 allows engraftment across MHC barriers. *Whole splenocytes from control (□) or ProGP-1 (■) treated B6 ptprc^a xC3H/HeJF1 donors were transplanted into recipient B6D2F1 mice conditioned with anti-CD3 mAb and 200 cGy TBI (n=6 per group). Percentage of donor cells engrafted was measured by flow cytometric analysis of peripheral blood 2, 4, 8 weeks post-SCT. Donor engraftment was measured using the following calculation [(% of CD45.1⁺ cells/% of CD45.1⁺ + % CD45.2⁺ cells) x 100]. **P< 0.01, *P<0.05.*

In order to confirm the establishment of mixed chimerism in both myeloid and lymphoid lineages, we phenotyped donor cells two weeks after SCT. As shown in Fig. 3, 13 ± 6% and 70 ± 7% of the engrafted donor population were of T cell and myeloid origin respectively.

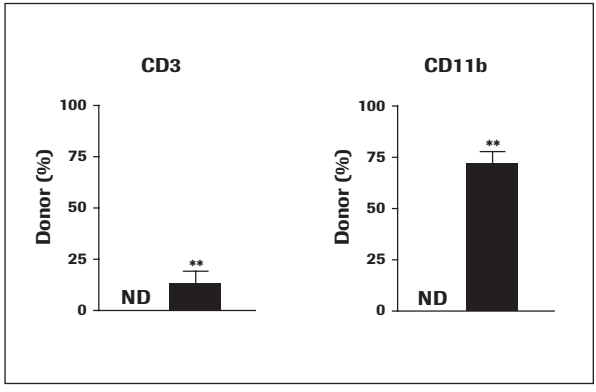

Figure 3. Multi-lineage engraftment following stem cell mobilisation with ProGP-1. *Whole splenocytes from control (□) or ProGP-1 (■) treated B6 ptprc^a xC3H/HeJF1 donors were transplanted into recipient B6D2F1 mice conditioned with anti-CD3 mAb and 200 cGy TBI (n = 6 per group). Percentage of CD3 and CD11b cells were quantified two weeks after SCT in the donor (CD45.1$^+$) population by flow cytometric analysis. **P< 0.01, N = not detected.*

Conclusions

These preliminary data demonstrate that transplantation of grafts from ProGP-1 treated donors result in stable mixed chimerism in both myeloid and T cell lineages following minimal recipient conditioning. In contrast, grafts from non-cytokine treated donors are promptly rejected. Despite robust engraftment, GVHD as assessed by clinical scoring was absent in all recipients of ProGP-1 treated grafts. These data suggest that the transplantation of these "regulatory" stem cell grafts from ProGP-1 treated donors may allow the establishment of mixed chimerism and donor-specific tolerance with conditioning intensity acceptable to prospective solid organ transplant recipients.

Ongoing work now involves grafting of stable mixed chimeras with donor or third party skin and cardiac grafts to confirm the presence of donor-specific tolerance. In addition we are undertaking histopathological analysis of GVHD target organs three months after stem cell transplantation to definitively exclude the presence of GVHD. At this time, recipient T cell responses to host, donor and third party antigen-presenting cells will be assessed to confirm the presence of donor-specific tolerance. We have proven in principle the feasibility of using "regulatory" stem cell grafts to overcome graft rejection and establish donor-specific tolerance following a clinically acceptable non-toxic conditioning regimen. The ability to establish chimerism with feasible donor cell doses following these low TBI doses in conjunction with depleting Ab represents a significant advance on previous reports. If the ongoing grafting studies are now successful, this represents a novel and promising approach for the induction of donor-specific tolerance prior to solid organ transplantation.

Publications

1. Sykes M. Mixed chimerism and transplant tolerance. *Immunity* 2001; 14:417–24.
2. van Pel M, Hilbrands L, Smits D, van Breugel DW, van Eck M, Boog CJ. Permanent acceptance of both cardiac and skin allografts using a mild conditioning regimen for the induction of stable mixed chimerism in mice. *Transpl Immunol* 2003; 11:57–63.
3. Petrus MJ, Williams JF, Eckhaus MA, Gress RE, Fowler DH. An immunoablative regimen of fludarabine and cyclophosphamide prevents fully MHC-mismatched murine marrow graft rejection independent of GVHD. *Biol Blood Marrow Transplant* 2000; 6:182–9.
4. Luznik L, Engstrom LW, Iannone R, Fuchs EJ. Posttransplantation cyclophosphamide facilitates engraftment of major histocompatibility complex-identical allogeneic marrow in mice conditioned with low-dose total body irradiation. *Biol Blood Marrow Transplant* 2002; 8:131–8.
5. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood* 2001; 98:3456–64.
6. Sykes M, Szot GL, Swenson KA, Pearson DA. Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nat Med* 1997; 3:783–7.
7. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Schaffer J, Sayegh MH, Sykes M. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* 2000; 6:464–9.
8. MacDonald KP, Rowe V, Filippich C, Thomas R, Clouston AD, Welply JK, Hart DN, Ferrara JL, Hill GR. Donor pretreatment with progenipoietin-1 is superior to G-CSF in preventing graft-versus-host disease after allogeneic stem cell transplantation. *Blood* 2003; 101:2033–42.
9. MacDonald KP, Rowe V, Clouston A, Welply JK, Kuns RD, Ferrara JL, Thomas R, Hill GR. Cytokine expanded myeloid precursors function as regulatory antigen-presenting cells and promote tolerance through IL-10-producing regulatory T cells. *J Immunol* 2005; 174:1841–50.
10. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. J *Exp Med* 1989; 169:493–502.
11. Hiruma K, Hirsch R, Patchen M, Bluestone J, Gress R. Effects of anti-CD3 monoclonal antibody on engraftment of T-cell-depleted bone marrow allografts in mice: host T-cell suppression, growth factors, and space. *Blood* 1992; 79:3050–8.

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

1. Introduction

We proposed to investigate naturally arising regulation mechanisms of alloreactivity by focusing on self-reacting regulatory T cells and interleukin (IL)-6, which play central roles in the negative and positive cross-talk between innate and adaptive immunity. The ultimate goal is to attempt to create a feasible and drug-free strategy for tolerance induction. Our approach took the view that some allografts are occasionally accepted in absence of immunosuppression. As $CD4^+CD25^+$ regulatory T cells have been shown to inhibit allograft rejection by adoptive transfer experiments in experimental settings of lymphopenia and/or immunosuppression, we first investigated their influence on the outcome of spontaneously accepted allografts in non-immunosuppressed mouse recipients.

2. Results

2.1 Natural $CD25^+$ Treg are critically involved in spontaneous acceptance of male skin grafts in HY TCR transgenic female recipients.

As already shown, 100% of female CBA/ca A1(M).RAG-$1^{-/-}$ mice, which express an anti-HY transgenic TCR rejected congenic male skin graft 15 days after transplantation. On the contrary, A1(M)RAG^+ females did not reject male skin grafts (Fig. 1). Given the known absence of $foxP3^+$, $CD25^+$ T cells in the A1(M).RAG-$1^{-/-}$, we examined the effect of $CD25^+$ T cell depletion on male skin graft survival in A1(M)RAG^+ mice. Pre-transplant injection of PC61 mAb resulted in skin graft rejection in the vast majority of the A1(M)RAG^+ recipients (Fig. 1). These results provide evidence that tolerance of male skin grafts in female transgenic A1(M)RAG^+ mice critically depends on natural $CD25^+$ Treg cells.

2.2 $CD25^+$ T cell depletion prevents acceptance of (C57BL/6 x bm12)F1 skin grafts in semi-allogeneic MHC class II disparate C57BL/6 recipients.

We investigated the role of natural $CD4^+CD25^+$ regulatory T cells in a combination where bm12 donors and C57BL/6 recipients differ for 3 amino acids in the unique major histocompatibility complex (MHC) class II molecule (I-A) they express. This disparity is sufficient to elicit acute rejection of bm12 skins by C57BL/6 recipients. We observed that (C57BL/6 x bm12)F1 skin grafts, which express both donor and recipient I-A were spontaneously accepted

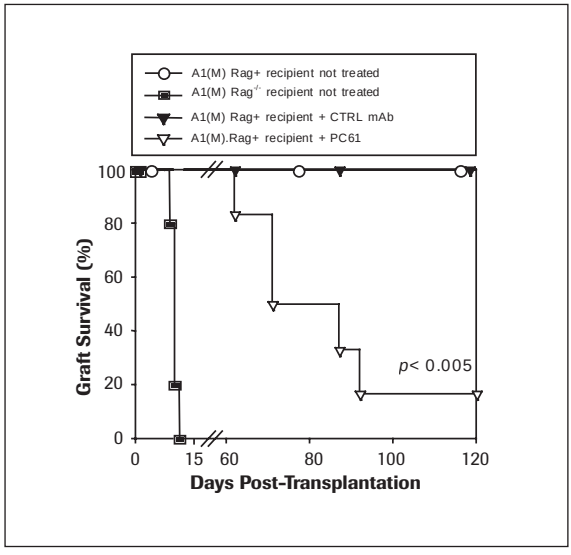

Figure 1. CD25-depletion of female A1(M)RAG^{+} recipient mice triggers rejection of male CBA/Ca skin grafts. *A1(M). RAG$^{-/-}$ mice which do not contain CD25^{+} natural Treg cells (■, n=5) were grafted with male CBA/Ca RAG1$^{-/-}$ skin. Male skin graft survival in untreated A1(M). RAG^{+} is shown (○, n=5). A1(M). RAG^{+} (▽, n=6) mice were depleted of CD25^{+} T cells through repeated injections of 1 mg of anti-CD25 mAb at day 17, 15 and 13 before transplantation. Control A1(M). RAG^{+} (▼, n=6) mice were injected with 1 mg of isotype-matched indifferent mAb according to the same schedule. All mice were grafted with male CBA/Ca RAG1$^{-/-}$ skin at day 0.*

in about 50% of C57BL/6 recipients (Fig. 2). Mice were depleted of natural CD25^{+} Treg cells by thymectomy followed by 3 injections of PC61 mAb, a regimen that resulted in almost complete disappearance of CD25high T cells in spleen and lymph nodes. Seventeen out of 18 CD25^{+} T cell depleted animals acutely rejected their allografts within 20 days whereas thymectomized mice injected with a control mAb behaved no differently to intact mice. Rejected allografts displayed a dense eosinophil infiltrate. In parallel, we found that natural CD25^{+} Treg cell depletion did not modify the rapid rejection of single MHC class II disparate bm12 skin allografts (Fig. 2).

2.3 CD25^{+} T cells control rejection of bm12 heart allografts in C57BL/6 recipients.

In contrast to skin transplants, heart allografts in the C57BL/6-bm12 strain combination undergo low-grade and delayed rejection, perhaps as a consequence of a favourable balance for regulation rather than aggressive T cell effector deployment. For this purpose, bm12 heart allografts were transplanted in C57BL/6 recipients depleted or not of natural CD25^{+} Treg cells. The median survival time in thymectomized mice injected with a control mAb was 27.5 (16-28) days. Mice injected with the PC61 mAb rejected more rapidly with a median graft survival time of 8 (7-13) days ($p < 0.05$), also implicating a regulatory action of natural CD25^{+} Treg in this combination. At the histological level, acutely rejected cardiac allografts in natural CD25^{+} Treg cell-depleted mice displayed large necrotic areas and infiltration by eosinophils and mononuclear cells. Acute rejection was accompanied with both Th1 and Th2 cytokine mRNAs in rejected hearts, whereas mRNA coding for the inhibitor of transcription FOXP3 was up-regulated in persistent allografts.

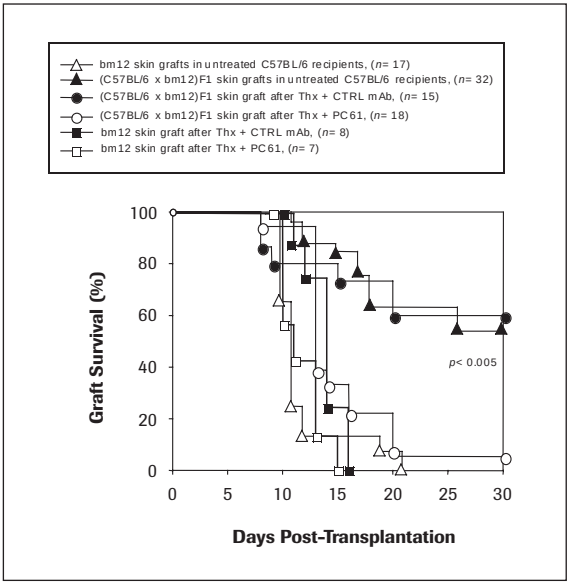

Figure 2. CD25⁺ T cell-depletion affects (C57BL/6xbm12)F1 skin allograft survival but not single MHC class II disparate bm12 skin grafts. *bm12 and (C57BL/6xbm12) F1 skin graft survival in uninjected non-thymectomized animals are shown (Δ, n=17 and ▲, n=32, respectively). Five-week old C57BL/6 recipient mice were thymectomized and depleted of CD25⁺ T cells with 3 consecutive injections of 1 mg PC61 mAb at days 11, 9 and 7 before transplantation. Control animals were thymectomized and injected with isotype-matched indifferent mAb. At day 0, CD25-depleted animals were grafted with either a (C57BL/6xbm12)F1 skin (○, n=18) or a bm12 skin (□, n=7). At the same time, control mice were grafted with either a (C57BL/6xbm12)F1 skin (●, n=15) or a bm12 skin (■, n=8).*

2.4 Natural CD25⁺ Treg cells control the production of both Th1 and Th2 cytokines in primary MLC.

The histological pictures of MHC class II-disparate grafts rejected by mice depleted of natural Treg cells suggested the involvement of Th2-type responses, as graft eosinophilic infiltrates were previously shown to depend on IL-5 and IL-4. This led us to investigate the impact of Treg CD25⁺ cells on the production of Th1- and Th2-type cytokines in MLC. For this purpose, we first added graded numbers of C57BL/6 natural CD25⁺ Treg cells to MLC between purified C57BL/6 CD4⁺CD25⁻ T cells as responders and either bm12 or (bm12xC57BL/6)F1 stimulators. Natural CD25⁺ Treg cells inhibited in a dose-dependent manner the production of IL-2, IFN-γ, and IL-13 in response to both types of stimulators (Fig. 3A). Concomitantly, FOXP3 expression increased with growing concentrations of CD25⁺ Treg cells.

We next compared the responses in primary MLC of CD4⁺ T cells obtained from either intact mice or natural Treg cell-depleted mice (Fig. 3B). *In vivo* depletion of natural CD25⁺ Treg cells in C57BL/6 mice enhanced the capacity of their CD4⁺ T cells to secrete IL-2, IFN-γ, IL-13 and IL-5 in response to either bm12 or (bm12xC57BL/6)F1 bone marrow-derived TNF-α stimulated dendritic cells (DCs), providing further evidence that natural CD25⁺ Treg cells control the development of both Th1 and Th2 alloreactive responses. In other experiments, we used immature DCs as stimulator cells. Immature DCs should be more inclined to promote regulatory processes than activated DCs. However, comparable results were obtained with a slightly more marked effect in cultures with (bm12xC57BL/6)F1 DCs as compared with fully-MHC class II disparate DCs.

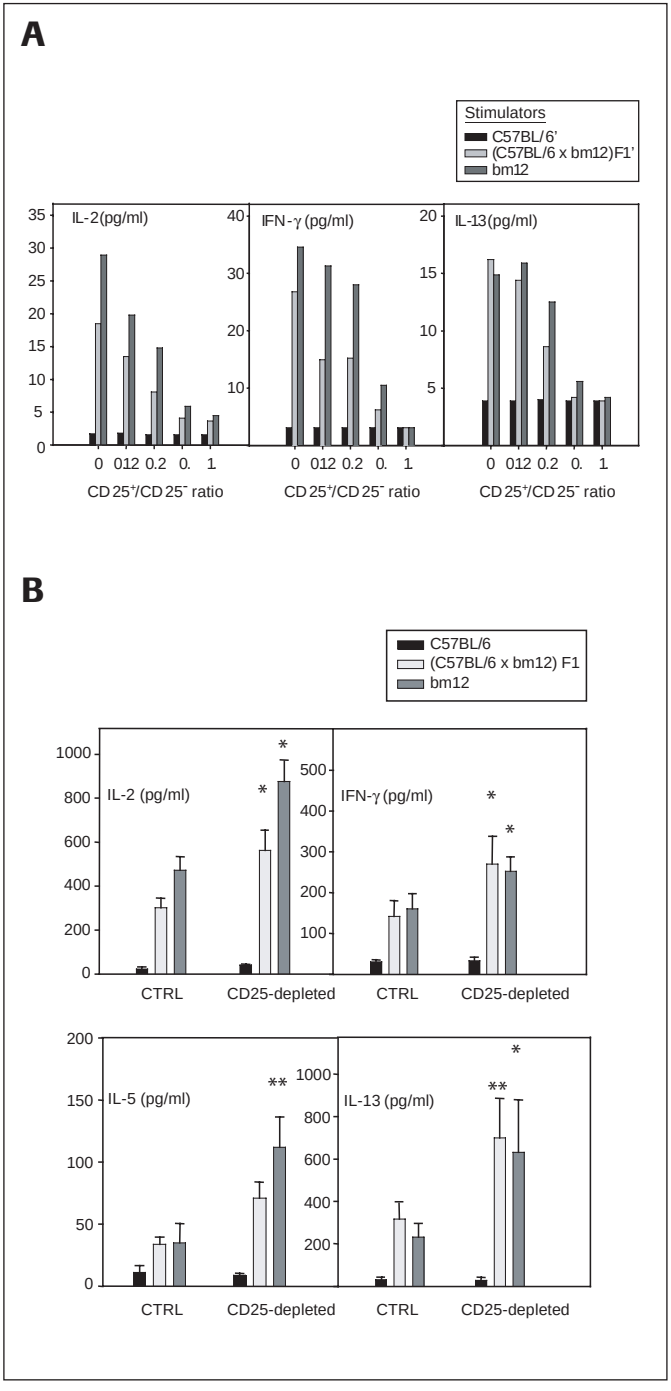

Figure 3. Natural CD25⁺ Treg cells control the production of both Th1 and Th2 cytokines in primary MLC. A) *Graded numbers of C57BL/6 natural CD25⁺ Treg cells were added to MLC between purified C57BL/6 CD4⁺CD25⁻ T cells as responders and either syngeneic C57BL/6 (in black), bm12 (light-grey) or (bm12xC57BL/6)F1 (dark-grey) T cell depleted irradiated spleen cells as stimulators. All the T cells (CD25⁺ or CD25⁻) came from naïve animals. IL-2 production at 48 hours (left panel), IFN-γ (middle panel) and IL-13 productions (right panel) at 72 hours were measured. Cytokine productions by natural CD25⁺ Treg cells in the presence of syngeneic, bm12 or (bm12xC57BL/6)F1 stimulators were undetectable (not shown).* ***B)*** *Primary MLC of CD4⁺ T cells obtained from either control mice (CTRL) injected with isotype-matched indifferent mAb (n=16) or natural Treg cell-depleted mice (referred as CD25-depleted, n=12). In each condition, 2x 10⁶ purified CD4⁺ T cells were used as responders and 6x 10⁵ TNF-α stimulated bone marrow-derived DCs as stimulators according to material and methods [C57BL/6 in black, bm12 in light-grey and (bm12xC57BL/6)F1 in dark-grey]. IL-2 production was quantified at 48 hours; IFN-γ IL-13 and IL-5 productions were measured at 72 hours. Results were expressed as mean ±SEM. *, p< 0.05; **, p< 0.01.*

3. Conclusion and perspectives

Our results clearly show that natural $CD25^+$ Treg cells control Th1- as well as Th2-type allohelper T cell responses and thereby influence the fate of allografts in non-immunosuppressed recipients. We are currently investigating the interactions between $CD25^+$ Treg and MHC class II molecules, particularly, the role of self-MHC class II but also the cross-reactivity with allo-MHC class II molecules. These results will confirm or invalidate the concept depending on whether matching MHC class II molecules between donor and recipients should facilitate regulatory processes and allo-tolerance induction. Furthermore, the role of IL-6 in those regulatory processes is also under current investigation.

The results of this work are *in press* in Transplantation as a rapid communication[1].

Publication

1. Benghiat S, Graca L, Braun MY, Detienne S, Moore F, Buonocore S, Waldmann H, Goldman M, Le Moine A. Critical influence of natural regulatory $CD25^+$ T cells on the fate of allografts in absence of immunosuppression. *Transplantation* 2005 (Rapid communication, *in press*).

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Induction of Transplantation Tolerance by Endogenous CD8α^+ Dendritic Cells

Introduction

Dendritic cells (DC) are rare white blood cells that exist throughout the body and serve as immune sentinels. After encountering a "danger" signal in the form of microbes, inflammatory molecules or allergens, DC ingest fragments of foreign material and transform into potent stimulatory cells. Albeit rare, DC are ascribed to have a large number of highly specialized functions that provide a critical, regulatory link between innate and adaptive immunity. Through intimate cell-to-cell contact, DC trigger other cells of the immune system to make vigorous responses, in particular, the induction of killer T cells, the activation of cytolytic NK cells and the production of neutralizing antibodies by B cells. Importantly, DC are equally active in mediating immune tolerance; the induction of central tolerance, and the maintenance/regulation of peripheral tolerance.

Until recently the discrete functions of DC were thought to reflect their activity at distinct stages of maturation. Immature DC are extremely well-equipped and anatomically positioned for efficient antigen capture, uptake and processing. In contrast, mature DC with their high expression of costimulatory molecules, and constitutive surface expression of MHC antigens, are specialized for antigen presentation and T cell activation. Similar to other leukocyte families, however, recent studies have identified subpopulations of DC with distinct phenotypes. DC subpopulations have been recognized in many species, including rodents, primates and humans and it is increasingly apparent that they exhibit functional specializations. For example, plasmacytoid DC (B220 DC) produce large amounts of the type-1 interferons, IFNα and IFNβ, that stimulate anti-viral immune responses following exposure to viruses. CD8α DC are the primary thymic DC and are involved in the maintenance of peripheral tolerance under resting conditions. Following activation, however, CD8α DC drive the development of type-1 T cells (IFNγ-producing) necessary for resistance to microbes and anti-tumour immunity. In contrast, CD11b DC elicit the differentiation of type-2 T cells (IL-4-producing) needed to fight extracellular parasites.

Aims

Mouse DC expressing CD8α as a homodimer (CD8α DC) impair host, anti-donor immune responses and promote the extended survival of fully allogeneic heart transplants in the absence of conventional immunosuppression[1]. Our goals are to determine the mechanism(s) through which CD8α donor DC subpopulations differentially initiate and terminate immune reactivity. We hypothesize that $CD8\alpha^+$ donor DC specifically impair host immune reactivity to alloantigens, and thereby prolong allograft survival through the activity of a regulatory T cell or the direct deletion of alloreactive T cells.

Results

The aim of our initial work was to determine the mechanism through which CD8α DC mediated their allo-regulatory activity. To examine this activity *in vivo*, we imported a transgenic mouse strain (2C; C57Bl/6; $H2^b$), that would enable us to model the recipient's response to direct presentation of donor alloantigen following adoptive transfer of DC subsets. The majority of T cells from the 2C mouse express a T cell receptor (TCR) specific for the allogeneic MHC class I antigen L^d. First, the 2C mice were embryo re-derived into the specific pathogen-free barrier facility of Robarts Research Institute. Next, we produced and affinity purified the clonotypic monoclonal antibody (mAb) 1B2 from hybridoma supernatant that specifically recognises the 2C TCR. Purified 1B2 (anti-L^d) was then biotinylated or directly conjugated to the fluorophore, FITC.

Our initial experiments (as described in our preliminary progress report) considered only CD8α DC and CD11b DC. We have since repeated many of these studies to include the less abundant B220 DC which was recently reported to also promote extended transplant survival in a mouse model[2]. Briefly, DC were mobilized from BALB/c mice ($H2^d$) with the recombinant human (rh) haematopoietic growth factor Flt3 ligand, administered 15 µg/day/ip for 10 consecutive days (Amgen). In untreated mice DC are rare (≤ 0.1% leukocytes), however, administration of rhFlt3 ligand promotes an expansion of phenotypically and functionally normal DC of approximately 500-fold. DC were routinely purified to greater than 90% purity using a mAb labelling and flow cytometric cell sorting (FACs) strategy.

The stimulatory activity of mouse DC subsets was assessed *in vitro*, using the allogeneic MLR ($H2^d$ DC → anti-L^d 2C T cells). After two, three or four days of coculture CD8α DC, and CD11b DC were found to be equivalent and potent stimulators of 2C T cell proliferation. B220 DC were significantly less stimulatory; their activity resembled that of bulk BALB/c spleen cells. Consistent with these data, CD8α DC and CD11b DC express equivalent levels of MHC class I antigens and costimulatory molecules, while B220 DC express markedly less. Thus, neither their phenotype, nor *in vitro* allostimulatory activity, could explain the capacity of CD8α DC to impair anti-donor immunity and promote allograft survival.

Next, we investigated the allostimulatory activity of mouse DC subsets *in vivo*. 2C T cells were negatively selected and injected intravenously into syngeneic recipients. Purified CD8α DC, CD11b DC or B220 DC were injected subcutaneously into the hind footpad or flank. Draining popliteal and/or inguinal lymph nodes were removed, processed into a single cell suspension and labelled with mAb to detect 2C T cells. CD8α DC mediated a loss of 2C T cells in a time-dependent manner compared with CD11b DC. The number of 2C T cells was not different in the recipients of B220 DC compared with PBS injected mice.

The activation state of 2C T cells present in the draining lymph nodes was assessed following administration of CD8α DC or CD11b DC. First their relative cycles of proliferation were measured by the stepwise loss of the fluorescent tracer CFSE. CD8α DC and CD11b DC both induced strong proliferation in 2C T cells which underwent similar numbers of cell-division cycles. Moreover, the expression profiles of T cell activation molecules, including CD25 and CD28 were also remarkably similar. Combined, these data indicate that CD8α DC and CD11b DC are both capable of stimulating the proliferation and equivalent activation of alloreactive 2C T cells *in vivo*.

We considered several possibilities for CD8α DC-mediated loss of 2C T cells including death via apoptosis, inhibition of further expansion due to a regulatory population, or recirculation to alternate tissues. We tested the deletion hypothesis first. 2C T cells were injected intravenously into syngeneic recipients followed by subcutaneous injection of CD8α DC or CD11b DC. The draining lymph nodes were collected and death via apoptosis in 2C T cells was assessed using Annexin V versus 7AAD staining. Administration of donor CD8α DC was associated with a high proportion of apoptotic 2C T cells.

The differential role of CD8α DC and CD11b DC in the initiation and regulation of T cell activation has a chequered history. CD8α DC have long been touted as “the regulatory DC”, however, few studies have substantiated this claim. CD8α DC were initially reported to be poor stimulators of T cell proliferation, due to the induction of CD4 T cell apoptosis via the Fas-Fas Ligand pathway, or their failure to induce adequate IL-2 production in CD8 T cells[3,4]. Latter reports found that CD8α DC were efficient activators of allogeneic and peptide antigen-specific T cells[5-8]. Microenvironmental stimuli were found to profoundly affect IL-12 production by CD8α DC and hence their ability to skew Th1 and Th2 cell differentiation[9,10]. Most recently, CD8α DC are reported to produce indoleamine 2,3 dioxygenase (IDO) which enables them to degrade the local tryptophan supply and thereby impair T cell proliferation[11].

Although capable of efficiently stimulating T cell proliferation *in vitro*, our data supports the contention that CD8α DC are also capable of immune regulation. Further, this new data supports our hypothesis that CD8α DC prolongs vascularized graft survival through the deletion of alloantigen specific T cells. It is unclear however, whether CD8α DC mediate this activity

directly or through the participation of a third-party. Moreover, the microenvironmental conditions which differentially modulate the immunogenic and tolerogenic activity of CD8α DC are yet to be determined.

Publications

1. O'Connell PJ, Li W, Wang Z, Specht SM, Logar AJ, Thomson AW. Immature and mature CD8alpha$^+$ dendritic cells prolong the survival of vascularized heart allografts. *J Immunol* 2002; 168(1):143-54.
2. Coates PT, Duncan FJ, Colvin BL, Wang Z, Zahorchak AF, Shufesky WJ, Morelli AE, Thomson AW. *In vivo*-mobilized kidney dendritic cells are functionally immature, subvert alloreactive T-cell responses, and prolong organ allograft survival. *Transplantation* 2004; 77(7):1080-9.
3. Suss G, Shortman K. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J Exp Med* 1996; 183(4):1789-96.
4. Kronin V, Winkel K, Suss G, Kelso A, Heath W, Kirberg J, von Boehmer H, Shortman K. A subclass of dendritic cells regulates the response of naive CD8 T cells by limiting their IL-2 production. *J Immunol* 1996; 157(9):3819-27.
5. Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, Maliszewski CR. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc Natl Acad Sci U.S.A.* 1999; 96(3):1036-41.
6. Maldonado-Lopez R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C, Thielemans K, Leo O, Urbain J, Moser M. CD8α^+ and CD8α^- subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*. *J Exp Med* 1999; 189(3):587-92.
7. Morelli AE, O'Connell PJ, Khanna A, Logar AJ, Lu L, Thomson AW. Preferential induction of Th1 responses by functionally mature hepatic (CD8alpha$^-$ and CD8alpha$^+$) dendritic cells: association with conversion from liver transplant tolerance to acute rejection. *Transplantation* 2000; 69(12):2647-57.
8. O'Connell PJ, Morelli AE, Logar AJ, Thomson AW. Phenotypic and functional characterization of mouse hepatic CD8 alpha$^+$ lymphoid-related dendritic cells. *J Immunol* 2000;165(2):795-803.
9. Huang LY, Reis e Sousa C, Itoh Y, Inman J, Scott DE. IL-12 induction by a TH1-inducing adjuvant *in vivo:* dendritic cell subsets and regulation by IL-10. *J Immunol* 2001; 167(3):1423-30.
10. O'Connell PJ, Son YI, Giermasz A, Wang Z, Logar AJ, Thomson AW, Kalinski P. Type-1 polarized nature of mouse liver CD8alpha$^-$ and CD8alpha$^+$ dendritic cells: tissue-dependent differences offset CD8alpha-related dendritic cell heterogeneity. *Eur J Immunol* 2003; 33(7):2007-13.
11. Mellor AL, Munn D, Chandler P, Keskin D, Johnson T, Marshall B, Jhaver K, Baban B. Tryptophan catabolism and T cell responses. *Adv Exp Med Biol* 2003; 527:27-35.

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Donor-Engineered Dendritic Cells to Generate Tolerogenic Regulatory T Cells for Renal Transplantation

Aims

Here we have proposed to characterize *in vitro* phenotypically and functionally regulatory T cells generated by immature dnIKK2-engineered DCs and to explore *in vivo* their capacity to control the effector arm of the immune response and induce tolerance to a solid organ graft from the same DC donor. Aims of the project were as follows:

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

This was originally designed as a 3-year project, however only 1 year was founded by the ROTRF. The results of the first year are reported below.

Results

Transfection with dnIKK2 inhibits DC allostimulatory function.

To obtain stably immature DCs, bone marrow (BM) cells from BN rats cultured with GM-CSF, IL-4 and rat serum were transfected with AdV-dnIKK2 (dnIKK2-DCs) at day 9. DCs transfected with AdV0 were used as controls (AdV0-DCs). The dose of AdV-dnIKK2 was titrated (200, 100 and 50 MOI) to find out the best transfection condition with the lowest toxic effect. A high percentage of cells transfected with 200 and 100 MOI AdV-dnIKK2 underwent apoptosis, as demonstrated by TUNEL reaction assay, whereas at a relatively low MOI (50), TUNEL assay revealed a low percentage of apoptotic cells (20%), which was comparable to that found in AdV0-transfected DCs (15%). The latter dose was used for the subsequent transfection experiments.

Both dnIKK2-DCs and AdV0-DCs expressed CD11c (Fig. 1) at comparable levels and were negative for lymphocyte and macrophage markers (CD45RA, CD3, ED1 and ED3). The intensity of MHCII expression and percentage of B7-2 positive cells were significantly ($P<0.05$) lower in dnIKK2-DCs than in AdV0-DCs (Fig. 1). Quantification of IL-12p40 mRNA expression by real-time PCR showed a reduction of expression in dnIKK2-DCs (0.3) when compared to the calibrator (not infected DCs), whereas IL-12p40 expression in AdV0-DCs (1.24) was comparable to the calibrator. No IL-10 mRNA was found in dnIKK2-DCs nor in AdV0-DCs.

	CD11c		MHC II		B7-2	
	n	**% positive cells**	**% positive cells**	**MFI**	**% positive cells**	**MFI**
AdV0-DCs	5	71 ± 5	65 ± 7	138 ± 28	64 ± 9	41 ± 4
dnIKK2-DCs	5	68 ± 6	61 ± 8	86 ± 18°	26 ± 6°	33 ± 5
° $P<0.05$ vs AdV0-DCs						

Figure 1. Phenotype analysis of BM-derived DCs.

While dnIKK2 expression was undetectable in AdV0-DCs, dnIKK2 expression in dnIKK2-DCs (50 MOI) was 1.562-fold that of the calibrator (human PBMCs). Densitometric analysis of EMSA demonstrated that transfection with 50 MOI AdV-dnIKK2 inhibited DC nuclear NF-κB activity by more than 70% as compared with AdV0-DCs.

BN dnIKK2-transduced DCs exhibited markedly impaired ability to stimulate naïve allogeneic LW T cells during primary MLR as compared with AdV0-DCs both at 1:100 ($P<0.01$ vs MLR with AdV0-DCs, $n=5$, Fig. 2) and 1:1000 ratios ($P<0.01$ vs MLR with AdV0-DCs, $n=5$, Fig. 2).

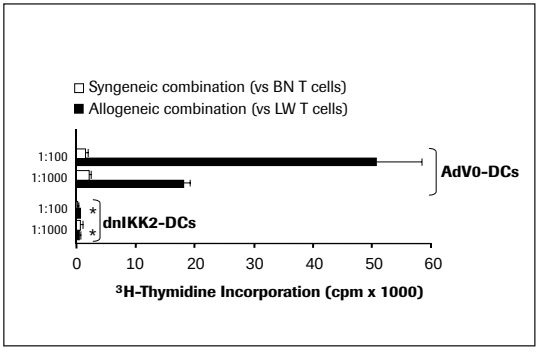

Figure 2. Primary MLR. *BM-derived DCs, from BN rats, transfected with 50 MOI AdV0 (AdV0-DCs) or AdV-dnIKK2 (dnIKK2-DCs) were used as stimulators in MLR with lymph node cells ($1x10^6$) from either naïve BN rats (syngeneic combination) or naïve LW rats (allogeneic combination) at 1/100 or 1/1000 ratio. Proliferation was measured by incorporation of ^{3}H-thymidine after a 4-day culture and expressed as cpm. Unlike AdV0-DCs, dnIKK2-DCs significantly failed to induce allogeneic T cell proliferation. Results are mean ± SE of five experiments. *P<0.01 vs AdV0-DCs.*

To evaluate whether T cells pre-exposed to dnIKK2-DCs were hyporesponsive to secondary stimulation by allogeneic APCs, $CD4^+$ T cells were purified at the end of primary MLR and used in secondary MLR with mature allogeneic BN AdV0-DCs. $CD4^+$ T cells primed with BN AdV0-DCs showed a robust response to the same allogeneic AdV0-DCs (n=3, Fig. 3). The proliferative response further increased in response to IL-2 (50 U/ml, $P<0.05$ vs no IL-2, n=3, Fig. 3). In contrast, $CD4^+$ T cells pre-exposed to dnIKK2-DCs showed only a marginal proliferation upon stimulation with the same allogeneic AdV0-DCs ($P<0.05$ vs $CD4^+$ T cells primed with AdV0-DCs, n=3, Fig. 3) and addition of IL-2 failed to reverse the hyporesponsiveness (n=3, Fig. 3). Thus, priming with dnIKK2-DCs irreversibly inhibited the capacity of antigen-driven proliferation in $CD4^+$ T cells. This effect was specific of pre-exposure to dnIKK2-DCs and was not merely the consequence of lack of allogeneic stimulation, as documented by data that LW $CD4^+$ T cells cultured with medium alone for 4 days vigorously proliferated upon stimulation with BN AdV0-DCs (35,620 ±7,300 cpm, n=3).

Finding that $CD4^+$ T cells pre-exposed to dnIKK2-DCs strongly proliferated in response to anti-CD3 plus anti-CD28 antibodies indicated that T cells were viable and functional (Fig. 3).

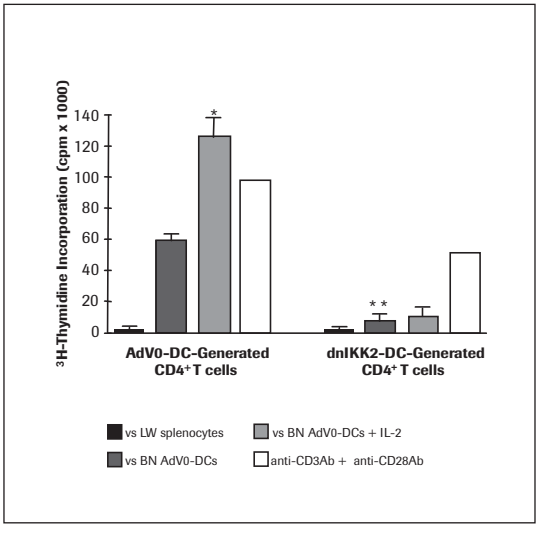

Figure 3. Secondary MLR. *Secondary MLR was performed with purified LW $CD4^+$ T cells (by immunomagnetic sorting) obtained at the end of primary MLR with either AdV0-DCs or dnIKK2-DCs. $CD4^+$ T cells ($1x10^6$) were cultured with LW irradiated splenocytes ($1x10^6$) as control combination or with BN AdV0-DCs ($1x10^4$) in the presence or absence of IL-2 (50 U/ml) for 3 days. Proliferation was measured by incorporation of ^{3}H-Thymidine and expressed as cpm. $CD4^+$ T cells pre-exposed to dnIKK2-DCs were unresponsive to a second allogeneic stimulation, even in the presence of IL-2. Results are mean ± SE of three experiments. *$P<0.05$ vs no IL-2, **$P<0.01$ vs $CD4^+$ T cells obtained from primary MLR with AdV0-DCs. In one experiment (white bar) cells ($1x10^5$) were stimulated with immobilized anti-CD3 mAb (10 μg/ml) plus anti-CD28 mAb (1 μg/ml).*

dnIKK2-DCs induced the formation of T regulatory cells.

To evaluate whether hyporesponsive $CD4^+$ T cells generated by exposure to allogeneic dnIKK2-DCs had regulatory properties, their effect on the activation of syngeneic (LW) T cells by irradiated splenocytes (from alloantigen-specific BN) was assessed in 4-day co-culture experiments (n=5). $CD4^+$ T cells generated from primary MLR with dnIKK2-DCs were

capable of almost completely suppressing, at 1:2 ratio, the proliferative response of naïve syngeneic T cells toward BN antigens ($P<0.05$ vs naïve MLR, Fig. 4). The suppressive effect was observed also at 1:10, $1:10^2$ and $1:10^3$ ratios (Figs 5,6) confirming a potent regulatory activity. We define these $CD4^+$ T cells as Treg. Of interest, the suppressive effect of Treg was already evident after 1-day co-culture (Fig. 6). Since exogenous IL-2 failed to reverse the absence of proliferation (Fig. 5) we can assume that Treg did not induce a state of anergy in syngeneic naïve T cells.

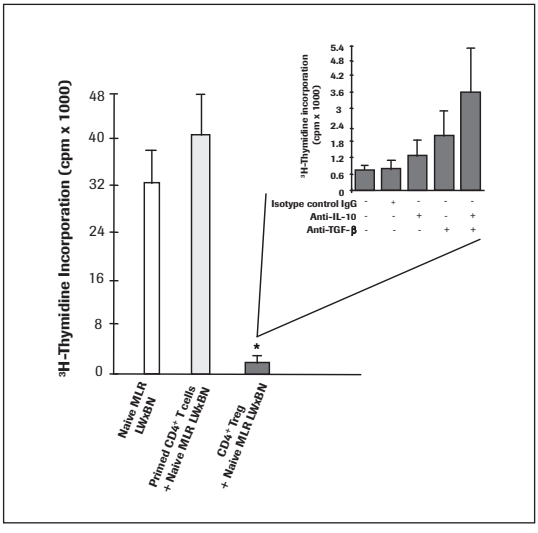

Figure 4. Co-culture MLR. *Co-culture MLR was performed by culturing for 4 days a naïve allogeneic MLR ($1.5x10^6$ LW lymph node cells as responders plus $1.5x10^6$ BN irradiated splenocytes as stimulators) in the presence or in the absence of LW $CD4^+$ T cells (at 1/2 ratio with naïve responder cells) obtained at the end of primary MLR with either AdV0-DCs (primed $CD4^+$ T cells) or dnIKK2-DCs ($CD4^+$ Treg). *$P<0.05$ vs naïve MLR and co-culture MLR with primed $CD4^+$ T cells. The upper right of figure shows co-culture MLR with $CD4^+$ Treg with or without anti-IL-10 (2 μg/ml) and/or anti-TGF-β (40 μg/ml) antibodies. Results are mean ± SE of five experiments.*

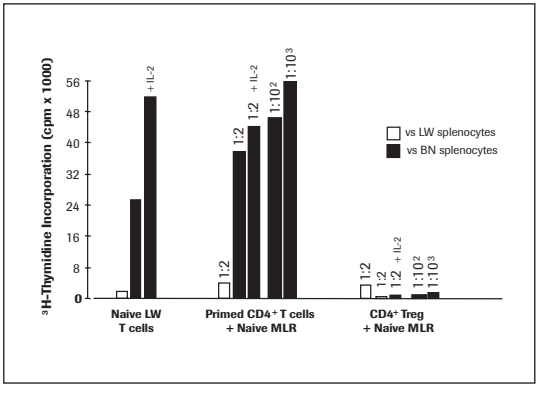

Figure 5. Co-culture MLR. *Co-culture MLR was performed by culturing for 4 days a naïve MLR in the presence or absence of primed $CD4^+$ T cells or $CD4^+$ Treg (at 1/2, $1/10^2$ and $1/10^3$ ratios with naïve responder cells). For the 1/2 ratio, the effect of exogenous IL-2 (50 U/ml) was tested. One representative experiment of three is shown. Proliferation was measured by incorporation of ^{3}H-Thymidine and expressed as cpm.*

By contrast $CD4^+$ T cells generated from primary MLR with AdV0-DCs (primed $CD4^+$ T cells) stimulated a naïve MLR in co-culture (Figs 4-6). $CD4^+$ T cells cultured for 4 days in resting condition had no effect on the proliferation of a naïve MLR in co-culture when added at both 1:10 and $1:10^3$ ratio, (Fig. 6) confirming that Treg were not resting T cells.

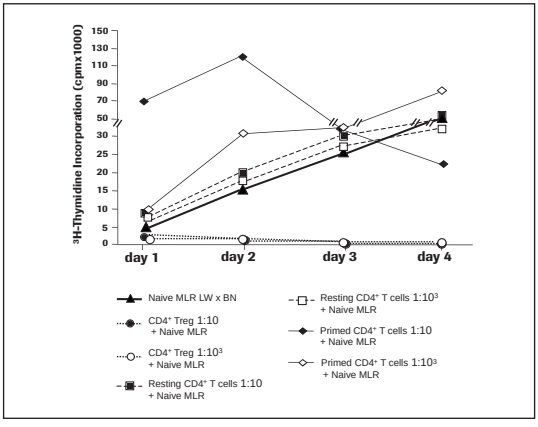

Figure 6. Co-culture MLR time-course. *Co-culture MLRs were performed by culturing a naïve MLR for different time points in the presence or absence of AdV0-DC-generated $CD4^+$ T cells (primed $CD4^+$ T cells), dnIKK2-DC-generated Treg ($CD4^+$ Treg) or resting $CD4^+$ T cells (at 1/10 or $1/10^3$ ratio with naïve responder cells). Proliferation was measured by incorporation of ^{3}H-Thymidine (added at the indicated time for additional 18 hours) and expressed as cpm. One representative experiment of three is shown.*

dnIKK2-DC-generated T regulatory cells act through the release of soluble factors.

We next evaluated whether cell-to-cell contact was required for regulatory activity by performing co-cultures using a transwell system (n=3). As shown in Figure 7, $CD4^+$ T cells generated from primary MLR with dnIKK2-DCs completely blocked the proliferative response of autologous LW T cells to BN stimulators in the bottom compartment.

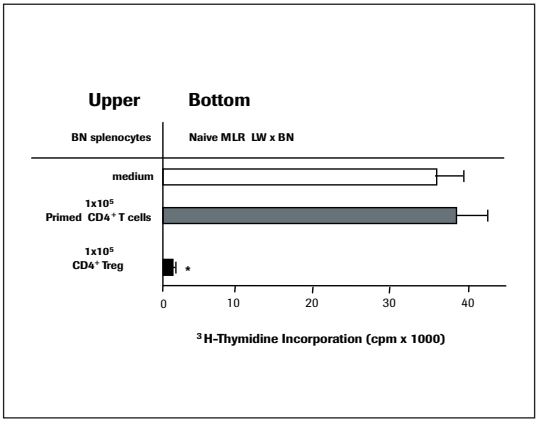

Figure 7. Transwell co-culture MLR. *A naïve MLR ($1x10^6$ LW lymph node cells as responders plus $1x10^6$ BN irradiated splenocytes as stimulators) was cultured for 4 days in the bottom compartment of a transwell system whereas irradiated splenocytes from BN rats were placed in the upper compartment with or without $CD4^+$ T cells ($1x10^5$) obtained from primary MLR with either AdV0-DCs (primed $CD4^+$ T cells) or dnIKK2-DCs ($CD4^+$ Treg). Proliferation was measured by incorporation of ^{3}H-Thymidine and expressed as cpm. Data are mean ± SE of three experiments. *$P<0.01$ vs medium alone and AdV0-DC-generated $CD4^+$ T cells.*

To confirm that regulatory function of Treg was mediated by soluble factors, a conditioned medium harvested at the end of co-cultures was added to a naïve MLR (n=3). We found that supernatant of co-cultures with dnIKK2-DC-generated Treg was capable of completely inhibiting an allogeneic naïve MLR till a dilution of 1:10^6 (Fig. 8). By contrast, conditioned medium from co-culture with primed CD4$^+$ T cells had no effect on a naïve MLR (Fig. 8).

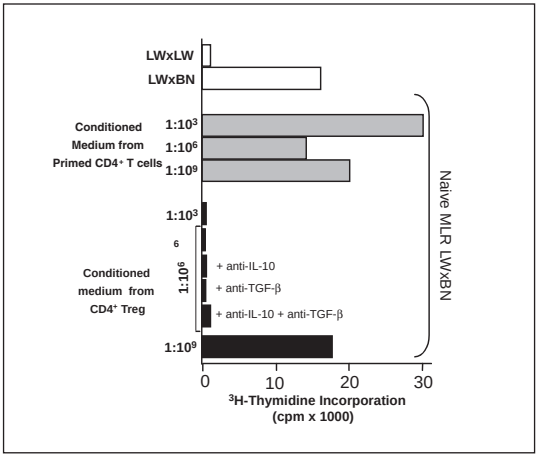

Figure 8. Effect of Treg-conditioned medium on a naïve MLR. *A naïve MLR (1x10^6 LW lymph node cells plus 1x10^6 BN irradiated splenocytes) was performed in the presence or absence of conditioned medium (diluted 1/10^3, 1/10^6 and 1/10^9 of the total well volume) harvested at the end of co-culture MLR with either AdV0-DC-generated CD4$^+$ T cells (primed CD4$^+$ T cells) or dnIKK2-DC-generated Treg (CD4$^+$ Treg). For the 1/10^6 dilution, the role of IL-10 and TGF-β was evaluated by adding anti-IL-10 and/or anti-TGF-β mAbs at the beginning of MLR. Proliferation was measured by incorporation of ^{3}H-Thymidine and expressed as cpm. One representative experiment of three is shown.*

We then analyzed by real-time PCR the pattern of cytokine expression in Treg as compared with primed CD4$^+$ T cells (n=4). Expression levels in syngeneic LW CD4$^+$ T cells cultured for 4 days with medium alone (resting) were taken as control (calibrator: 1). As shown in Figure 9, Treg had higher expression of IL-10 as compared with primed CD4$^+$ T cells and resting CD4$^+$ T cells, TGF-β expression was higher than in AdV0-DC-generated CD4$^+$ T cells and comparable to that of naïve resting CD4$^+$ T cells. Expression levels of IL-2 and IFN-γ were intermediate between those of primed CD4$^+$ T cells and those of resting CD4$^+$ T cells. IL-4 expression was detected in primed CD4$^+$ T cells and resting CD4$^+$ T cells but not in Treg. The above pattern of cytokine expression distinguishes Treg from both primed CD4$^+$ T cells and resting syngeneic CD4$^+$ T cells.

To determine whether IL-10 and TGF-β production by Treg mediated their regulatory function, co-culture experiments were repeated in the presence of anti-IL-10 and/or anti-TGF-β blocking antibodies. As shown in Figure 4, the combination of the two antibodies, but not the single ones, only partially reverted the suppressive effect of Treg on a naïve allogeneic MLR. Similar results were obtained when anti-IL-10 and/or anti-TGF-β blocking antibodies were added to a naïve MLR performed in the presence of conditioned medium harvested at the end of co-culture MLR with Treg (Fig. 8). Collectively these data suggest that soluble mediators other than IL-10 and TGF-β are involved in the regulatory function of dnIKK2-DC-generated Treg.

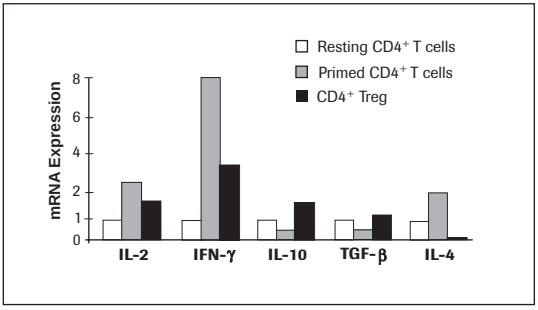

Figure 9. Cytokine expression by real-time PCR. *Cytokine (IL-2, IFN-γ, IL-10, TGF-β, IL-4) mRNA analysis in AdV0-DC-generated CD4⁺ T cells (primed CD4⁺ T cells) and dnIKK2-DC-generated Treg (CD4⁺ Treg) was performed by real-time PCR. The cDNA content in each sample was calculated by ΔΔCT technique, using as calibrator the cDNA expression in CD4⁺ T cells cultured for 4 days with medium alone (resting CD4⁺ T cells). One representative experiment of four is shown.*

Tolerogenic potential of dnIKK2-DCs *in vivo*

To evaluate whether dnIKK2-DCs had a tolerogenic potential *in vivo*, $10x10^6$ dnIKK2-DCs or $10x10^6$ AdV0-DCs from BN rats were infused in naïve LW rats 7 days before a kidney BN allotransplantation, without any immunosuppressive therapy.

While donor AdV0-DCs pre-treatment accelerated kidney allograft rejection (survival time: 5.5 ± 0.8 days, $n=4$, vs 7 ± 1 days in transplanted untreated rats, $n=3$), infusion of donor dnIKK2-DCs prolonged kidney allograft survival (survival time: 16.6 ± 0.7 days, $P<0.05$ vs untreated and AdV0-DC treated rats, $n=3$) in fully MHC-mismatched recipients.

Conclusions

Results of this project documented that immature DCs, obtained through transfection with dnIKK2-encoding adenoviral vector (dnIKK2-DCs), failed to induce proliferation of allogeneic T cells *in vitro* and prolonged allograft survival *in vivo*. DnIKK2-DC-generated CD4⁺ T cells acquired strong regulatory capacity. Indeed, when co-cultured (at up to $1/10^3$ ratio) with a naïve MLR they potently suppressed T cell proliferation and addition of IL-2 did not reverse hyporesponsiveness. The regulatory effect was cell-to-cell contact-independent since it was also observed in a transwell system. The supernatant from co-cultures with dnIKK2-DC-generated Treg potently inhibited an allogeneic naïve MLR till a dilution of $1/10^6$, indicating the release of potent immunoregulatory soluble factors. Neutralizing antibodies to IL-10 and TGF-β did not reverse suppression, excluding a role of these cytokines in Treg-mediated suppression.

Mechanisms by which dnIKK2-DCs promote the emergence of Treg, mediators involved in the regulatory function of these Treg and their tolerogenic potential *in vivo* are under investigation.

Publication

1. Tomasoni S, Aiello S, Cassis L, Noris M, Longaretti L, Cavinato RA, Azzollini N, Pezzotta A, Remuzzi G, Benigni A. Dendritic cells genetically engineered with adenoviral vector encoding dnIKK2 induce the formation of potent CD4⁺ T-regulatory cells. *Transplantation* 2005; 79(9):1056-61.

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Disordered Thromboregulation in Xenotransplantation

Introduction

Xenotransplantation is the transplantation of viable cells, organs or tissues between species. Although this modality of transplantation has been proposed as a solution to the shortage of human donor organs and cells for the treatment of organ failure by allotransplantation, many obstacles exist to the application of this therapy. Hyperacute rejection (HAR) of discordant xenografts can be now effectively managed, but vascularized discordant xenografts are still subject to acute vascular rejection, alternatively referred to as delayed xenograft rejection (AVR/DXR[1-3]). This form of rejection is associated with vascular-based injury and is associated with xenoreactive antibody deposition. The consequent development of thrombocytopenia and consumptive coagulopathy (CC) may ultimately evolve to a bleeding disorder[3-5]. The infusion of discordant (porcine) hematopoietic cells in primates to study tolerance by induction of mixed chimerism is also associated with widespread vascular injury, known as thrombotic microangiopathy that is associated with end-organ injury[6-10].

The mechanisms underlying the consumptive coagulopathy and thrombotic microangiopathy in both these settings have been unclear. Antibody-mediated vascular injury with loss of the natural thromboregulatory factors expressed by endothelium, such as the vascular ectonucleotidase (termed CD39/vascular nucleoside triphosphate diphosphohydrolase-1; CD39/NTPDase1) and increased levels of inflammatory mediators or cytokines within the vascularized xenograft could promote vascular thrombosis[11,12]. Molecular incompatibilities can also be shown between primate coagulation factors and porcine anticoagulant proteins. These combinations might include thrombin and natural anticoagulants, e.g. thrombomodulin (TM); or between factor Xa and tissue factor pathway inhibitor (TFPI). Both of these natural anticoagulants are expressed on porcine endothelium and leukocytes[12-15].

We hypothesize that such molecular barriers between species would further exacerbate the proinflammatory and thrombotic reactions after xenotransplantation of a vascularized graft or post-infusion of xenogeneic cells. We have therefore considered a genetic approach to overexpress relevant human antithrombotics (CD39/NTPDase1) and anticoagulants (TM and TFPI) in xenogeneic cells and to generate transgenic animals (mice and ultimately pigs) to test this hypothesis.

Specific Aims

1. To generate porcine cells overexpressing human TM, TFPI or CD39 and to test these for anticoagulant and/or antithrombotic properties, ultimately in thrombotic microangiopathy *in vivo.*
2 To generate transgenic mice expressing human CD39, TM or TFPI, to determine any deleterious phenotype and to test these animals in thrombotic inflammatory models, including xenograft rejection *in vivo.*

Results

Thrombotic microangiopathy and cellular modifications:

Baboon recipients of porcine bone marrow-derived cells develop microangiopathic hemolytic anemia with variable levels of thrombotic microangiopathy, predominantly involving lung, kidneys and brain[7,8,16]. Mechanisms of vascular injury are mediated by inflammatory pathways induced by xeno-cellular infusions, that may be further exacerbated by molecular incompatibilities with respect to TM and TFPI, and by loss of CD39[17]. Standard antithrombotic (heparin and prostacycline) and anti-inflammatory (steroids) agents do ameliorate thrombotic microangiopathy, but do not fully prevent vascular injury[7,9,10].

Mechanisms of this form of vascular injury in primates have been examined in more detail[8,9,18]. *In vitro,* porcine, but not baboon, bone marrow-derived cells induce aggregation of baboon platelets in a dose-dependent manner[16,18]. Immunohistological examination of derived cell-platelet aggregates confirm the incorporation of porcine leukocytes[8]. Aggregation can be fully abrogated by the addition of eptifibatide a GPIIbIIIa antagonist, and prevented by anti-P-selectin and anti-CD154 monoclonal antibodies that recognize adhesion receptors on activated primate platelets[18]. We have also shown that stringent purification of the porcine bone marrow precursor cells (PBPC) mitigate against thrombotic microangiopathy *in vivo*[8-10].

Effects of xenogeneic PBPC on endothelial cells (EC) were also examined. Porcine cells directly activate primate/human EC, as suggested by an increase in surface adhesion markers: ICAM-1, VCAM-1, and E-selectin[19]. We have also recently shown that in contrast to Gal-positive PBPC, porcine cells that have decreased expression of Gal do not induce aggregation of baboon platelets nor activate human EC[20]. The induction of tolerance through mixed hematopoietic cell chimerism in the pig-to-primate model may be facilitated when cells derived from GalT-KO α-galactosyltransferase-null pigs are studied in more detail.

The alternative approach has been to generate stable xenogeneic cell cultures to upregulate expression of these human thromboregulatory factors *in vitro* and to ultimately test these cells in functional assays and *in vivo*. *In vitro* studies have been done successfully for CD39 and TM (see below); primary porcine and human cultures have been also modified by somatic recombination using viral vectors and functional effects determined by *in vitro* analyses[21-23].

Human TFPI was initially problematical but has been modified to incorporate a GPI linker (see below). We have evaluated the development of porcine cell lines overexpressing TM, TFPI and CD39 following recombinant retroviral and adenoviral infections[23-25]. Our published data show that *ex vivo* infection by recombinant adenovirus vectors can result in vascular expression of a potential therapeutic agent[24]. For technical reasons and because of the early benefits seen with pharmacological modalities (as detailed above), the work dealing with genetic modification of PBPC has not yet been translated to large animal xenotransplant models to test these cells for anticoagulant and/or antithrombotic properties in baboons *in vivo.*

Xenograft rejection, vascular inflammation and transgenesis:

The long-term goal is to develop genetically engineering pigs that overexpress human anticoagulants and thromboregulatory factors. We have commenced the generation and characterization of transgenic mice expressing CD39/NTPDase, TM and TFPI to determine the feasibility of this approach and to evaluate any deleterious phenotype. The H2-Kb promoter used produces a pattern of expression of the transgene that includes high levels on heart, lung and pancreatic islets (Fig. 1).

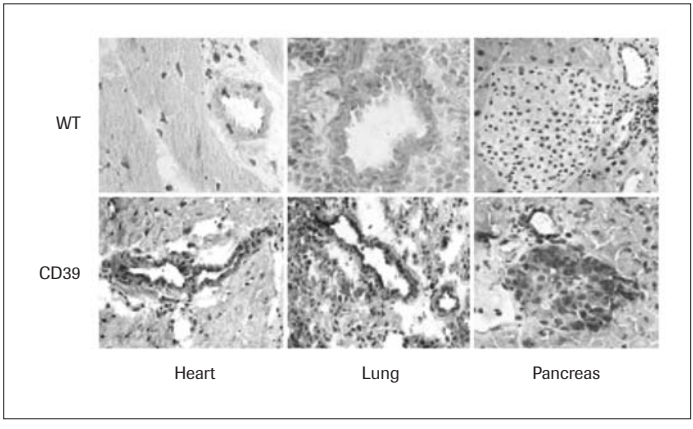

Figure 1. Expression of human CD39/NTPDase1 in transgenic mouse tissues. *High levels of CD39 antigen are shown in heart, lung, and in pancreatic islets.*

CD39/NTPDase1

The cDNA in pcDNA3/CD39 was cloned into pCMVpuro for transfection into SVAP cells. When transiently transfected into COS cells, CD39 can be detected by FACS with approximate efficiency of 60%[23]. Substantive increases in biochemical NTPDase activity have been measured by generation of free inorganic phosphate from ADP (or ATP) by transfected vs. mock-transfected SVAP cells[17,26]. Physiological functions of CD39 have also been determined by inhibition of ADP-mediated platelet aggregation[17,26], as measured in a dual sample platelet

aggregometer *in vitro*. Human CD39 was cloned into pEA (H2-Kb promoter) for microinjection and several lines validated that overexpress this transgene. CD39 transgenic mice exhibited a modified hemostatic system with impaired platelet aggregation to collagen and ADP *in vitro*, prolonged bleeding times, and resistance to prothrombotic stimuli. Importantly, the transgenic mice displayed no overt bleeding tendency under normal circumstances and coagulation profiles (INR and the aPTT) were normal[21].

The expression of human CD39 in transgenic mice (Fig. 1), however, did result in a distinct anticoagulant phenotype, with prolonged bleeding times and resistance to induced thrombosis, without apparent deleterious effects. Overexpression of human CD39 protected transgenic mouse cardiac grafts against Gal antibody-mediated rejection processes and ameliorated collagen and ADP agonist-induced pulmonary thromboembolism[21,27,28]. Results from this murine model are favorable and encouraging for the ultimate development of transgenic swine, using published techniques[29].

Thrombomodulin (TM)

The human cDNA was cloned into pCMVpuro for transfection as above. It has been demonstrated that overexpression of TM results in activated protein C generation, using techniques as published previously[25,30]. The native human TM was then cloned into pEA (H2-Kb promoter) for microinjection and four transgenic mice have been generated to date. All these mice express human TM. Again, these mice do not have grossly abnormal hemostasis and are being bred to generate sufficient mice for full characterization of the phenotype. The TM transgenics have normal coagulation assays (activated partial thromboplastin time and thrombin time) and normal whole blood and washed platelet aggregation times in response to collagen and thrombin (not shown). However, they have significantly prolonged tail bleeding times (not shown). Like the CD39 transgenic mice[21], they are resistant to intravascular coagulation following intrajugular injection of collagen (Fig. 2): 14/17 (82%) TM transgenic mice survived compared to 9/19, (47%) of non-transgenic control mice, in this systemic thromboembolism model[21].

Tissue Factor Pathway Inhibitor

The pcDNA3/TFPI was initially cloned into pCEP4 and transfected cells were used to study the specific anticoagulant activity by inhibition of factor Xa activity in an indirect assay (TF-dependent pathways)[31,32]. Minimal TFPI expression was demonstrated. Given that this anticoagulant may be lost from the cell surface with shedding of heparan sulphate, TFPI was then cloned with a glycosyl phosphatidylinositol (GPI) anchor, as we have done for von Willebrand factor A1 domains[33]. This approach has had more success and the validated GPI-linked TFPI has been used to generate transgenic mice. A single male transgenic mouse was generated (to date) and the offspring analyzed. The derived mice have normal tail bleeding times; coagulation assays (activated partial thromboplastin time and thrombin time) with

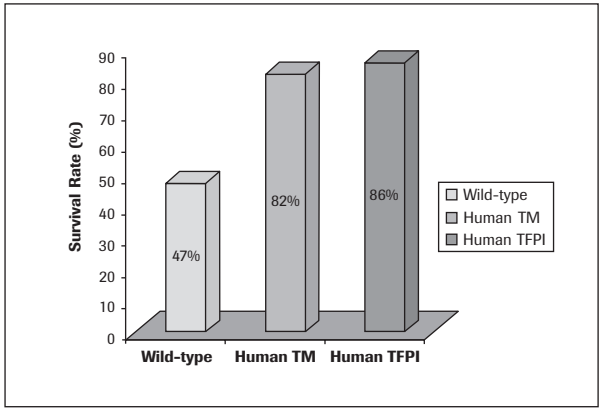

Figure 2. Thromboembolism model. *Transgenic mice overexpressing TM and TFPI have increased rates of survival (% rate on vertical axis) following injection of 0.75 μg collagen per gram mouse body weight.*

normal whole blood and washed platelet aggregation times in response to collagen and thrombin (not shown). The transgenic mice showed significantly greater survival (12/14, 86%) than non-transgenic controls (9/19, 47%), following intrajugular collagen injection (Fig. 2).

Conclusions

This application to ROTRF provided funding over 2 years to aid in the investigation of the mechanisms of vascular injury in thrombotic microangiopathy, to examine upregulation of selected human thromboregulatory factors in xenogeneic cells and to finally generate transgenic mice overexpressing human anti-thrombotic and anti-coagulant proteins. This work was all performed in close collaboration with AJ d'Apice, PJ Cowan and KM Dwyer who derived the transgenic mice. Together, we have examined the utility of this approach in vascular thromboembolism and in transplantation models. Our data suggest the potential utility of this approach in correcting disordered thromboregulation associated with conditions of vascular inflammatory stress.

Publications

1. Platt JL, Lin SS, McGregor CGA. Acute vascular rejection. *Xenotransplantation* 1998; 5:169-75. Review.
2. Bach FH, Winkler H, Ferran C, Hancock WW, Robson SC. Delayed xenograft rejection. *Immunology Today* 1996; 17(8):379-84. Review.
3. Bach FH, Robson SC, Ferran C, Winkler H, Milan MT, Stuhlmeier KM, Vanhove B, Blakely ML, van der Werf WJ, Hofer E, et al. Endothelial cell activation and thromboregulation during xenograft rejection. *Immunol Rev* 1994; 141(5):5-30. Review.
4. Ierino FL, Kozlowski T, Siegel JB, Shimizu A, Colvin RB, Banerjee PT, Cooper DK, Cosimi AB, Bach FH, Sachs DH, Robson SC. Disseminated intravascular coagulation in association with the delayed rejection of pig-to-baboon renal xenografts. *Transplantation* 1998; 66(11):1439-50.
5. Kozlowski T, Fuchimoto Y, Monroy R, Bailin M, Martinez-Ruiz R, Foley A, Xu Y, Awwad M, Fishman J, Andrews D, Ritzenthaler J, Sablinski T, Ierino FL, Sachs DH. Apheresis and column absorption for specific removal of Gal-α-1,3 Gal natural antibodies in a pig-to-baboon model. *Transplant Proc* 1997; 29(1-2):961.

6. Buhler L, Basker M, Alwayn I, Goepfert C, Kitamura H, Kawai T, Gojo S, Kozlowski T, Ierino FL, Awwad M, Sachs DH, Sackstein R, Robson SC, Cooper DK. Coagulation and thrombotic disorders associated with pig organ and hemopoietic cell transplantation in non-human primates. *Transplantation* 2000; 70:1323-31.
7 Buhler L, Goepfert C, Kitamura H, Basker M, Gojo S, Alwayn IP, Chang Q, Down JD, Tsai H, Wise R, Sachs DH, Cooper DK, Robson SC, Sackstein R. Porcine hematopoietic cell xenotransplantation in nonhuman primates is complicated by thrombotic microangiopathy. *Bone Marrow Transplant* 2001; 27(12):1227-36.
8. Alwayn IPJ, Buhler L, Appel JZ 3rd, Goepfert C, Csizmadia E, Correa L, Harper D, Kitamura H, Down J, Awwad M, Sackstein R, Cooper DK, Robson SC. Mechanisms of thrombotic microangiopathy following xenogeneic hematopoietic progenitor cell transplantation. *Transplantation* 2001; 71(11):1601-9.
9. Appel JZ, Alwayn IPJ, Buhler L, DeAngelis HA, Robson SC, Cooper DKC. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. I. The roles of individual components of a transplantation conditioning regimen and of pig peripheral blood progenitor cells. *Transplantation* 2001; 72(7):1299-1305.
10. Appel JZ, Alwayn IPJ, Correa LE, Cooper DKC, Robson SC. Modulation of platelet aggregation in baboons: implications for mixed chimerism in xenotransplantation. II. The effects of cyclophosphamide on pig peripheral blood progenitor cell-induced aggregation. *Transplantation* 2001; 72(7):1306-10.
11. Buhler L, Yamada K, Kitamura H, Alwayn IP, Basker M, Appel JZ 3rd, Colvin RB, White-Scharf ME, Sachs DH, Robson SC, Awwad M, Cooper DK. Pig kidney transplantation in baboons: anti-Gal α 1-3Gal IgM alone is associated with acute humoral xenograft rejection and disseminated intravascular coagulation. *Transplantation* 2001; 72(11):1743-52.
12. Sachs DH, Sykes M, Robson SC, Cooper DKC. Xenotransplantation. *Adv Immunol* 2001; 79(79):129-223. Review.
13. Robson SC. Disordered regulation of coagulation and platelet activation in xenotransplantation. In: Xenotransplantation. Basic Research and Clinical Applications. Jeffrey Platt, ed. Humana Press 2002; 215-46. Review.
14. Robson SC, Goodman D. Leukocyte adhesion and activation in xenografts. Birkhauser Verlag, Basel, Switzerland, 1999.
15. Robson S, Schulte am Esch II J, Bach F. Factors in xenograft rejection. *Ann NY Acad Sci* 1999; 875:261-76.
16. Alwayn IPJ, Buhler L, Basker M, Goepfert C, Kawai T, Kozlowski T, Ierino F, Sachs DH, Sackstein R, Robson SC, Cooper DK. Coagulation/thrombotic disorders associated with organ and cell xenotransplantation. *Transplant Proc* 2000; 32(5):1099.
17. Robson SC, Kaczmarek E, Siegel JB, Candinas D, Koziak K, Millan M, Hancock WW, Bach FH. Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J Exp Med* 1997; 185(1):153-63.
18. Alwayn IPJ, Appel JZ, Goepfert C, Buhler L, Cooper DKC, Robson SC. Inhibition of platelet aggregation in baboons: therapeutic implications for xenotransplantation. *Xenotransplantation* 2000; 7(4):247-57.
19. Appel JZ, Newman D, Awwad M, Gray HSK, Down J, Cooper DKC, Robson SC. Activation of human endothelial cells by mobilized porcine leukocytes *in vitro* – implications for mixed chimerism in xenotransplantation. *Transplantation* 2002; 73(8):1302-9.
20. Knosalla C, Giovino MA, Harper D, Kaczmarek E, Gollackner B, Cooper DK, Robson SC. Relative effects of GAL+ and GALlow/- porcine hematopoietic cells on primate platelet aggregation and endothelial cell activation: implications for the induction of mixed hematopoietic chimerism in the pig-to-primate model. *Xenotransplantation* 2004; 11:72-7.
21. Dwyer KM, Robson SC, Nandurkar HH, Campbell DJ, Gock H, Murray-Segal LJ, Fisicaro N, Mysore TB, Kaczmarek E, Cowan PJ, d'Apice AJ. Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. *J Clin Invest* 2004; 113(10):1440-6.
22. Robson SC. Acute vascular rejection/delayed xenograft rejection and consumptive coagulopathy in xenotransplantation. *Curr Opin Org Transplant* 2003; 8(1):76-82.
23. Robson SC, Enjyoji K, Goepfert C, Imai M, Kaczmarek E, Lin Y, Sevigny J, Warny M. Modulation of extracellular nucleotide-mediated signaling by CD39/nucleoside triphosphate diphosphohydrolase-1. *Drug Dev Res* 2001; 53(2-3):193-207.

24. Imai M, Takigami K, Guckelberger O, Kaczmarek E, Csizmadia E, Bach FH, Robson SC. Recombinant adenoviral mediated CD39 gene transfer prolongs cardiac xenograft survival. *Transplantation* 2000; 70(6):864-70.
25. Kopp CW, Grey ST, Siegel JB, McShea A, Vetr H, Wrighton CJ, Esch JSA, Bach FH, Robson SC. Expression of human thrombomodulin cofactor activity in porcine endothelial cells. *Transplantation* 1998; 66(2):244-51.
26. Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC. Identification and characterization of CD39 vascular ATP diphosphohydrolase. *J Biol Chem* 1996; 271(51):33116-22.
27. Dwyer K, Robson S, Nandurkar H, Mysore T, Murray-Segal L, Cowan P, d'Apice AJF. *Xenotransplantation* Sept 2003; 10(5):482.
28. Dwyer KM, Robson SC, Nandurkar HH, Mysore TB, Kaczmarek E, Cowan PJ, D'Apice AJF. *Am J Transplant Suppl May* 2003; 3(5):468.
29. Boquest AC, Grupen CG, Harrison SJ, McIlfatrick SM, Ashman RJ, d'Apice AJ, Nottle MB. Production of cloned pigs from cultured fetal fibroblast cells. *Biol Reprod* 2002; 66:1283-7.
30. Siegel JB, Grey ST, Lesnikoski BA, Kopp CW, Soares M, Esch J, Bach FH, Robson SC. Xenogeneic endothelial cells activate human prothrombin. *Transplantation* 1997; 64(6):888-96.
31. Kopp CW, Siegel JB, Hancock WW, Anrather J, Winkler H, Geczy CL, Kaczmarek E, Bach FH, Robson SC. Effect of porcine endothelial tissue factor pathway inhibitor on human coagulation factors. *Transplantation* 1997; 63(5):749-58.
32. Kopp CW, Robson SC, Siegel JB, Anrather J, Winkler H, Grey S, Kaczmarek E, Bach FH, Geczy CL. Regulation of monocyte tissue factor activity by allogeneic and xenogeneic endothelial cells. *Thromb Haemost* 1998; 79(3):529-38.
33. Esch JSA, Cruz MA, Siegel JB, Anrather J, Robson SC. Activation of human platelets by the membrane-expressed A1 domain of von-Willebrand-factor. *Blood* 1997; 90(11):4425-37.

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Gene Expression in Tolerogenic Dendritic Cells

Harnessing of natural tolerance processes remains one of the most desirable goals in transplantation[1]. While the exact dendritic cell (DC) subset/relative maturation state involved remains controversial[2], pharmacological agents can be used to generate defined populations of immune-modulated DCs *in vitro*[3-6]. The major goals of this project were to:

1. Use serial analysis of gene expression (SAGE) of pharmacologically-modified DCs to determine diagnostic gene-expression patterns, and also individual genes associated with tolerance.
2. Establish systems to probe the relevance to DC function, in particular with respect to tolerance, of candidate genes, identified both 'dogma-free' from SAGE and directed by literature.

1. Characterising pharmacologically modified DC using SAGE[7,8]

Data have been generated from murine DCs counter-modulated by three pharmacological agents, IL-10, TGFβI and 1α,25-dihydroxyvitamin D3 (VD3). In each case, the concentration of agent and the length of exposure required to drive bone marrow DC (bmDC) to a state in which they are unable to stimulate in an allogeneic mixed leukocyte reaction have been experimentally determined. LPS does not rescue these cells to render them immunogenic. SAGE libraries have been generated (Table I).

Our SAGE resource now includes ~50 immune related libraries (Table I). We have confirmed the reliability of inter-library and inter-laboratory library comparisons and determined the effective limit of short SAGE libraries as ~30 000 tags[7]. We have incorporated new SAGE technology generating tags extended by 7 bp; effecting increased transcriptome penetration, improved tag-to-gene annotation and simplified cloning of novel transcripts[7,9-11]. Global comparisons have identified transcripts associated with non-immunogeneic DCs and pharmacologically modified bmDCs (data not shown). While the functional relevance remains elusive, the relative expression of CCL6 has been confirmed by real-time PCR and Western blot analyses and represents a potential marker of DC tolerogenicity.

Cell population	Library source	Approx. tag numbers	NCBI GEO accession no.
bmDC	Waldmann	21 789	GSM3833
bmDC +LPS	Waldmann	13 085	GSM3832
bmDC +LPSlong	Waldmann*	31 588	
bmDC +IL10	Waldmann	30 455	GSM3834
(bmDC +IL10)+LPS	Waldmann	31 135	GSM3835
bmDC +vitD3	Waldmann	27 368	GSM3836
bmDC +TGF-β	Waldmann*	31 495	
ESF116-derived immature DC, iesDC	Waldmann	30 738	GSM3830
ES116-derived mature DC, esDC +LPS	Waldmann	31 751	GSM3831
ESF116, embryonic stem cell line	Waldmann	15 112	GSM3829
R1, embryonic stem cell line	SAGEMAP	137 906	GSM580
Splenic B cells	Waldmann	26 836	GSM3837
NIH 3T3 fibroblast line	SAGEMAP	28 531	(SAGENet)
Normal brain granular cells	SAGEMAP	61 526	GSM767

Plus, additional libraries including those detailed in Cobbold et al[7].

*Long SAGE libraries

Libraries that have been generated or expanded during the period of ROTRF funding are shaded grey

Table I. Summary of SAGE gene-profiling database.

Pairwise comparisons have demonstrated that IL-10 actively 'conditions' DCs to alter the quality of their subsequent response to stimuli. While their ability to initiate local inflammation and bacterial clearance is promoted, accompanied by a co-ordinate (presumably protective) increase in anti-inflammatory agents, their ability to activate and recruit T cells in response to microbial stimuli is apparently constrained[8]. How these observations relate to particular subsets of DCs and biological scenarios *in vivo* remains to be established. Global gene changes in response to VD3 and TGFβI suggest that these agents also act in a manner that is subtler than mere blocking of maturation.

A custom 70-mer oligonucleotide microarray has now been generated. In addition to transcripts identified from the literature and from parallel studies investigating genes associated with regulatory T cells, transcripts shown by SAGE to be associated with non-immunogenic DC populations are included. Rapid, simultaneous analysis of these transcripts will now be facilitated across multiple samples, including those obtained from *in vivo* tolerance models

and variously stimulated IL-10, VD3 and TGFβI modulated DC to investigate the extent and similarity of their immunological skewing.

2. Establishing relevance of candidate genes to DC function[8,12-18]

i) Use of embryonic stem cell-derived DC (esDC) to analyse candidate gene function

We previously demonstrated the differentiation of embryonic stem (ES) cells along the DC lineage[19]. Genetic manipulations can be achieved at the ES cell level, avoiding inherent difficulties associated with manipulating primary bmDC or the investment of generating manipulated mice as a source of bone-marrow[13]. The esDC undergo significant expansion *in vitro*, are phenotypically stable over time and retain the capacity to mature in response to LPS[13,19]. Comparative SAGE analysis of the parent ES cell line and differentiated esDC, (Table I), have provided further supportive data for the integrity of this system[7,12,17]. These SAGE libraries are now available for subtractive purposes to identify genes responsive to a transfected candidate-transcript.

The feasibility of generating stable lines of genetically-modified esDC has been verified using EGFP[13]. These EF1α-EGFP-esDC maintained their capacity for maturation in response to LPS, with no associated loss of transgene expression. We have demonstrated that there is no perturbation of induced CCR7 expression (real-time PCR), and the genetically modified esDC retain their migration patterns *in vivo*. Bicistronic plasmid expression vectors based on the strong constitutive promoter EF1α have been established.

Achieving RNA interference in esDC will require long-term stable expression of short hairpin RNAs (shRNAs). To this aim we have cloned shRNA sequences targeted against IL-12 p40 (Ambion) to the pol II based expression vector pSilencer 4.1-CMV neo (Ambion). Knockdown of IL12-p40 is predicted to skew the esDC from a conventional Th1 to a Th2 biased response[19].

Significant progress has been made towards establishing an *in vivo* readout to access the tolerogenicity of modified esDC using TCR transgenic A1xRAG$^{-/-}$ mice. These mice are specific for the male antigen Dby presented in the context of H2E^{k} and provide a model of transplant rejection by CD4^{+} T cells in response to a minor transplantation antigen[21]. CBA/Ca ES cell lines karyotypically male and female have previously been established and naïve female A1xRAG$^{-/-}$ T cells respond vigorously to male, but not female esDC. We have demonstrated that administration of immature or pharmacologically modified male CBA/Ca bmDC to female A1xRAG$^{-/-}$ mice prior to grafting skin from a male donor prevents rejection. This tolerance is antigen-specific, indefinite and dominant and involves an accumulation of FoxP3+ regulatory T cells in the accepted graft[18]. This system will be used to assess the impact of male esDC modifications on their ability to confer allograft survival and invoke tolerance.

ii) Investigation of specific candidate genes

Preliminary inspection of the modulated DC SAGE data indicates a number of tags for which there is no gene currently assigned. Particularly using the longer SAGE tags, it should be possible to clone the corresponding transcripts for functional investigation. We have characterised a novel CXCR2 ligand, DCIP-1, using such methodology, LPS induction of which was not perturbed by pre-incubation with IL-10[8].

DC lacking CD40 confer suppression involving the induction of antigen-specific regulatory T cells[22]. As such, esDC lacking CD40 expression offer potential as pre-tolerisation agents in future stem cell-derived cell and organ replacement strategies[14,16,17]. We have described five alternatively spliced forms of CD40, including a dominant negative type III isoform[15]. Preliminary data have indicated that esDC over-expressing this isoform are rendered incapable of T cell priming. We have also established shRNA constructs to target knockdown of CD40 and the most effective constructs will be used to transfect male and female ES cells.

Indoleamine 2,3-dioxygenase (IDO) is responsible for catabolism of tryptophan and has been implicated in dominant tolerance[23,24]. To investigate whether stable expression of IDO by esDC might confer protection from rejection and permit polarisation of T cells to a regulatory phenotype, we have generated male and female IDO-transfected esDC lines to be assessed in A1xRAG1$^{-/-}$ mice.

Agonist antibodies against glucocorticoid-induced tumor necrosis factor receptor (GITR) neutralise the suppressive activity of CD4^{+}CD25^{+} regulatory T cells[25]. Using a GITR-Fc fusion protein we have demonstrated a significant increase in binding to bmDC conditioned by exposure to IL-10. We have now cloned the murine GITR-ligand and confirmed that its interaction with GITR reverses suppression by CD4^{+}CD25^{+} T cells[15]. The relevance of GITRL expression on IL-10 modulated DC is as yet undetermined, but is increased following exposure to LPS. It remains to transfect GITR-L into ES cells and to establish the impact of the resulting esDC in the A1xRAG$^{-/-}$ allograft rejection model.

Publications

1*. Waldmann H, Cobbold S. Exploiting tolerance processes in transplantation. *Science* 2004; 305(5681):209-12.
2. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* 2002; 99(1):351-8.
3. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002; 99(7):2468-76.
4. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 2001; 98(12):6800-5.
5. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol* 2004; 4(1):24-34.
6. Streilein JW, Masli S, Takeuchi M, Kezuka T. The eye's view of antigen presentation. *Hum Immunol* 2002; 63(6):435-43.

7*. Cobbold SP, Nolan KF, Graca L, Castejon R, Le Moine A, Frewin M, Humm S, Adams E, Thompson S, Zelenika D, Paterson A, Yates S, Fairchild PJ, Waldmann H. Regulatory T cells and dendritic cells in transplantation tolerance: molecular markers and mechanisms. *Immunol Rev* 2003; 196:109-24.
8*. Nolan KF, Strong V, Soler D, Fairchild PJ, Cobbold SP, Croxton R, Gonzalo JA. Rubio A, Wells M, Waldmann H. IL-10-conditioned dendritic cells, decommissioned for recruitment of adaptive immunity, elicit innate inflammatory gene products in response to danger signals. *J Immunol* 2004; 172(4):2201-9.
9. Akmaev VR, Wang CJ. Correction of sequence-based artifacts in serial analysis of gene expression. *Bioinformatics* 2004; 20(8):1254-63.
10. Lu J, Lal A, Merriman B, Nelson S, Riggins G. A comparison of gene expression profiles produced by SAGE, long SAGE, and oligonucleotide chips. *Genomics* 2004; 84(4):631-6.
11. Saha S, Sparks AB, Rago C, Akmaev V, Wang CJ, Vogelstein B, Kinzler KW, Velculescu VE. Using the transcriptome to annotate the genome. *Nat Biotechnol* 2002; 20(5):508-12.
12*. Fairchild PJ, Nolan KF, Waldmann H. Probing dendritic cell function by guiding the differentiation of embryonic stem cells. *Methods Enzymol* 2003; 365:169-86.
13*. Fairchild PJ, Nolan KF, Cartland S, Graca L, Waldmann H. Stable lines of genetically modified dendritic cells from mouse embryonic stem cells. *Transplantation* 2003; 76(3):606-8.
14. Fairchild PJ, Cartland S, Nolan KF, Waldmann H. Embryonic stem cells and the challenge of transplantation tolerance. *Trends Immunol* 2004; 25(9):465-70.
15*. Tone M, Tone Y, Adams E, Yates SF, Frewin MR, Cobbold SP, Waldmann H. Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc Natl Acad Sci U S A* 2003; 100(25):15059-64.
16*. Fairchild PJ, Nolan KF, Cartland S, Waldmann H. Embryonic stem cells: a novel source of dendritic cells for clinical applications. *Int Immunopharmacol* 2005; 5(1):13-21.
17 Fairchild PJ et al. Cell replacement therapy and the evasion of destructive immunity. *Stem Cell Rev.* (In press)
18*. Yates SF et al. Vitamin D3 reinforces the tolerogenic phenotype of immature dendritic cells. *Nat Med. (Manuscript in preparation)*
19. Fairchild PJ, Brook FA, Gardner RL, Graca L, Strong V, Tone Y, Tone M, Nolan KF, Waldmann H. Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr Biol* 2000; 10(23):1515-8.
20. Kumar R, Conklin DS, Mittal V. High-throughput selection of effective RNAi probes for gene silencing. *Genome Res* 2003; 13(10):2333-40.
21. Zelenika D, Adams E, Mellor A, Simpson E, Chandler P, Stockinger B, Waldmann H, Cobbold SP. Rejection of H-Y disparate skin grafts by monospecific CD4+ Th1 and Th2 cells: no requirement for CD8+ T cells or B cells. *J Immunol* 1998; 161(4):1868-74.
22. Martin E, O'Sullivan B, Low P, Thomas R. Antigen-specific suppression of a primed immune response by dendritic cells mediated by regulatory T cells secreting interleukin-10. *Immunity* 2003; 18(1):155-67.
23*. Mellor AL, Chandler P, Baban B, Hansen AM, Marshall B, Pihkala J, Waldmann H, Cobbold S, Adams E, Munn DH. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. *Int Immunol* 2004; 16(10):1391-401.
24. Grohmann U, Fallarino F, Puccetti P. Tolerance, DCs and tryptophan: much ado about IDO. *Trends Immunol* 2003; 24(5):242-8.
25. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; 3(2):135-42.

Publications arising in association with this work

26 Fairchild PJ, Cartland S, Nolan KF, Waldmann H. Embryonic stem cells and the challenge of transplantation tolerance. *Trends Immunol* 2004; 25:465-70.
27 Fairchild PJ, Robertson NJ, Cartland S, Nolan KF, Waldmann H. Cell replacement therapy and the evasion of destructive immunity. *Stem Cell Rev.* (*In press*)
28 Paterson AM, Yates SF, Nolan KF, Waldmann H. The new immunosuppression: intervention at the dendritic cell-T-cell interface. *Curr Drug Targets - Immune, Endocrine and Metabolic disorders. (In press)*

All publications marked with *

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Use of a Death Decoy Protein DcR3/TR6 to Treat Organ Graft Rejection

With the financial support of the one-year ROTRF grant, we have published three articles in 2004 in high impact journals[1-3].

We have also significantly advanced our research on organ transplantation using DcR3/TR6 transgenic (Tg) mice. Initially, we used the CMV promoter to drive TR6 expression, but the expression was lost in the adulthood. We then generated actin promoter-driven TR6 Tg mice. The TR6 Tg expression in these mice was of high level and stable. TR6 Tg was superior to adenovirus-mediated TR6 expression, because the former had long-term stable TR6 secretion. We decided to use the TR6 Tg model for our study. *In vitro*, we cultured isolated TR6 Tg T cells with solid phase anti-CD3 and anti-CD28, or employed mixed lymphocyte culture, to compare their proliferation with that of wild-type T cells. *In vivo*, we used FITC ear painting to measure the delayed-type hypersensitivity (DTH), which is essential in graft rejection. The lymphocyte proliferation and DTH of TR6 Tg mice were all significantly decreased compared to that of the wild-type mice. These results indicate that soluble TR6 effectively dampens cellular immune responses, and imply possible therapeutic applications of soluble TR6 in controlling graft rejection.

We backcrossed the TR6 Tg mice into the C57BL/6 background for eight generations. We titrated suboptimal cyclosporin A (CsA) dosage in mouse heart transplantation. We examined the chronic graft rejection in TR6 Tg mice transplanted with BALB/c heart in the presence of suboptimal CsA. These mice had reduced pathological findings of chronic rejection in their vascular system, compared to CsA administration alone. These results demonstrate that soluble TR6 is useful in reducing chronic graft rejection.

Publications

1. Wu Y, Han B, Luo H, Shi G, Wu J. Dipeptide boronic acid, a novel proteasome inhibitor, prevents islet-allograft rejection. *Transplantation* 2004; 78:360-6.
2. Luo H, Yu G, Tremblay J, Wu J. EphB6-null mutation results in compromised T cell function. *Clin Invest* 2004; 114:1762-73.
3. Yu G, Luo H, Wu Y, Wu J. EphrinB1 is essential in T-cell-T-cell co-operation during T-cell activation. *Biol Chem* 2004; 279:55531-9.

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Prevention of Phagocyte-Mediated Xenograft Rejection by Targeting Immune Inhibitory Receptors

Specific Aim

To determine whether or not augmentation of CD47-CD172a and/or CD200-CD200R interactions can attenuate porcine xenograft destruction by macrophages.

CD172a, an immune inhibitory receptor on macrophages, plays a critical role in preventing self-destruction by macrophages. Cells that express no or low levels of CD47, the ligand for CD172a, are phagocytosed by macrophages. Moreover, the strength of CD47-delivered inhibitory signals also regulates the susceptibility of antibody- and complement-opsonized cells to phagocytosis. Recently, the interaction of CD200, a membrane protein expressed widely throughout the body, with its receptor (CD200R) on macrophages was demonstrated as necessary in restraining the activation of tissue macrophages. It has been reported that the absence of a CD200-CD200R interaction leads to accelerated activation and expansion of tissue macrophages, and that macrophage activity can be downregulated if their surrounding cells express high levels of CD200. Thus, we hypothesize that the species specificity in CD47 and/or CD200 may account for the high susceptibility of xenografts to cytotoxicity by macrophages.

In the past year, we have obtained considerably interesting results that provide significant insights into the understanding of the role of CD47 in controlling macrophage activation and phagocytosis of xenogeneic cells. We have established various methods for measuring phagocytosis including flow cytometry- and confocal microscope-based *in vitro* assays and adoptive cell transplantation assays for assessing phagocytosis *in vivo*, and used these assays to investigate the role of CD47-CD172a interaction in xenograft rejection by macrophages.

1. Porcine CD47 does not efficiently interact with the mouse CD172a.

CD172a contains intracellular immune receptor tyrosine-based inhibitory motifs (ITIMs). CD172a activation upon CD47 engagement results in tyrosine phosphorylation of ITIMs, leading to the recruitment and activation of protein tyrosine phosphatases. To determine whether or not porcine CD47 can interact with mouse CD172a, we compared CD172a

tyrosine phosphorylation in bone marrow (BM)-derived macrophages after contact with porcine, CD47 KO and wild-type (WT) mouse RBCs. Incubation of macrophages with WT mouse RBCs resulted in significant CD172a tyrosine phosphorylation. However, neither CD47 KO mouse nor porcine RBCs were able to stimulate CD172a tyrosine phosphorylation compared to control macrophages that were incubated in media alone (data not shown).

We also measured the effect of anti-mouse CD172a backing mAb (P84) on phagocytosis of porcine cells by mouse macrophages using an *in vitro* phagocytic assay. Previous studies have shown that P84 can augment phagocytosis of $CD47^{+/+}$ target cells by blocking CD47-CD172a interactions in WT mice. P84 should not affect the phagocytosis of pig RBCs by mouse macrophages if porcine CD47 fails to cross-react with murine CD172a. In these experiments, mouse macrophages were incubated in media with or without P84 for 20 minutes prior to the addition of target cells (i.e., CD47 KO mouse, WT mouse, and porcine RBCs). In the cultures without P84, $CD47^{-/-}$ mouse RBCs and pig RBCs were more efficiently phagocytosed by macrophages compared to the background (i.e., the percentage of WT macrophages engulfing WT mouse RBCs; $P<0.01$). Consistent with previous studies, blocking CD172a with P84 led to a significant increase in engulfment of WT mouse RBCs, but did not affect phagocytosis of CD47 KO mouse or porcine RBCs by WT macrophages (data not shown). Together, the above described results (data not shown) indicate that pig CD47 cannot deliver inhibitory signals to mouse macrophages through CD172a receptor.

The lack of functional interaction between porcine CD47 and mouse CD172a was also suggested by *in vivo* experiments. It has been shown that macrophages in CD47 KO mice, for unknown reasons, are adapted and do not phagocytose $CD47^{-/-}$ cells. Thus, we compared the survival of porcine cells after being injected into CD47 WT versus KO B6 mice. CFSE-labeled pig RBCs were injected into WT or $CD47^{-/-}$ mice; blood was collected from the recipient mice at various times and the levels of injected porcine RBCs were measured by flow cytometric analysis. While porcine RBCs were completely rejected in both WT and CD47 KO mice, the clearance of porcine RBCs from blood was significantly delayed in CD47 KO mice whose macrophages do not phagocytose $CD47^{-/-}$ mouse cells (data not shown). We acknowledge that anti-pig xenoresponses by T cells, B cells, and NK cells also contribute to the rejection of pig RBCs in the mouse recipients. However, the dramatic difference in the clearance of pig RBCs between WT and CD47 KO mice suggests that macrophages are essential to the early rejection of cellular xenografts.

2. Mouse CD47 expression on pig cells reduces their susceptibility to phagocytosis by mouse macrophages.

To determine whether expression of murine CD47 on pig cells may confer protection from phagocytosis by mouse macrophages, we have generated mouse CD47-expressing

(mCD47) porcine cell lines by transfection of porcine B lymphoma-like cells (LCL-13271) with a mouse CD47 expressing plasmid (data not shown). We compared the survival and expansion of murine CD47-transfected (LCL-mCD47) and null-neo-transfected control (LCL-neo) cells in cultures containing mouse macrophages. LCL-mCD47 and LCL-neo cells were labeled with different fluorescent dyes (red or green) and co-cultured at a 1:1 ratio in the presence and absence of mouse macrophages. The cultures were harvested daily for 3 days and the percentages of LCL-mCD47 and LCL-neo cells in the cultures were determined by flow cytometry. The ratio of LCL-mCD47 to LCL-neo cells was significantly increased in the presence of mouse macrophages but remained unchanged in the absence of macrophages (data not shown).

Taken together, our results implicate that the lack of a proper interaction between donor CD47 and the recipient CD172a increases the susceptibility of xenogeneic cells to phagocytosis. Thus, genetic manipulation of donor CD47 to improve its interaction with the recipient CD172a may provide a novel approach to preventing macrophage-mediated xenograft rejection.

3. Establishment of human CD47-expressing porcine cell lines for determining the effect of human CD47 expression on phagocytosis of porcine cells by human macrophages.

Full length of human CD47 cDNA in a pEF-Bos-Myc vector (pEF-Bos-Myc-hCD47, kindly provided to us by Dr. Ohdan at the Hiroshima University, Japan) was cloned into an eukaryotic expression vector pCDNA-3 to generate a human CD47-expression plasmid. After the orientation and sequence of the human CD47 cDNA insert was confirmed by DNA sequencing, the plasmid construct was used to transfect a porcine B lymphoma-like cell line, LCL13271, to establish human CD47-expressing porcine cell lines. As described above, mouse CD47 expression on LCL13271 cells reduces their susceptibility to phagocytosis by mouse macrophages. To date, we have performed two transfection experiments. However, for unknown reasons, all G418-resistant clones we have obtained showed no detectable surface expression of human CD47. We are currently performing molecular analyses to determine whether the lack of CD47 surface expression was due to the transfection failure or to a defect in human CD47 transcription or expression in porcine cells. If human CD47 cDNA cannot be detected in porcine cell transfectants by DNA PCR, we will need to generate a new human CD47-expression construct using different expression plasmids. However, if porcine cell transfectants contained human CD47 cDNA (i.e., positive by DNA PCR), but failed to transcript (i.e., negative by RT-PCR) or express (i.e., negative by Western and FACS analyses), we will try other porcine cell lines or generate chimeric CD47 that contains the extracellular portion of human CD47 and the transmembrane and intracellular portions of porcine CD47 for these studies.

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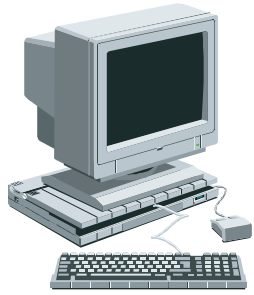

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