R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

October 2002

The Roche Organ Transplantation Research Foundation

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

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

The funding of the Foundation consists of a donation from F. Hoffmann-La Roche Ltd, with an initial sum of 25 million Swiss francs over the first five years and a renewal donation of 15 million Swiss francs over the following three years. The funds are disbursed 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

For this eighth funding cycle we are pleased to announce the distribution of a total of 2.1 million Swiss Francs (CHF) to 12 scientists from around the world, with the majority coming from the United States but also from the United Kingdom, France, Canada, Italy and Australia.

I would like to introduce two new members to the ROTRF Board of Trustees: Dr. Jim Woody from Roche Bioscience in Palo Alto, USA, and Prof. Jean-Paul Soulilou of Nantes, France, who already served on our Scientific Advisory Committee. At the same time I would like to thank Prof. Hans Wigzell of Stockholm, Sweden, and Dr. Robert Booth of Palo Alto, USA, for their work and commitment for the Board of Trustees. Prof. Wigzell and Dr. Booth were both on the board since the founding of the ROTRF and both left the board in 2002.

The Progress and Final Reports published in this Biannual Report demonstrate the high quality of work that has been carried out over the last few years with the support of the ROTRF. Many papers have been published in respected international journals and we are very hopeful that the number of papers being published will grow over the next few years. All this is thanks to the continued support of Roche, which has extended its commitment to the international transplant community with an additional CHF 15 million (as already publicised in our last Biannual Report).

The ROTRF continues to fulfil its mission to advance the science of solid organ transplantation. Much of the credit for the success goes to the scientists who send their projects to the ROTRF and to the excellent reviews of the Scientific Advisory Committee (SAC), whose members will be introduced in this Biannual Report.

In summary, the Foundation represents a unique achievement: an internationally refereed competition in transplantation sciences founded by our benefactor, but run by the scientific community. The mission of the ROTRF is the advancement of the science of organ transplantation and the needs of the people who require this service. We would like to express our gratitude to all those who have supported the ROTRF over the last four years, especially Roche for funding this charity, and the scientists who serve as trustees and advisors. Thanks as well to Dr. Sabine Stotz who provides an excellent service in operating our office.

On behalf of the Board of Trustees

Phil Halloran

2. Facts and Figures

Funding Cycle VIII – Letter of Intent Submission in April 2002

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

The ROTRF received 108 letters of intent from scientists around the world. Almost half of the applications came from the United States and Canada (46%), while 45% came from Europe, the major countries being the UK (13%), France (7%) and Germany (7%). Australia and Japan accounted for the remaining 9% of the applicants.

All the letters of intent were reviewed by the Scientific Advisory Committee for scientific quality and originality, and based on these reviews the Board of Trustees invited the 20 top-ranked applicants to prepare full paper submissions.

ROTRF grants were awarded to 12 applicants: five from the USA, two each from Canada and the UK, and one each from Italy, France and Australia.

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

Statistics on Applications to the ROTRF

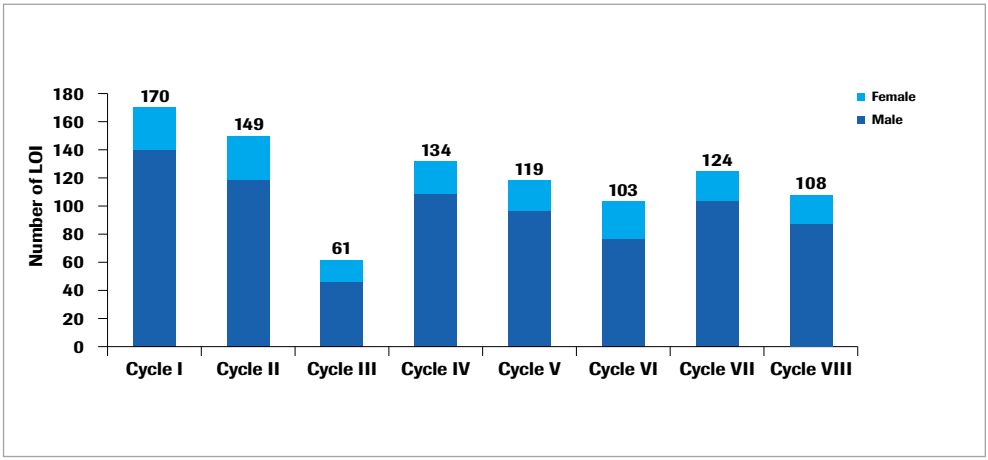


Figure 1. *Number and gender of applicants who submitted letters of intent (LOI) to the ROTRF during the first eight ROTRF funding cycles.*

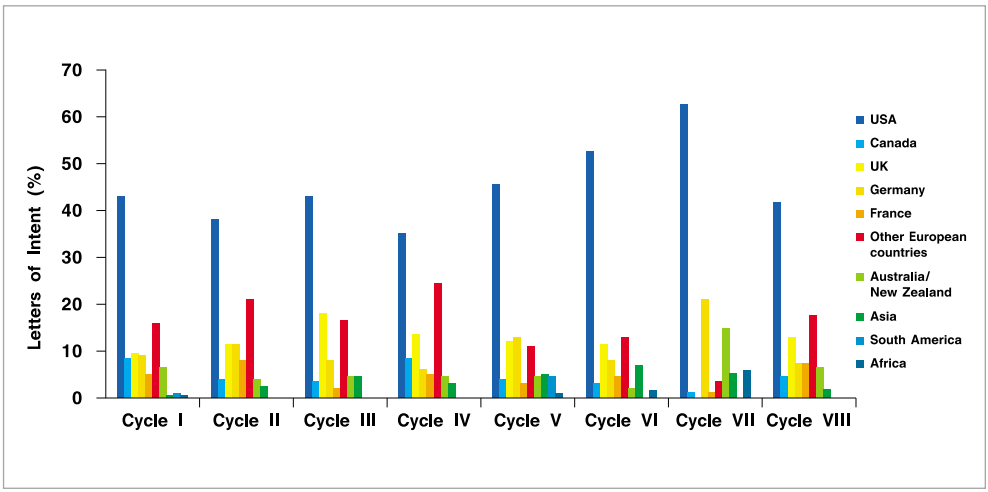
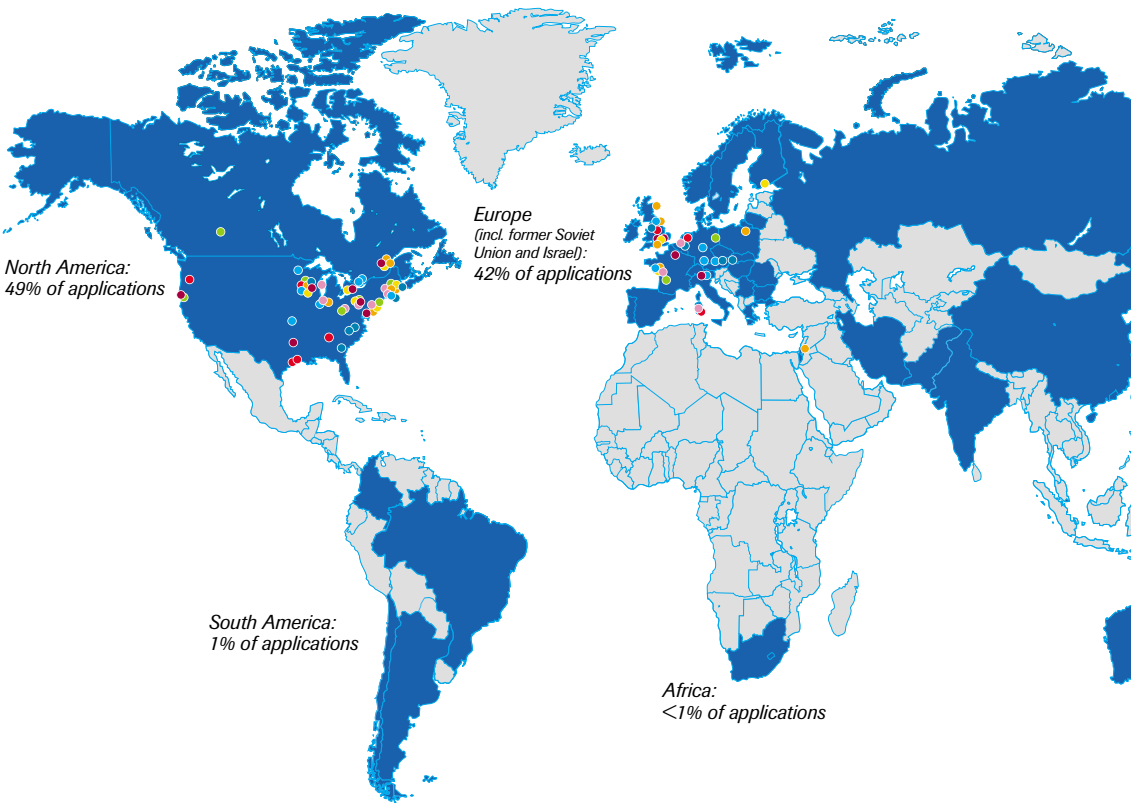
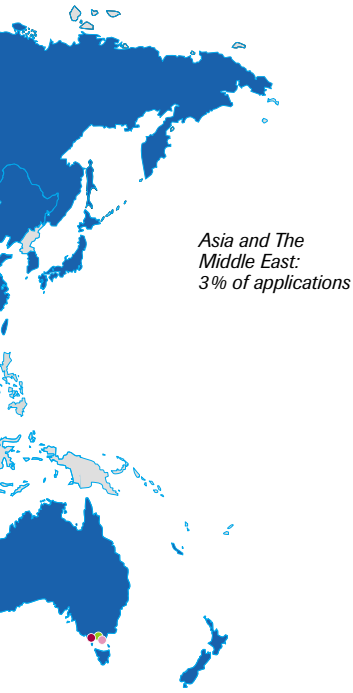


Figure 2. *Geographical distribution of the applicants who submitted letters of intent (LOI) during the first eight ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of the ROTRF applications worldwide





Asia and The Middle East: 3% of applications

Australia: 5% of applications

at least one application ever received

no application received

Cycle I Grantees

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

Cycle II Grantees

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

Cycle III Grantees

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

Cycle IV Grantees

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

Cycle V Grantees

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

Cycle VI Grantees

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

Cycle VII Grantees

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

Cycle VIII Grantees

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

3. Grant Awards in Cycle VIII

Dr. Tausif Alam, Principal Investigator
Dr. Michael MacDonald, Consultant
Dr. James Malter, Consultant
Dr. John Young, Consultant

University of Wisconsin Medical School, Madison, USA

Glucose-Regulated Improved Insulin Production from Hepatocytes

Insulin-dependent diabetes mellitus (IDDM) is caused by selective autoimmune destruction of insulin-producing ß-cells of endocrine pancreas. Two therapies are currently available for IDDM and both have serious limitations. The first therapy, based on daily insulin injections, inadequately controls hyperglycemia and consequently does not prevent the long-term damage associated with the disease. The second therapy, transplantation of the whole pancreas or of pancreatic islets precisely regulates blood sugar levels, but limited availability of organs and lifelong use of immunosuppression limit the usefulness of this therapy. Advances in cellular and molecular engineering now make it possible to attempt replacing the function of a ß-cell by a non-ß-cell, engineered to provide insulin only when needed.
Our novel approach is based on recipient's own liver cells, engineered for glucose-regulated *de novo* synthesis and secretion of insulin, eliminating the need to duplicate a complex and incompletely understood mechanism of regulated insulin secretion of ß-cells. The preliminary studies performed in cell culture and in diabetic rats support our hypothesis. The proposed use of vectors that allow long-term expression and optimization of our gene constructs will probably correct hyperglycemia in diabetic rats and lay the foundation for IDDM treatment by insulin gene-therapy in man. Using our approach, the diabetic recipients of insulin-gene-engineered hepatocytes may experience a transient, mild hyperglycemia in the minutes after eating, but should be able to avoid the chronic hyperglycemia that typically occurs with insulin injections alone, avoiding severe diabetes-associated complications. Success in our approach will provide a basis for future gene-therapy-based IDDM treatment that could be administered before the development of hyperglycemia-related serious complications. Furthermore, the use of autologous liver cells may eliminate the necessity for immunosuppression.

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

In addition to the well-publicized "embryonic stem cells", pre-implantation-stage embryos also contain stem cells that give rise to placental structures. From early rat embryos, we have recently isolated stem cells of unknown identity that are unlikely to be embryonic stem cells and might be placental. These rat cells show the unusual ability to avoid destruction by the immune system of immunologically different rats, which is reminiscent of the ability of the embryo to avoid immune rejection by the mother. We term this property "stem cell immune resistance". Moreover, when rat stem cells from strain A were injected into strain B, and then hearts were transplanted from strain A to strain B, the hearts were no longer rejected. No medication was needed. Thus, these stem cells may confer tolerance of normally incompatible organ transplants. Mouse pre-implantation stem cells are much better known than stem cells from rats or any other species, but it is unknown whether they are immune resistant. Here, we want to assess how widespread the phenomenon of immune resistance is among extraembryonic and embryonic mouse stem cells, and at the same time determine the identity of our rat stem cells. Knowing the cellular scope of the new phenomenon of immune resistance will allow its systematic exploration and practical use in organ transplantation and tissue repair.

Dr. Kenneth Fang, Principal Investigator
Mr. Anthony Cruz, Research Associate

University of California, San Francisco, USA

Role of Mast Cells in Bronchiolitis Obliterans in a Tracheal Allograft Model of Lung Transplantation

Long-term survival of lung transplant recipients is limited by the development of airway scarring known as bronchiolitis obliterans, which causes irreversible airway narrowing and ventilatory failure. Acute and chronic graft rejection, ischemia-reperfusion injury, and cytomegaloviral infections may contribute to the development of the disease. However, current immunosuppressive regimens do not prevent the disease, and understanding of molecular pathways contributing to bronchiolitis obliterans remains poor. Lung transplant tissues demonstrate an overabundance of mast cells in the vicinity of the airways and lymphocytic infiltrates, which correlates with the grade of rejection or extent of bronchiolitis obliterans. How mast cells participate in pathways of airway remodeling leading to the development of bronchiolitis obliterans remains unknown. Clarification of cell signaling pathways and mechanisms regulating mast cell degranulation of stored mediators may identify new pharmacologic targets that may attenuate the progression or prevent the onset of bronchiolitis obliterans. To study the role of mast cells, a tracheal transplantation model using donor and recipient mice tracheas will be used to examine the participation of mast cells in immunologic responses. Use of mice with spontaneous or engineered genetic mutations will permit transplantation experiments to determine the necessity of active Kit receptor tyrosine kinase, a mast cell signaling molecule, and whether mast cells are essential for the development of bronchiolitis obliterans. Mice with genetically engineered deficiencies or defects in proteases will permit investigation of the roles of serine proteases and metalloproteinases in the mast cell response to transplantation and the development of airway scarring. Comparing data of transplanted tracheas from mutant mice with those from wild-type mice should demonstrate whether mast cells and degranulation of serine proteases contribute to scarring and disease development, and whether activity of metalloproteinases influences mast cell behavior by regulating signaling mediated by the Kit receptor.

Dr. Marina Noris, Principal Investigator
Prof. Giuseppe Remuzzi, Co-Investigator
Dr. Carlo Alberto Redi, Co-Investigator
Dr. Maurizio Zuccotti, Co-Investigator

Mario Negri Institute for Pharmacological Research, Bergamo, Italy

Donor Stem Cell Infusion to Induce Allospecific Graft Tolerance

Donor-specific tolerance, defined as a state of systemic non-responsiveness to an allograft without the need for anti-rejection drugs is becoming increasingly the 'holy grail' of transplant physicians. Transplantation of allogeneic hematopoietic cells into irradiated hosts can lead to life-long donor-specific chimerism that is linked with permanent tolerance of donor organs or tissue transplants. In this regard, we recently documented that pre-transplant infusion of donor bone marrow cells or donor peripheral blood leukocytes into rats allowed indefinite survival of a subsequent kidney allograft. The above strategies however, expose graft recipients to the risk of graft-versus-host disease (GVHD). In addition, donor MHC-mismatched hematopoietic grafts are usually acutely rejected unless the host is vigorously myeloablated or immunosuppressed. Data are now emerging that the use of donor embryonic stem cells would circumvent the above hurdles. Given their immature immunological status these cells would not cause GVHD and would circumvent acute rejection of the allospecific immune response of the recipient. However, the use of embryo-derived stem cells is still far from being clinically applicable to solid organ transplantation, due to strong ethical issues and the limited availability of embryo-derived stem cells and solid organ grafts from the same donor. On the other hand, a large number of totipotent stem cells could be acquired by the 'nuclear transfer' technique, which consists of the genetic re-programming of the nucleus of the somatic differentiated cell. In this project we will assess in a mouse model of heart allografts, whether donor stem cells derived by 'nuclear transfer' can be applied to induce tolerance after solid organ transplantation.

Dr. Peta J. O' Connell, Principal Investigator
Dr. Joaquin Madrenas, Co-Investigator
Dr. Robert Zhong, Consultant
Dr. Li Zhang, Consultant

Robarts Research Institute, London, Canada

Induction of Transplantation Tolerance by Endogenous CD8α^+ Dendritic Cells

Dendritic cells (DC) are rare leukocytes uniquely specialized for immune surveillance and the induction of primary immune responses. Traditionally regarded as the instigators of rejection, it is now recognized that either donor or host DC, particularly those that are immature, can also modulate immune reactivity and the induction of tolerance to foreign and self antigens. Recent proposals attribute this dichotomy of DC function in part to the existence of distinct DC subsets, although it is likely that the state of DC maturation and/or activation and local environmental factors also play significant roles. DC can be divided into distinct subpopulations that differ in phenotype, function and microenvironmental location. Although human and mouse DC subtypes are phenotypically disparate, there is clear evidence for similar functional specializations. Mouse CD8α^+ DC (distinct from classically described myeloid DC) are the principal DC subtype identified in the thymus and have been implicated in the regulation of central and peripheral tolerance. Consistent with these reports, our preliminary observations have demonstrated that CD8α^+ DC possess an innate capacity to impair donor-specific reactivity and prolong organ allograft survival. The immune-regulatory effect is observed using CD8α^+ DC isolated directly from donor spleen, without requiring *in vitro* culture or sophisticated manipulation such as genetic engineering. We hypothesize that CD8α^+ donor DC suppress allo-antigen specific responses and prolong allograft survival in the absence of exogenous immunosuppression, either through the induction of regulatory T cells, and/or the deletion of alloreactive T cells. Testing of this hypothesis will place the investigators in a powerful position to develop a DC-based, alloantigen-specific strategy for the reliable induction of tolerance.

Dr. Julian Pratt, Principal Investigator
Prof. Steven Sacks, Head of Department

King's College, University of London, London, UK

T Cell Costimulation through Complement Receptors in Kidney Transplantation

We propose that complement activated and deposited at the site of tissue injury promotes T cell activation, which can destroy transplanted organs. In this way damaged tissues in the graft could directly present sufficient signals to host T cells even in the absence of other molecules thought to be required to activate T cells. The activation of complement specifically at the site of tissue injury either through the classical or alternative pathway could therefore label the donor organ as damaged. T cells could then respond if they directly engage their specific antigen expressed by the "foreign" tissue of the graft. We hope to identify complement receptors expressed by T cells that can promote T cell activation and T cell responses independently of other pathways, such as CD28. Based on our preliminary data we expect to show that complement can mediate costimulation to T cells in the absence of and equivalent to costimulation through the usual pathways such as CD28. We will test our hypothesis by transplanting kidneys from allogeneic donors into C3-receptor-deficient hosts and hope to show prolongation of graft survival. These experiments will test the involvement of complement receptors in triggering an immune response in graft rejection.
This work could contribute to understand how innate immunity directs T cell responses. We hope to define a mechanism of injury that could assist self/non-self discrimination by adaptive immunity, now thought to be required in order to initiate an immune response. Transplantation activates complement either through ischemia-reperfusion injury or the involvement of immunoglobulins, and we have already shown that it is complement produced by the transplanted kidney itself that is most active in this process. We hope our study will define a new mechanism by which host T cells become activated so that therapeutic options can be investigated in the future.

Dr. Hamid Rabb, Principal Investigator
Dr. Lorraine Racusen, Consultant
Dr. Peter Heeger, Consultant
Dr. Sam Mohapatra, Consultant

Johns Hopkins University School of Medicine, Baltimore, USA

Mechanisms of T Cell Modulation of Renal Ischemia Reperfusion Injury

Kidney ischemia is the main cause of the initial poor function of an organ transplant. Kidney ischemia predisposes to acute rejection, which in turn predisposes to chronic rejection – the major long-term cause of graft loss. There is no specific treatment for kidney ischemia. Recently, we and others have identified that the circulating T cell is an important mediator of kidney ischemic damage. This is somewhat of a surprise finding, because the T cell is known to be important in rejection, but not in organ procurement injury. The observation that the T cell is a modulator of kidney ischemia has now been well verified in experimental models. However, the underlying mechanisms for this are unknown. We therefore propose to begin to elucidate these underlying mechanisms. Based on our preliminary data using a mouse model of kidney ischemia, we hypothesize that a major subset of T cells, called Th1, functionally mediates the T-cell-mediated kidney injury, and that T-cell-mediated injury requires engagement of the T cell receptor on its surface.

We plan to test this hypothesis using an established mouse model of kidney ischemia, various strains of generated mutant mice, sensitive measures of kidney function and molecular and cellular detection techniques. Our results will have implications towards the understanding of organ procurement injury and may help lead to new therapies. In addition, given the novel focus of our proposal, our results will also extend and challenge our basic understanding of the immune system.

Dr. Parmjeet Randhawa, Principal Investigator
Dr. Sidney Finkelstein, Co-Investigator

University of Pittsburgh, Pittsburgh, USA

Polyomavirus BK, JC and SV40 in the Kidney: Pathogenesis, Early Diagnosis and Improving Graft Outcome

The kidney is a common site for latent infection by polyomavirus. In healthy subjects with a functioning immune system, this latent infection is not associated with any symptoms. However, in kidney transplant recipients who receive potent immunosuppressive drugs to prevent rejection of the graft, latent virus can reactivate and result in a disease called polyomavirus nephropathy. At present, this disease is diagnosed at a rather late stage, and frequently results in loss of the transplanted organ.

This grant proposal will focus on early diagnosis of currently unrecognized and milder forms of this disease using the polymerase chain reaction (PCR), a technique that can amplify minute quantities of virus to a level that can be detected easily in the laboratory. Once a diagnosis of viral nephropathy has been made, physicians reduce the dosage of immunosuppressive drugs. This allows the patient's immune system to recover and try to get rid of the viral infection. We have recently started giving a drug called cidofovir to help combat polyomavirus infection in the kidney. Unfortunately, this drug can be quite toxic to the kidney if given for a prolonged period. The PCR test described above will allow us to determine quickly when polyomavirus has been eliminated from the patient's kidney, and thus minimize exposure to this potentially harmful drug. We will also use virus growing in the laboratory to identify less toxic alternative drugs for possible clinical use in patients with polyomavirus nephropathy.

Finally, the PCR test will be used to understand the changes in viral genetic configuration that allow transformation of silent or latent viral infection to an active disease, which interferes with optimal functioning of the transplanted kidney.

Dr. Benoît Salomon, Principal Investigator
Dr. José Cohen, Co-Investigator
Aurélie Trenado, Research Associate
Dr. Sylvain Fisson, Research Associate
Dr. Benoît Barrou, Research Associate
Dr. Frédéric Charlotte, Research Associate

Hôpital de la Pitié Salpêtrière, Paris, France

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

Significance and goal of our research

Solid organ transplantation necessitates the administration of non-specific immunosuppressive treatments. In some patients, these only imperfectly prevent graft rejection and can have severe side effects. Alternative treatments that induce specific tolerance to the graft are thus desirable. The goal of our project is to test this possibility by using a population of immunoregulatory $CD4^+CD25^+$ T cells (T_{reg}) that we and others have shown to be involved in the regulation of auto-immune diseases and graft-versus-host disease.

How can our goal be reached?

After transplantation, alloantigens (histocompatibility antigens) from the graft are presented either by donor (direct pathway) or recipient (indirect pathway) antigen-presenting cells. Indirect evidence suggests that prevention of allograft rejection by T_{reg} may be achieved by using cells previously selected to recognize specifically donor-type alloantigens through the direct and/or indirect pathways. In preliminary experiments, we were able to expand and select *in vitro* T_{reg} specific for alloantigens presented by the direct pathway that function *in vivo* to induce specific tolerance. In our research proposal, we will optimize this culture and set up new conditions for the generation of T_{reg} specific for alloantigens presented by the indirect pathway. These two types of T_{reg} will then be tested in parallel for their ability to control allograft rejection in mouse models of skin and islet transplantation. All these culture conditions should be transferable to the human setting. Thus, we will adapt the protocol for *in vitro* selection of alloantigen-specific T_{reg} in humans.

Possible impact of our research in medicine

The generation of alloantigen-specific T_{reg} may be used in the future in organ transplantation to induce long-term acceptance of the graft. This new therapeutic strategy may allow a reduction in the doses of the immunosuppressive drugs, thus limiting adverse events.

Prof. Mauro Sandrin, Principal Investigator

Austin Research Institute, Heidelberg, Australia

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

Advances in surgical and immunosuppressive techniques have led to organ transplantation as the method of choice for the treatment of many diseases. However, the number of suitable donors is dwindling, due to many factors, but largely as a result of the reduction in deaths from car accidents. Xenotransplantation, the transplantation of organs from species other than humans, is now seen as a viable solution to the world-wide problem of lack of supply of suitable human donors. The pig is the most suitable for a variety of reasons. However, the problem is that all humans contain natural antibodies to the pig, which would lead to rejection within a few minutes because the antibodies bind to the transplant and induce its rapid destruction (so called "hyperacute rejection"). Recent studies from our laboratory have indicated that most, if not all, of the antibodies react with the sugar galactose, which is present on many molecules on the surface of transplanted pig tissues. Our studies have indicated very large amounts of this material in pig blood vessels – guaranteeing the early rejection of transplanted organs such as kidney, heart and liver. The production of pigs that do not express galactose is an important pre-requisite for successful xenotransplantation. While mice that lack this sugar have been produced, and indeed form the basis of our models, the technology to perform this in pigs is not available. We have recently described an alternative strategy to reduce the amount of galactose expressed by transgenic animals, a technique that is suitable for the production of transgenic pigs. We will examine a number of different transgenic mice expressing a combination of genes that reduce the expression of galactose and determine the effects of these modifications on organ and cell transplantation. These studies will be the prelude to the production of pigs that could be used for human transplantation.

Prof. Herman Waldmann, Principal Investigator
Dr. Kathleen Nolan, Research Associate
Dr. Paul Fairchild, Research Associate
Dr. Stephen Cobbold, Research Associate
Mr. Mark Frewin, Research Associate
Mrs. Sue Humm, Research Associate

Oxford University, Oxford, UK

Gene Expression in Tolerogenic Dendritic Cells

The immune system is geared to react to dangerous foreign microbes. Such microbes are displayed or "presented" to the immune system on specialised antigen-presenting cells which, based on their morphology, are known as dendritic cells. Rejection of transplanted organs results from graft antigens being wrongly perceived as "dangerous" because the dendritic cells displaying them are driven into alert mode. Recent evidence suggests that dendritic cells that are either immature, quiescent or pharmacologically restrained may indeed not immunise the host, but instead direct the host immune system towards permanently tolerating that tissue.

Harnessing tolerance processes is one of the most desirable goals in transplantation because it would spare the use of immunosuppressive drugs that penalise the whole immune system, risking infections and cancer, as well as the many other side effects associated with these drugs.

For this reason we would like to know the differences in the genes expressed by tolerogenic populations of DC compared with their activated counterparts. Such differentially expressed genes should provide clues as to the proteins that determine immunity rather than tolerance.

The host laboratory has created a wide range of "gene libraries" of dendritic cell populations as a baseline for such an analysis. The aim of this proposal is to expose dendritic cells to a range of pharmacological agents that are known to promote tolerance, and to determine through construction of new libraries, which gene products are lost and which gained. By testing candidate genes or their inhibitors in simple readouts of immune responsiveness it should be possible to establish which sets of gene changes are important to tolerance. This information may lead to improved strategies for modifying the way the immune system perceives transplants so as to ensure that it becomes reprogrammed to recognise them as "friendly".

Dr. Jiangping Wu, Principal Investigator

Notre-Dame Hospital, University of Montreal, Montreal, Canada

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

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

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

4. Progress Reports of ROTRF Grantees

Dr. Maria-Luisa Alegre, Principal Investigator
Dr. J. Richard Thistlethwaite, Research Associate
Dr. Ping Zhou, Research Associate

University of Chicago, Chicago, USA

Role of Costimulatory Molecules in Cardiac Allograft Rejection

The rejection of solid organ allografts in fully mismatched recipients is dependent on T cell activation, and blockade of specific costimulatory receptors on T cells leads to the acceptance of some but not all allogeneic tissues. However, the biochemical signaling pathways necessary in T cells for promoting allograft rejection or tolerance are not fully understood. T cell stimulation leads to the nuclear translocation of several transcription factors, including nuclear factor of activated T cells (NFAT), activating protein 1 (AP-1), and nuclear factor κB (NF-κB). Of these transcription factors, NF-κB, is a prime candidate for regulating transcription of genes central to the allogeneic immune response, as its activation has been linked to cell survival, differentiation, and cytokine production in different systems. mRNA levels of the different NF-κB subunits are upregulated in allogeneic grafts during acute rejection episodes. Furthermore, graft survival is prolonged following treatment with decoy NF-κB oligonucleotides, and cardiac allografts are permanently accepted in mice with impaired NF-κB activation in T cells. Using mice expressing a mutant IκBα superrepressor transgene restricted to the T cell lineage that reduces NF-κB activation in T cells (IκBαΔN-Tg mice), we have investigated the role of normal NF-κB activation in the acute rejection of fully allogeneic heart and skin grafts.

Results

1. Skin but not cardiac allografts are rejected in IκBαΔN-Tg T cells

To determine whether normal activation of NF-κB in T cells is necessary for acute rejection of different allografts, B6 mice expressing a non-degradable form of IκBα under the control of the Lck promoter, IκBαΔN-Tg mice, were transplanted with fully allogeneic BALB/c heart or skin grafts. In contrast to wild-type mice, cardiac allografts were not rejected in IκBαΔN-Tg recipients (Figure 1). Histology and immunohistochemistry analyses performed on accepted heart grafts from IκBαΔN-Tg recipients on day 100 post-transplant revealed hearts with little cellular

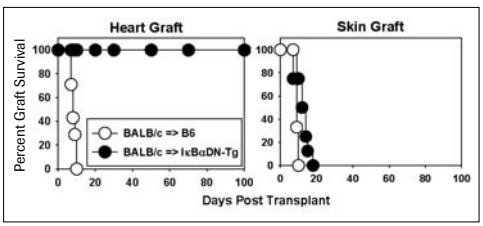


Figure 1. *Skin but not cardiac allografts are rejected in IκBαΔN-Tg recipients.*

infiltration (data not shown). The numbers of $CD4^+$ infiltrating T cells were similar to those found in normal untransplanted hearts, and $CD8^+$ T cells were absent. Cardiac graft acceptance was not specific to the $H\text{-}2^d/H\text{-}2^b$ strain combination, as C3H ($H\text{-}2^k$) hearts transplanted into IκBαΔN-Tg mice ($H\text{-}2^b$) were also accepted long-term (data not shown). In contrast, IκBαΔN-Tg mice effectively rejected skin allografts, albeit with slightly delayed kinetics compared with wild-type mice (Figure 1). Together, these results indicate that IκBαΔN-Tg T cells are capable of effectively responding to alloantigens *in vivo,* and suggest that the immune responses elicited by heart and skin allografts are distinct and differentially require NF-κB activation in T cells.

2. Depletion of $CD8^+$ T cells does not prevent skin allograft rejection in IκBαΔN-Tg mice

The subsets of T cells necessary to reject skin versus heart allografts may differ, as depletion of $CD4^+$ T cells prevents cardiac allograft rejection in this strain combination, but not skin allograft rejection. Therefore, it was possible that activation of $CD8^+$ T cells did not depend as much on NF-κB translocation and that this subset was sufficient to reject skin allografts. To determine if skin graft rejection in IκBαΔN-Tg mice was due to $CD8^+$ T cells, B6 and IκBαΔN-Tg mice were treated with the anti-CD8 depleting mAb YTS on days −2 and +11 post BALB/c skin transplantation. This regimen resulted in the absence of detectable $CD8^+$ T cells for over 2 weeks and a further 70–80% reduction for 30 days (data not shown), and was effective at preventing rejection in CD28-deficient animals. Anti-CD8 treatment did not prevent skin allograft rejection, although the mean survival time was slightly prolonged in both B6 and IκBαΔN-Tg mice (Figure 2). Together with the fact that IκBαΔN-Tg mice have reduced numbers of peripheral $CD8^+$ T cells, these data suggest that differences in the survival of heart and skin allografts in IκBαΔN-Tg recipients are not likely to be explained by dependence of skin rejection on $CD8^+$ T cells.

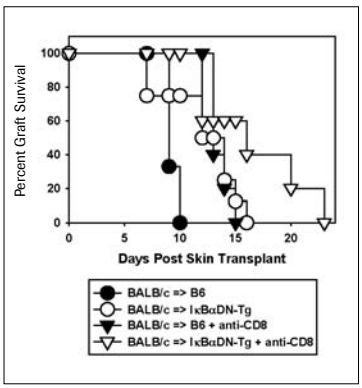


Figure 2. *Depletion of $CD8^+$ T cells does not induce skin graft survival in IκBαΔN-Tg mice.*

3. Transferred $CD4^+$ IκBαΔN-Tg T cells fail to promote cardiac allograft rejection in nude recipients

IκBαΔN-Tg mice have reduced numbers of peripheral T cells. Thus, it was possible that the absolute number of IκBαΔN-Tg $CD4^+$ T cells was insufficient for rejection of cardiac allografts but not for that of the more immunogenic skin grafts. To address this possibility, $CD4^+$ T cells were purified from B6 and IκBαΔN-Tg and adoptively transferred into syngeneic T cell-deficient nude B6 mice previously transplanted with BALB/c hearts. Transfer of

0.1×10^6 wild-type T cells was sufficient to induce rejection of cardiac allografts in the majority of the mice and transfer of 5×10^6 T cells resulted in rejection in all recipients (Figure 3). In contrast, even 25×10^6 IκBαΔN-Tg $CD4^+$ T cells could not trigger cardiac allograft rejection, demonstrating at least a 5-fold inferiority of NF-κB-impaired T cells. This finding indicates that lack of heart rejection in IκBαΔN-Tg animals is not due to the reduced number of $CD4^+$ T cells seen in those mice. These data also confirm that lack of cardiac allograft rejection in IκBαΔN-Tg mice can be accounted for by an effect on T cells alone and is not due to alterations in other cell populations.

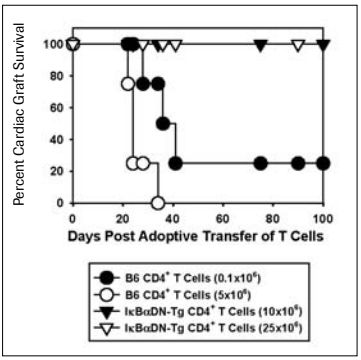


Figure 3. *Transferred $CD4^+$ IκBαΔN-Tg T cells fail to promote cardiac allograft rejection in nude recipients.*

4. Costimulation does not prevent tolerance to cardiac allograft in IκBαΔN-Tg mice

It was conceivable that diminished CD28 costimulation was occurring in the setting of a cardiac allograft compared with a skin allograft, perhaps due to decreased expression of B7 ligands by cardiac APCs compared with Langherans cells. To determine if increased CD28 costimulation could promote cardiac allograft rejection in mice with impaired T cell-intrinsic NF-κB activation, IκBαΔN-Tg mice were treated with an agonistic anti-CD28 mAb for 7 days at the time of transplantation with BALB/c hearts. As a control, B7-1/B7-2-deficient mice that fail to reject cardiac allografts were also treated with anti-CD28. Consistent with previous results, administration of anti-CD28 mAb to B7-deficient mice promoted cardiac allograft rejection (Figure 4). In contrast, administration of anti-CD28 failed to induce cardiac allograft rejection in IκBαΔN-Tg mice. This result indicates that poor CD28 ligation does not explain the failure of IκBαΔN-Tg mice to reject allogeneic hearts, and supports the argument that NF-κB activation downstream of CD28 engagement is necessary for the costimulatory function of CD28 *in vivo*.

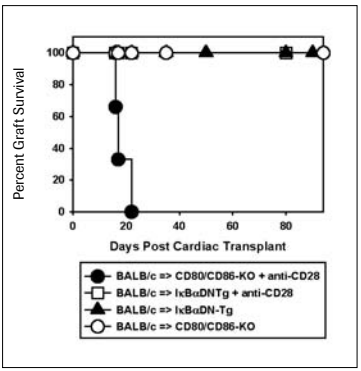


Figure 4. *CD28-mediated costimulation does not promote cardiac allograft rejection in IκBαΔN-Tg mice.*

Challenge with skin allografts promotes cardiac allograft rejection in IκBαΔN-Tg mice, but challenge with cardiac allografts results in donor-specific tolerance

Skin and cardiac allografts followed different fates in IκBαΔN-Tg mice. To address whether the acceptance of cardiac allografts depended on a failure of productive T cell priming, BALB/c hearts were transplanted into IκBαΔN-Tg mice that had previously rejected BALB/c skin. Unlike non-manipulated IκBαΔN-Tg animals, these skin-challenged IκBαΔN-Tg mice

promptly rejected cardiac allografts of donor origin (Figure 5, left panel). This result indicates that prior exposure to skin allografts effectively primes T cells for subsequent rejection of cardiac transplants under conditions that would otherwise result in cardiac graft acceptance.

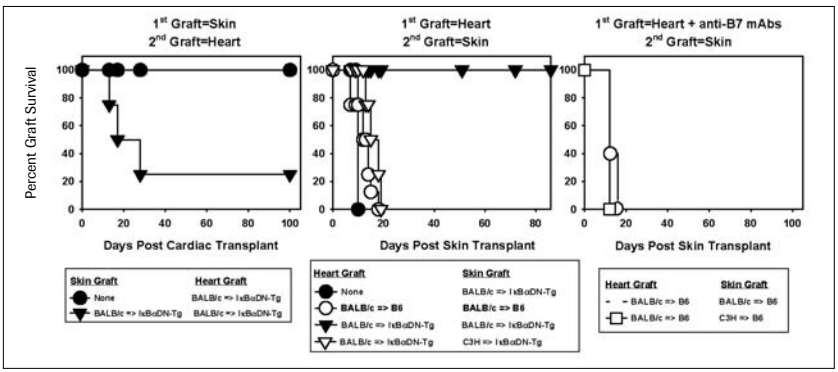


Figure 5. *Initial exposure to cardiac allografts results in acceptance of second party skin grafts in IκBαΔN-Tg mice.*

Thus, cardiac allografts appeared less capable of effectively priming T cells than skin allografts in IκBαΔN-Tg mice. It was conceivable that IκBαΔN-Tg mice were in fact rendered systemically tolerant following acceptance of a primary heart allograft. To investigate this possibility, we examined the survival of donor or third-party skin grafts in IκBαΔN-Tg that had accepted a cardiac allograft. Wild-type and IκBαΔN-Tg mice were left untreated or were transplanted with BALB/c cardiac allografts, and subsequently grafted with donor (BALB/c) and third-party (C3H) allogeneic skin on each flank. IκBαΔN-Tg mice that had been previously transplanted with BALB/c hearts now accepted skin grafts of donor but not third-party origin (Figure 5, middle panel). Thus, T cell interaction with cardiac alloantigens, in an NF-κB-impaired setting, resulted in donor-specific tolerance to highly immunogenic skin tissue, suggesting that IκBαΔN-Tg T cells that had encountered a cardiac allograft were not ignorant of the graft, but rather had become hyporesponsive or had been deleted. This result was very different from what was observed in B6 animals made tolerant to BALB/c heart allografts by treatment with anti-B7-1 + anti-B7-2 mAbs. We have previously shown that this treatment also results in cardiac allograft acceptance for longer than 100 days. However, both BALB/c and C3H skin grafts placed in anti-B7-treated B6 mice 50 days post heart transplant were rapidly rejected (Figure 5, right panel), indicating that tolerance in these latter animals is more limited than that observed in similarly transplanted IκBαΔN-Tg animals.

5. Reduced mixed lymphocyte reactions by splenocytes from tolerant IκBαΔN-Tg mice

To investigate T cell responses from these tolerant animals, mixed lymphocyte reactions were performed. B6 and IκBαΔN-Tg mice were left untreated or transplanted with BALB/c cardiac allografts. On day 50, some mice received BALB/c skin allografts and all animals

were sacrificed 2 weeks later. Splenocytes from these animals were incubated with syngeneic or allogeneic T-depleted irradiated splenocytes as stimulators and ^{3}H-thymidine incorporation was measured on day 5. As expected, the proliferative response of unmanipulated IκBαΔN-Tg splenocytes to alloantigen was somewhat reduced compared with B6 splenocytes (Figure 6). However, skin grafting resulted in increased ^{3}H-thymidine incorporation by both B6 and IκBαΔN-Tg splenocytes. A similar result was observed in splenocytes from wild-type B6 mice transplanted with heart and skin allografts. In striking contrast, splenocytes from IκBαΔN-Tg mice that received skin grafts after heart transplantation exhibited a dramatic decrease in T cell proliferation to alloantigen. This was not due to lack of T cells in the spleen of transplanted animals as the proportion and total number of T cells per spleen were similar in unmanipulated and transplanted animals (data not shown). These data indicate reduced alloreactivity in the spleen of tolerant IκBαΔN-Tg mice.

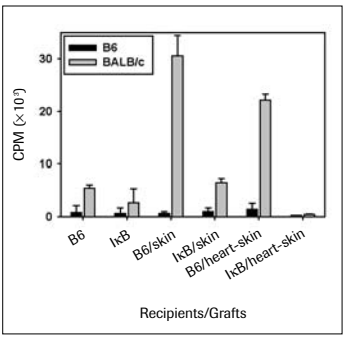


Figure 6. *Reduced MLR in splenocytes from tolerant IκBαΔN-Tg mice.*

6. Organ vascularization during initial exposure to alloantigen is not necessary for inducing donor-specific tolerance in IκBαΔN-Tg mice

One major distinction between cardiac and skin transplantation is that heart allografts are revascularized during the transplantation procedure, whereas skin allografts are progressively colonized by growing capillaries. Soluble antigen has been shown to promote T cell tolerance rather than T cell priming. Thus, it was possible that abdominal cardiac allografts induced tolerance during impaired T cell-intrinsic NF-κB activation because alloantigens are shed in the circulation. To address whether cardiac tissue in a non-vascularized form would also result in antigen-specific tolerance, fragments of neonatal hearts were transplanted subcutaneously under the skin of the ear of wild-type or IκBαΔN-Tg mice. Allogeneic but not syngeneic subcutaneous neonatal hearts were promptly rejected in wild-type recipients (Figure 7). In contrast, allogeneic neonatal cardiac grafts placed subcutaneously into IκBαΔN-Tg were accepted in

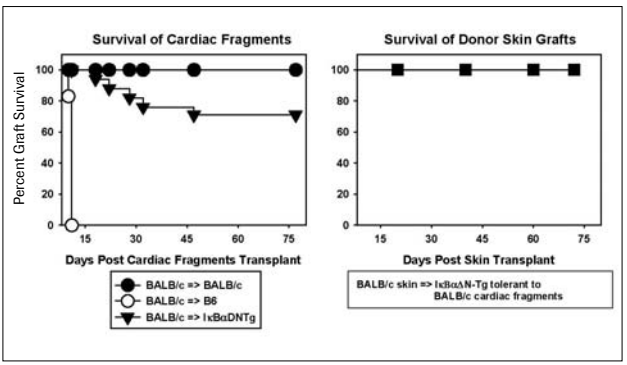


Figure 7. *Surgical organ vascularization is not necessary for inducing donor-specific tolerance in IκBαΔN-Tg mice.*

the majority of the animals, although some animals rejected their grafts. This result indicates that acceptance of cardiac allografts in IκBαΔN-Tg mice does not depend on organ vascularization.

To determine if tolerance was achieved in IκBαΔN-Tg mice challenged with subcutaneous allogeneic cardiac fragments, BALB/c skin was transplanted in the mice that had not rejected the heart pieces. Skin allografts were also permanently accepted in these animals (Figure 7, right panel). In addition, skin transplantation did not result in rejection of the allogeneic cardiac fragments (data not shown). This finding indicates that induction of tolerance in IκBαΔN-Tg mice required neither the transplantation of a vascularized whole heart nor the abdominal local environment of the initial graft. Taken together, these data indicate that impaired NF-κB activation in T cells favors the induction of tolerance to a highly immunogenic tissue.

Summary and Further Studies

In conclusion, we have found that normal NF-κB activation in T cells is required for acute rejection of cardiac but not skin allografts *in vivo.* Furthermore, exposure to alloantigen in the context of reduced NF-κB activation in T cells results in subsequent acceptance of donor but not third-party skin allografts, indicative of donor-specific tolerance. Therefore, reducing NF-κB activation in T cells during exposure to alloantigen in a context of limited immunogenicity may be a strategy by which to induce antigen-specific tolerance in the clinic.

We are in the process of identifying the underlying mechanisms that lead to graft acceptance and tolerance in this model of reduced NF-κB activation in T cells. We are exploring whether tolerance is due to deletion or hyporesponsiveness (intrinsic or extrinsic) of alloreactive T cells. Furthermore, we are currently investigating the allogeneic tissues and cell-types that are necessary during the initial exposure of NF-κB-impaired T cells to impart graft acceptance. Finally, we are investigating different inhibitors of NF-κB in normal mice to determine whether similar results can be achieved in normal animals initially treated to reduce NF-κB activation.

Publications

1. Szot GL, Zhou P, Rulifson I, Wang J, Guo Z, Kim O, Newell KA, Thistlethwaite JR, Bluestone JA, Alegre ML. Different mechanisms of cardiac allograft rejection in wildtype and CD28-deficient mice. *Am J Transplant* 2001; 1:38-46.
2. Guo Z, Meng L, Kim O, Wang J, Hart J, He G, Alegre ML, Thistlethwaite JR, Newell KA. CD8 T cell mediated rejection of intestinal allografts is resistant to inhibition of the CD40/CD154 costimulation pathway. *Transplantation* 2001; 71:1351-1354.
3. Guo Z, Wang J, Meng L, Wu Q, Kim O, Hart J, He G, Zhou P, Thistlethwaite, Jr JR, Alegre ML, Fu YX, Newell KA. Membrane lymphotoxin regulates CD8+ T cell-mediated intestinal allograft rejection. *J Immunol* 2001; 167:4796-4800.
4. Chin R, Zhou P, Alegre ML, Fu YX. Confounding factors complicate conclusions in aly model. *Nat Med* 2001; 7:1165.
5. Zhou P, Hwang KW, Palucki DA, Guo Z, Boothby M, Newell KA, Alegre ML. Impaired NF-κB activation permits tolerance to primary heart allografts and secondary skin grafts. *Am J Transplant,* in press.

Dr. Dirk Dittmer, Principal Investigator

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SCID-Mouse/Human Transplant Model for Gamma Herpes Virus Infection

The aim of this project is the study the effect of cytokines and immunosuppressive therapy on Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) and EBV pathogenesis. KSHV and Epstein-Barr virus are both associated with significant mortality and morbidity in transplant patients. We initially showed that interferon (IFN)-γ can reactivate KSHV from latency. But the contribution of these paracrine factors cannot be adequately studied *in vitro.* Rather, a system that models KSHV infection *in vivo* is needed. Therefore, we proposed to establish an animal model for KSHV- and EBV-associated lymphoma and to determine the impact of anti-viral agents, cytokines and immunosuppressive drugs on tumorigenesis. In this first year of funding, we have established the following prerequisites for our experiments:

(1) We established a colony of immunocompetent NOD/SCID mice at OUHSC. This colony is located outside the common animal facility and we are confident that this physical separation will allow us to pursue the experiments as planned.

To prove that we can successfully manipulate SCID mice we used a simplified experimental design. We injected KSHV-positive lymphoma cells (BCBL-1) directly into SCID and SCID/NOD mice. The BCBL-1 cell line was obtained from a pleural effusion and previously believed to grow only in the peritoneal cavity of mice.
However, we were able to show that given proper extracellular support (matrigel) we could grow subcutaneous tumors within two weeks (see Figure 1). Without matrigel we did not obtain any tumors. This demonstrates the strict requirement for extracellular or paracrine factors for the growth of KSHV-associated lymphoma. Now we can screen rapidly for these factors as well as include immunosuppressants, IFN-α or anti-viral agents in the matrix. These experiments will be conducted concordantly with our infection of SCID-human chimeras.

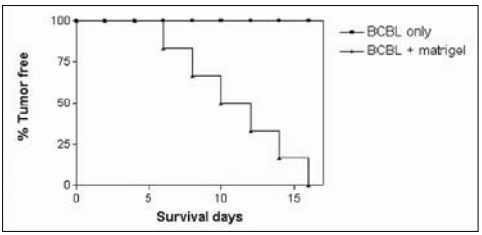

Figure 1

(2) We developed a real-time quantitative RT-PCR to measure KSHV viral load and virus specific-transcripts (*J Virol* 2002; 76:6213-6223). We are now in a position to quantify mRNA for every single one of the 80 genes that make up the KSHV genome. This is in effect a KSHV-specific DNA chip, except that rather than solid support, we are using solution PCR. This design offers two advantages: due to the power of PCR we can use minimal input samples; and we can look specifically for the various spliced mRNAs.

(3) We developed a similar real-time quantitative RT-PCR to measure EBV viral load and virus specific-transcripts (unpublished results).

(4) To prove that we could stratify IFN-α-treated from untreated lymphomas, we collaborated with Dr. William Harrington at the University of Miami Cancer Center. He previously showed that AZT and interferon-α induced apoptosis in EBV-positive lymphoma (*Cancer* Res 1999; 59:5514-5520). He recently treated a patient with a KSHV-positive lymphoma using the same regimen and sent us the cells for array analysis. Figure 2 shows the cluster analysis of KSHV transcription in lymphoma from a treated patient. Lanes A, B, C and D were treated with 10 µg/ml AZT + 1000 U/ml IFNα, while lanes F, G, H and I were treated only with AZT for 1, 2, 8 and 24 h, respectively. Lane E is an untreated control. KSHV mRNA transcription sorted the samples into two groups, that either were or were not exposed to IFNα.

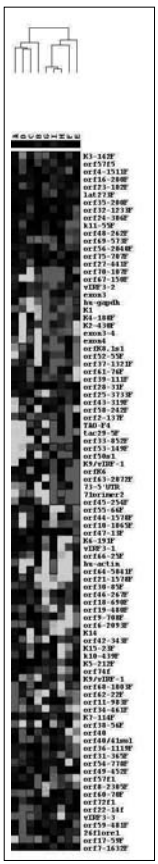

Figure 2

For the following two years, we will continue our work as proposed, namely to establish a reliable, easy to work with, *in vivo* model for KSHV. While we will still use NOD/SCID-cordblood reconstituted mice, we will also include KSHV-associated lymphomas, for which we now have an *in vivo* model and which show a clinical response to AZT and IFN. By implanting KSHV-positive tumor cells directly, we circumvented the problems that are associated with the notoriously weak infectivity of this virus.

Dr. Christiane Ferran, Principal Investigator

Beth Israel Deaconesss Medical Center, Boston, USA

Protective Effect of A20 Against Transplant-Associated Vasculopathy

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

We had 3 Specific Aims:

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

Specific Aim 2. To determine the role of A20 upon SMC apoptosis *in vitro.*

Specific Aim 3. To determine whether expression of A20 protects against TAV *in vivo.*

We have now completed most of the tasks planned in Specific Aims 1 and 2.

1. We have shown that A20 expression in SMC inhibits NF-κB activation in response to two pro-inflammatory stimuli relevant to TAV cytokines and Fas cross-linking. Inhibition of NF-κB activation inhibits in turn the up-regulation of the pro-atherogenic molecules ICAM-1 and MCP-1. In addition, we have demonstrated that expression of A20 in SMC inhibits SMC proliferation by increasing the levels of the p21 and p27 cyclin-dependent kinase inhibitor and by inhibiting phosphorylation of the retinoblastoma protein Rb.

2. We have also confirmed that expression of A20 in SMC increases their sensitivity to cytokine- and Fas-mediated apoptosis. This novel function is independent from inhibition of NF-κB activation. We are currently analyzing the impact of A20 upon, activation of caspases, alteration of mitochondrial membrane potential, cytochrome *c* release and cleavage of death substrates[1].

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

The work for Specific Aim 3 has not yet started. However, in collaboration with Drs. Uta Kunter and J. Floege from Aachen, Germany, we have been able to show that expression of A20 in the vessel wall of rat kidney allografts correlates with protection from transplant arteriosclerosis. This work adds strong indirect evidence for the protective function of A20 against TAV[3].

Publications

1. Kunter U, Floege J, von Jungensonn AS, et al. Expression of A20 in the vessel wall of rat-kidney allografts correlates with protection from transplant arteriosclerosis. *Transplantation* 2003;75 (1):3–9.
2. Longo CR, et al. A20 protects from CD40–CD40 ligand mediated endothetial cell activation and apoptosis. *Circulation;* in press.
3. Arrelo MB, et al. A20 sensitizes smooth muscle cells to apoptosis and inhibits their activation and proliferation. (Manuscript submitted).

Prof. Edward K. Geissler, Principal Investigator
Dr. Markus Guba, Co-Investigator
Dr. Markus Steinbauer, Research Associate
Dr. Christian Graeb, Research Associate

University of Regensburg, Regensburg, Germany

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

During the first period of our ROTRF grant we feel much progress has been gained to determine the potential for rapamycin and paclitaxel treatment of tumors in an organ transplant situation. Several different aspects of our project have been initiated and some of these data have already been incorporated into abstracts and journal articles. Below, we will discuss the areas where progress has been made.

Experiments have been performed to determine the more exact mechanism by which rapamycin mediates an antiangiogenic effect. In general, inhibition seems to be mediated primarily through a direct effect on endothelial cell activities, including VEGF-mediated cell proliferation, and differentiation into tubular structures[1]. We hypothesize that the primary VEGF intracellular signaling pathway (PI3K – mTOR – p70S6K) is blocked by rapamycin's activity, and we have recently confirmed this by Western blot analysis of p70S6K phosphorylation. An important point is that this VEGF-induced intracellular signaling pathway is blocked by concentrations of rapamycin at < 5 ng/ml, which is a normal serum concentration to achieve in human immunosuppression therapy.

In the grant we also proposed to test whether rapamycin affects the expression of TGF-β and HIF-1α. Our initial mRNA quantitation studies by real-time PCR do not show any effect of rapamycin on these potential regulatory molecules in either HUVECs or in various types of tumor cell. We also have assessed TGF-β protein secretion in the same cells and found no significant effect of rapamycin. Therefore, thus far, we only observe interference of rapamycin with the central PI3K – mTOR – p70S6K intracellular signaling pathway. Since this pathway is vital for angiogenesis, we are now exploring the potential effects on multiple aspects of new blood vessel generation.

One interesting observation we have made regarding the potential mechanism of rapamycin's action comes from work in the dorsal skin-fold chambers. What we have observed is that blood vessels within rapamycin-treated CT-26 adenocarcinomas show an abnormal pooling effect 9 days after cells are implanted into the dorsal skin-fold chambers. More specifically, tumor vessels in controls show typical chaotic interconnections, but the

vessels appear to function normally, as can be seen by the consistent blood flow throughout the tumor in the dorsal skin-fold chamber. In contrast, tumor vessels in rapamycin-treated mice show an inconsistent pattern of growth, where interconnections are few and vessels are highly variable in width. Further analysis of blood flow dynamics revealed that while 81.1 ± 11.5% of developing tumor vessels in control mice showed a positive flow, only 42.8 ± 13.3% of tumor vessels had a positive flow with rapamycin treatment (n = 3 mice for each group). These results suggest rapamycin can have an effect on the quality of the circulation within a tumor. These results have been presented at the National German Transplant Meeting and are to be published as a preliminary study[2]. How the circulatory effect of rapamycin is mediated is presently unknown; however, at least two possible explanations exist. First, rapamycin could affect the complex structural design of blood vessels, thereby permitting abnormal flow mechanics. A second possibility is that rapamycin influences blood coagulation, which could have an obvious effect on blood flow through tumor vessels. Further experimentation will be necessary to better dissect the mechanisms involved. Notwithstanding the awaited explanations, poor blood flow dynamics associated with rapamycin use in organ transplantation could contribute to its antiangiogenic-antitumor effect in a situation where a transplant patient has developed cancer. However, in another respect, wound healing problems and hepatic artery thrombosis, which have been reported with rapamycin usage after organ transplantation, could also be related to the circulatory phenomenon that we observe.

Rapamycin drug delivery has also become an important issue in our experiments. The effectiveness of low doses of drug signaled us to test whether rapamycin peak concentrations (>100 ng/ml) are required at all for an effective antiangiogenic effect. Therefore, we used an intraperitoneal osmotic pumping system to deliver rapamycin continuously at the same daily doses that we have recently published (1.5 mg/kg/d)[1]. Interestingly, CT-26 colon tumors in animals receiving rapamycin at a continuous level showed the greatest response (Fig. 1). Tumors in mice receiving a once-a-day dosing responded well to therapy, but the continuous dosing was better. Also, higher bolus dosing (4.5 mg/kg/every 3 days) actually produced a lesser effect on tumors. When we measured serum drug levels in mice with the osmotic pumps, rapamycin was present at a therapeutic amount of 15 ± 5 ng/ml (n = 6). These results clearly indicate that normal immunosuppressive

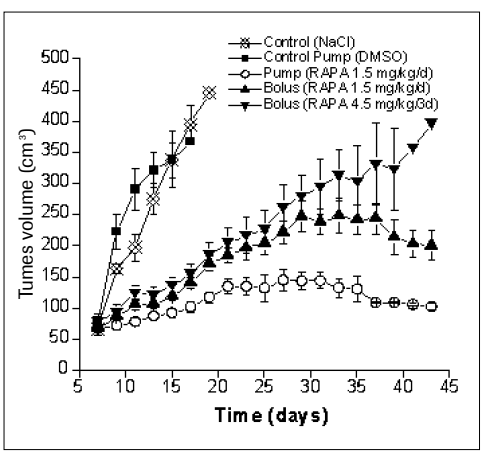

Figure 1.

levels of rapamycin are the most effective at reducing tumor growth – this could prove to be an important consideration for its clinical use. Moreover, these data suggest that clinically recommended immunosuppressive doses of rapamycin coincide with doses required for optimal tumor inhibition (at least in mice).

An important consideration for upcoming studies is models where tumors are present in animals with an organ transplant on conventional cyclosporine immunosuppression. Therefore, we have looked at tumor growth in the presence of cyclosporine, rapamycin, or the drug combination. Two tumor models were used. In the first model BALB/c mice were given subcutaneous implants of syngenic CT-26 colon adenocarcinoma cells, and cyclosporine or rapamycin therapy was initiated 7 days later. In a second model, C57BL/6 mice received subcutaneous implants of syngenic B16 melanoma cells and immunosuppressive treatment was initiated in the same way. Tumor growth was determined. Results in the BALB/c model show that control CT-26 tumors normally grew for 2 weeks before mice were sacrificed because of tumor complications (Fig. 2a). Cyclosporine-treated mice showed accelerated tumor growth before succumbing to tumor complications. In contrast, rapamycin treatment controlled tumor growth and allowed for longer-term survival. Similar growth inhibitory and promoting effects on B16 melanomas in C57BL/6 mice were observed with rapamycin and cyclosporine, respectively (Fig. 2b). Interestingly, in the CT-26 tumor model, rapamycin treatment reversed the tumor promoting activity of cyclosporine; in fact, these preliminary experiments suggest that the effect of rapamycin is even greater when cyclosporine is present (data not shown). At this time, we cannot explain this phenomenon and have not tested for the same effect in the B16-melanoma model. Certainly, these effects will need to be tested further in this study and should be considered when treating cancer in transplant patients.

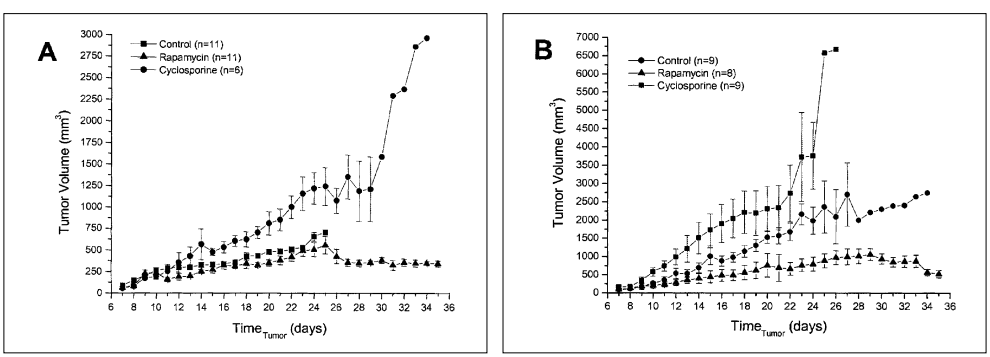

Figure 2.
(A) *Effect of cyclosporine and rapamycin on tumor growth in mice simultaneously bearing a subcutaneous CT-26 colon adenocarcinoma. BALB/c mice were treated intraperitoneally with saline, 20 mg/kg/day cyclosporine or 1.5 mg/kg/day rapamycin starting 7 days after tumor implantation.*
(B) *A similar experiment was performed in C57BL/6 mice bearing a B16 melanoma. Note that where error bars are no longer present for the tumor volume, only one animal remained alive in the experimental group.*

One of the primary objectives in our proposal is to determine whether taxol and rapamycin can work against hepatic tumors in a situation where organ transplantation could be potentially curative, but significant relapse rates remain a problem. Therefore, we have begun to examine the effects of paclitaxel on the growth of McA-RH7777 hepatoma cells and on liver allograft rejection. In initial experiments we have found that low concentrations (33–99 ng/ml) of paclitaxel strongly inhibit hepatoma cell proliferation *in vitro.* Furthermore, we have shown that paclitaxel reduces liver allograft rejection when used at doses as low as 0.5 mg/kg/d for 14 days. The results from this preliminary study were published recently[3]. In addition, we have now tested the effect of rapamycin on hepatoma cell proliferation, but actually find little inhibitory effect. It remains logical, therefore, to combine the antiangiogenic properties of rapamycin with the tumor-cell antiproliferative effects of paclitaxel to reduce cancer formation following liver transplantation for HCC. The development of an *in vivo* model to simulate the situation of a hepatocellular carcinoma is set to be initiated. Testing the effects of rapamycin and paclitaxel on tumor growth and transplant rejection will then begin.

Finally, we have continued to test the potency of the immunosuppressive effect of paclitaxel in a heterotopic rat heart transplant model. We have tested whether paclitaxel could reverse an ongoing immune response in transplant recipients. Therefore, we used a model where Lewis recipients received ACI heterotopic heart allografts, with initiation of paclitaxel or cyclosporine treatment on day 5. Allograft survival was determined in one group, and in a second group cytotoxic T lymphocyte (CTL) responses were determined and serum antidonor cytotoxic antibody levels were measured. Results showed that paclitaxel was as effective as cyclosporine at rescuing recipients from imminent allograft rejection (Table 1). Immunologically, paclitaxel reduced the allogeneic-CTL response (Fig. 3), but most impressively, the cytotoxic antibody response was nearly eliminated in rescued recipients (Table 2). Therefore, these data confirm and extend the potency of paclitaxel's immunosuppressive properties in an organ transplant situation. Data from this group of experiments is due to be published[4].

Table 1. *Effect of short-course, delayed, paclitaxel or cyclosporine treatment on the rescue of ACI heart allografts in Lewis recipients. Treatment with paclitaxel or cyclosporine was initiated on day 5 after heart transplantation. There was no significant difference between the paclitaxel- and cyclosporine-treated groups ($p = 0.13$).*

Treatment	Graft survival time (GST; days)	Mean GST (days)	*p* value vs control
None (control)	6, 6, 6, 6, 7, 7	6.3 ± 0.5	–
Paclitaxel	8, 13, 14, 14 17	13.2 ± 3.3	0.0014
Cyclosporine	6, 16, 17, 18, 18	15.0 ± 5.1	0.013

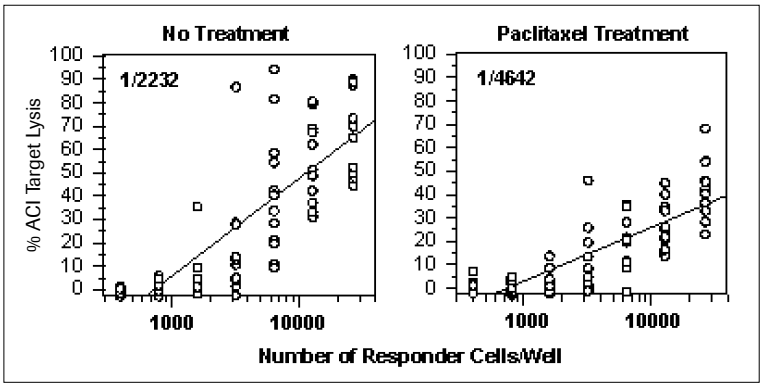

Figure 3.
Effect of delayed paclitaxel treatment on donor-reactive CTL in Lewis recipients of ACI heart allografts. ACI heart allografts were placed in Lewis recipients and paclitaxel treatment was initiated, as usual, on day 5 posttransplantation. Cervical lymph nodes were removed on day 7 posttransplantation and were tested in a limiting dilution assay for anti-ACI CTL precursors. The results above are from a representative animal either not treated, or treated, with paclitaxel. The relative amount of ACI target-cell killing is shown for each of the limiting-dilution cultures, as well as the calculated CTL precursor frequency (upper left corner of each graph). Similar results were obtained in 2 additional animals for each group.

Table 2. *Effect of paclitaxel and cyclosporine on the antibody response in Lewis recipients already undergoing acute rejection of ACI heart allografts.*

Treatment	Individual Animals in each Group	Titer[a] Day 7	Day 10
No Treatment	1	1250	1250
	2	>1250	>1250
	3	>1250	>1250
Paclitaxel Treatment (days 5–11)	1	<10	50
	2	<10	<10
	3	<10	250
	4[b]	1250	50
Cyclosporine Treatment (days 5–11)	1	>1250	250
	2	>1250	250
	3	>1250	250
	4	>1250	>1250

[a] *The titer represents the first dilution of serum where killing of ^{51}Cr-labeled ACI targets did not exceed 2% above background in the complement-mediated antibody cytotoxicity assay. The serum dilutions tested in the assay were 1:10, 1:50, 1:250 and 1:1250.*

[b] *This recipient was not rescued from allograft rejection (rejected on day 7).*

In general, we believe the information gained during the first 9 months of ROTRF support strengthens the likelihood that rapamycin and paclitaxel usage can help fight cancer in patients with an organ transplant. In the upcoming months, we hope to further these studies and determine if the combination of rapamycin and paclitaxel can be particularly effective in this situation.

Publications

1. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch K-W and Geissler EK. (2002) Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med,* 8:128-135.
2. Koehl G, Guba M, Seeliger H, Steinbauer M, Anthuber M, Jauch K-W and Geissler EK. Rapamycin treatment at immunosuppressive doses affects tumor blood vessel circulation. *Transplant Proc,* submitted as proceedings from the DTG meeting in Hannover, Germany.
3. Iesalnieks I, Tange S, Scherer MN, Graeb C, Frank E, Jauch K-W and Geissler EK. (2002) Paclitaxel promotes liver graft survival in rats and inhibits hepatocellular carcinoma growth *in vitro;* a potentially useful drug for transplant patients with liver cancer. *Transplant Proc,* 34:2316-2317.
4. Tange S, Scherer MN, Graeb C, Andrassy J, Justl M, Frank E, Jauch K-W and Geissler EK. Paclitaxel rescues rat heart allografts from rejection by inhibition of the primed antidonor humoral and cellular immune response: implications for transplant patients with cancer. *Transplant Int,* in press.

Prof. Kathryn Wood, Principal Investigator

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Identity of the Cells Responsible for Transplant Survival

The **objectives** of the project are

(i) to determine the phenotype, function and specificity of immunoregulatory $CD4^+$ T cells responsible for the maintenance of tolerance to alloantigens *in vivo* following pretreatment with alloantigen in combination with either anti-CD4 or anti-CD154.

(ii) to develop *in vitro* assays that enable the functional activity of immunoregulatory T cells to be correlated *in vivo* and *in vitro.*

Work on the project was initiated in the laboratory in October 2000. To date, we have made good progress towards the first objective (see below). A research assistant has now been appointed, enabling work on the second objective to begin.

Specific Aim 1 – To determine if $CD45RB^{low}CD4^+$ cells are also responsible for immune regulation in mice following treatment with anti-CD154.

We have shown that in the maintenance phase of specific unresponsiveness to alloantigens *in vivo* induced following pretreatment with donor alloantigens in combination with anti-CD4 therapy $CD45RB^{low}CD4^+$ cells contribute to long-term graft survival[1]. This work was then extended to determine whether anti-CD154 (CD40L) therapy had a similar capacity to facilitate the development of immunoregulatory T cells in the maintenance phase of the response[2].

Blocking CD40-CD154 interactions by administering anti-CD154 (MR1) at the time of transplantation was found to prolong the survival of cardiac allografts in a number of different donor-recipient combinations in mice. A short course of MR1 therapy enabled the development of $CD4^+$ T cells with regulatory properties that had the ability to maintain specific immunological unresponsiveness to the allograft[2]. The regulatory cells were characterised phenotypically and functionally and were shown to express high levels of CD25 and have specificity for donor alloantigen. Thus we have shown that both anti-CD4 and anti-CD154 therapy can generate donor alloantigen specific regulatory cells in the maintenance phase of the response after transplantation.

As part of this study, we also carried out an additional series of experiments not specified in the original application to investigate whether $CD25^+CD4^+$ induced as a result of anti-CD154 therapy could control the potential of $CD8^+$ T cells to initiate rejection. This is an important question in this context, as previous work by ourselves and others has shown that anti-CD154 therapy does not impact the ability of $CD8^+$ T cells to initiate rejection in the early phase of the response[3-6]. This could be because $CD8^+$ T cells are refractory to control by regulatory T cells or that in naive recipients there is an insufficient number of donor alloantigen-specific regulatory T cells to control the aggressive potential of $CD8^+$ T cells.

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

Specific Aim 3 – To determine the role of CTLA-4 in immune regulation by $CD45RB^{low}CD4^+$ cells.

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

Specific Aim 5 – To use additional cell surface markers to refine the phenotype of $CD45RB^{low}CD4^+$ regulatory T cells.

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

In ongoing studies we have been examining the ability of other cell surface markers to refine further the characterisation of regulatory T cells. To date we have ruled out CD122, the β chain of the IL-2R, as providing a useful additional marker[8]. In contrast, intracellular

expression of high levels of CTLA-4 (CD152) has been found to be useful as a strategy for enriching for $CD25^{+}CD4^{+}$ T cells with regulatory activity[7]. The search for more specific markers for regulatory T cells is ongoing.

References and Publications

1. Hara M et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. *J Immunol* 2001;166:3789-3796.
2. van Maurik A, Wood KJ & Jones N. Cutting Edge: $CD4^{(+)}CD25^{(+)}$ Alloantigen-Specific Immunoregulatory Cells That Can Prevent $CD8^{(+)}$ T Cell-Mediated Graft Rejection: Implications for Anti-CD154 Immunotherapy. *J Immunol* 2002;169:5401-5404.
3. Trambley J et al. Asialo $GM1^{+}$ $CD8^{+}$ T cells play a critical role in costimulation blockade resistant allograft rejection. *J Clin Invest* 1999;104:1715-1722.
4. Honey K, Cobbold S & Waldmann H. CD40 ligand blockade induces $CD4^{+}$T cell tolerance and linked suppression. *J Immunol* 1999;163:4805-4010.
5. Jones N et al. CD40-CD40L independent activation of $CD8^{+}$ T cells can trigger allograft rejection. *J Immunol* 2000;165:1111-1118.
6. van Maurik A, Wood KJ & Jones N. Impact of both donor and recipient strains on cardiac allograft survival after blockade of the CD40-CD154 costimulatory pathway. *Transplantation* 2002;74:740-743.
7. 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;168:1080-1086.
8. Karim M, Bushell A & Wood KJ. Regulatory T cells in transplantation. *Curr Opin Immunol* 2002;14:584-591.

5. Final Reports of ROTRF Grantees

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Dr. Peter J. Cowan, Co-Investigator
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Prevention of Rejection of Organ Transplants between Species by Anti-Oxidant Genes

Xenotransplantation is viewed as a potential solution to the chronic shortage of human organs for transplantation. With the availability of effective strategies to prevent hyperacute rejection of pig-to-primate xenografts, acute vascular rejection (AVR) is regarded as the current barrier to be overcome. Exactly what initiates AVR is unclear, but the key event appears to be injury to and activation of graft endothelial cells (EC) by multiple factors including reperfusion, binding of xenoreactive antibodies and complement, and interactions with activated platelets and infiltrating leukocytes. Ischemia reperfusion (IR) injury causes oxidative-stress-induced activation of graft ECs, resulting in a cascade of cytokine and adhesion molecule upregulation. The destructive agents in IR injury are reactive oxygen intermediates (ROIs). In addition, the predominant cell type infiltrating vascularised xenografts, namely macrophages, also generates ROIs, thus contributing further to the injury and activation of graft ECs. These molecules cause tissue injury and destruction by damaging DNA, proteins and cell membranes. Under normal physiological conditions, cells maintain an equilibrium between oxidant and antioxidant mechanisms by the action of antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. However, in situations such as AVR, the magnitude of ROIs generated at the surface of and within the xenograft endothelium may overwhelm the endogenous antioxidant enzymes, resulting ultimately in loss of graft function. The hypothesis of this application is that the transgenic expression of a "balanced combination" of antioxidant enzymes in the endothelium of a xenograft will afford protection from ROI-induced xenograft injury and will prolong xenograft survival.

Aims:

1. To prepare expression constructs for native and epitope-tagged versions of human copper zinc superoxide dismutase (CuZnSOD), glycosyl-phosphatidyl inositol-linked extracellular superoxide dismutase (GPI-linked ECSOD) and glutathione peroxidase (GPx) and to establish expression and activity of the enzymes *in vitro.*
2. To generate transgenic mice expressing combinations of the above enzymes and to show expression and activity of the enzymes *in vivo.*
3. To determine the functional effect of antioxidant enzyme overexpression.

Results transfection and *in vitro* testing

Constructs for native and epitope-tagged forms of CuZnSOD, GPI-linked ECSOD and GPx were generated and transiently transfected into COS cells. Expression of CuZnSOD and GPI-linked ECSOD was detected by flow cytometric analysis (FACS) of intact (GPI-linked ECSOD) or saponin-permeabilised (CuZnSOD) cells using specific antibodies to the anti-oxidant enzyme and/or the epitope tag (Figure 1). High levels of human CuZnSOD and ECSOD expression were detected. Comparable levels of expression were observed with native and epitope-tagged forms of the enzymes. Antibodies to GPx or the epitope tag were unsuccessful in detecting expression of GPx by either FACS or western blotting.

SOD activity in lysates from transfected cells was measured spectrophotometrically by monitoring the inhibition of nitrite formation from hydroxylammonium chloride. Native and epitope-tagged forms of CuZnSOD and GPI-linked ECSOD showed comparable increases in enzyme activity over vector-transfected cells (Figure 2).

GPx activity in lysates from transfected cells was also measured spectrophotometrically using glutathione reductase and NADPH in a coupled assay procedure. Similar increases in enzyme activity over vector-transfected cells were observed for both the native and epitope-tagged forms of GPx (Figure 2).

Generation and analysis of transgenic mice

Transgenic mice (single and triple combinations) were generated using a generic microinjection construct comprising the H2kb promoter, hybrid intron, cDNA and SV40 polyadenylation signal. The H2kb promoter was chosen because this promoter drives constitutive expression in adult tissues and cells, and appears to be particularly active in vascular endothelium (Table 1).

Mice were screened for transgenesis by PCR analysis of tail tip DNA and for expression of the antioxidant enzymes at the RNA level by reverse transcriptase PCR (Figure 3). Expression of CuZnSOD and GPI-linked ECSOD was also detected by western blotting using specific antibodies to their respective epitope tags (Figure 4).

Lung lysates from WT and transgenic mice were analysed for antioxidant enzyme activity. Transgenic mice expressing CuZnSOD or GPI-linked ECSOD showed total SOD activity up to 4.6-fold that of WT mice, while GPx expressing mouse lines showed GPx activity up to 2.2-fold that of control mice (Figure 5).

Functional effect of antioxidant enzyme overexpression

The functional effect of antioxidant enzyme expression was assessed using a modified Langendorff heart perfusion system. Hearts from WT and triple transgenic mice were removed and immersed in ice-cold perfusion buffer for a period of 30 minutes to simulate cold ischemia. Hearts were then cannulated via the aorta, connected to the Langendorff apparatus, and perfused in a retrograde manner at a pressure of 100–110 mmHg for a period of 60 minutes. Changes in heart rate (beats/min) and force (*g*) were monitored using a transducer attached to the apex of the heart. Work performed by the heart was calculated

as the product of force of contraction and heart rate. Triple transgenic hearts showed a greater protection at all time points compared with WT control hearts (Figure 6). We are currently using this model to examine double (CuZnSOD + GPx and GPI-linked ECSOD + GPx) and single (CuZnSOD, GPI-linked ECSOD, and GPx) transgenic hearts to determine which gene(s) are protective and whether protection is additive.

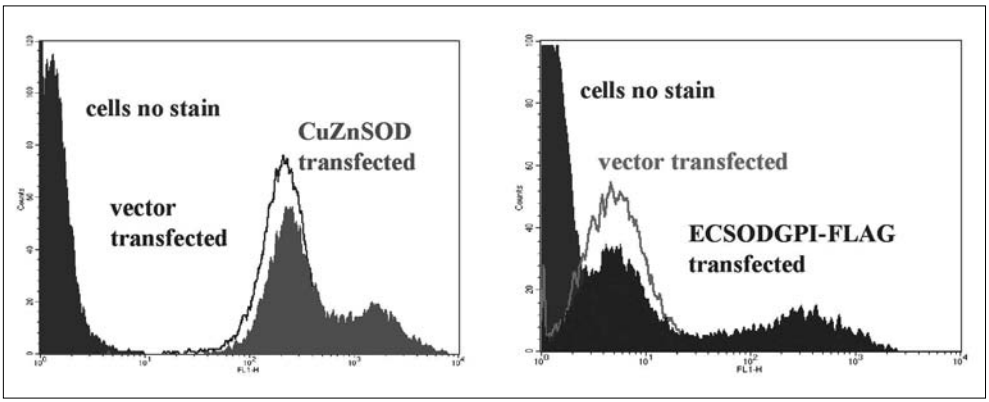


Figure 1. *Expression of antioxidant enzymes from transient transfections of COS cells. (A) Saponin-permeabilised transfected cells stained with mouse anti-human CuZnSOD followed by anti-mouse FITC. (B) Transfected cells stained with mouse anti-Flag followed by anti-mouse FITC.*

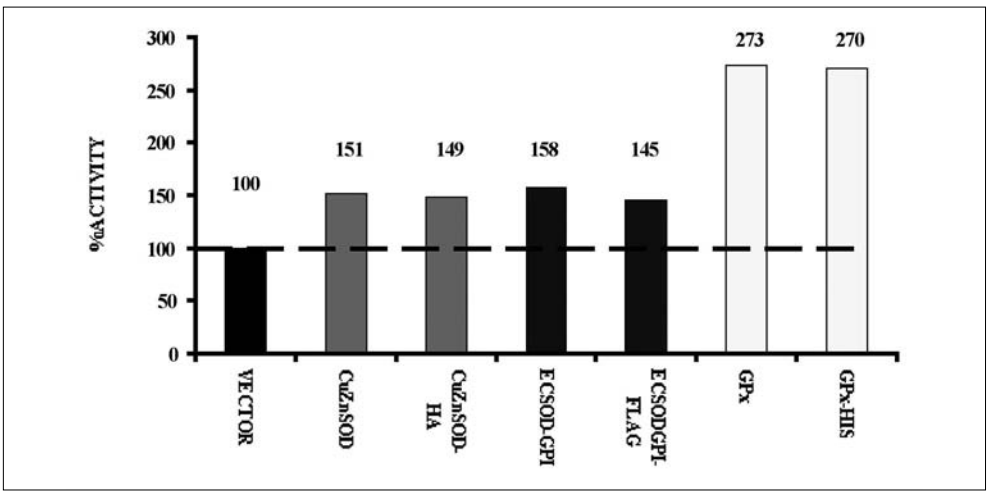


Figure 2. *Enzyme activity of transiently transfected COS cells. SOD and GPx activity are expressed as the % activity compared with vector-transfected cells (100%).*

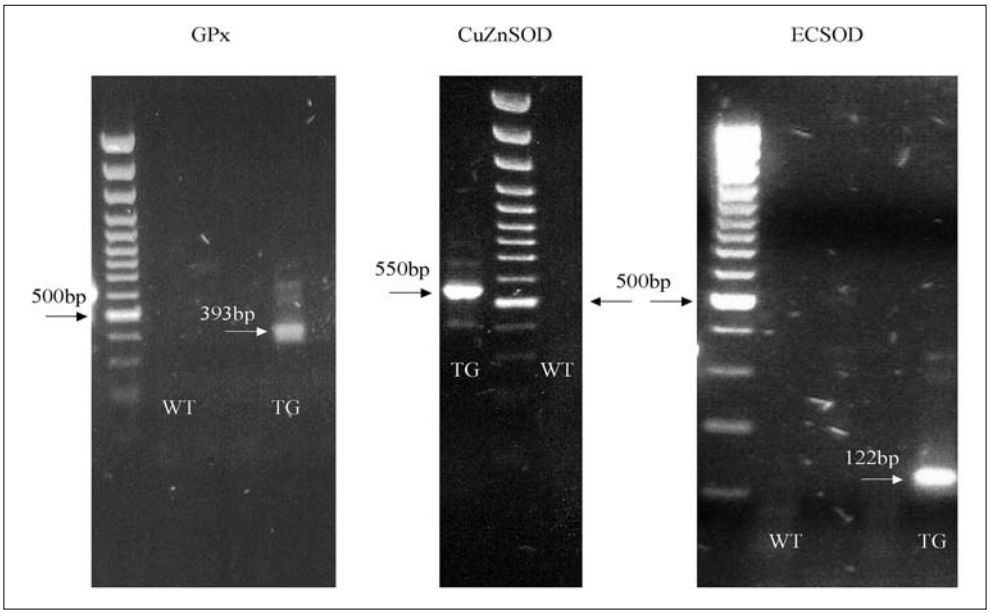


Figure 3. *Expression of antioxidant enzymes at the RNA level by RT-PCR. RNA was extracted from mouse blood and double stranded cDNA produced using gene-specific primers and Superscript II RNAse H-reverse transcriptase. Primers specific to the transgenes were used in a PCR reaction to detect the presence of the transcripts. Bands were seen for transgenic mice only.*

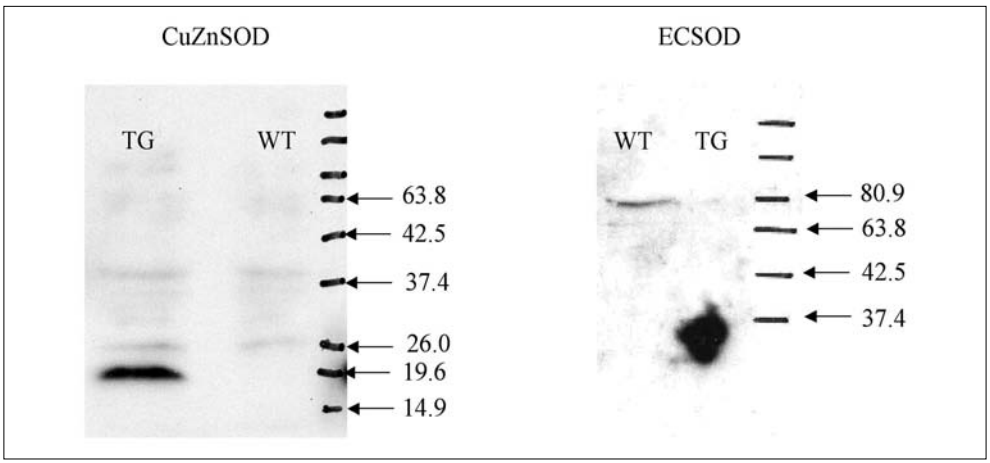
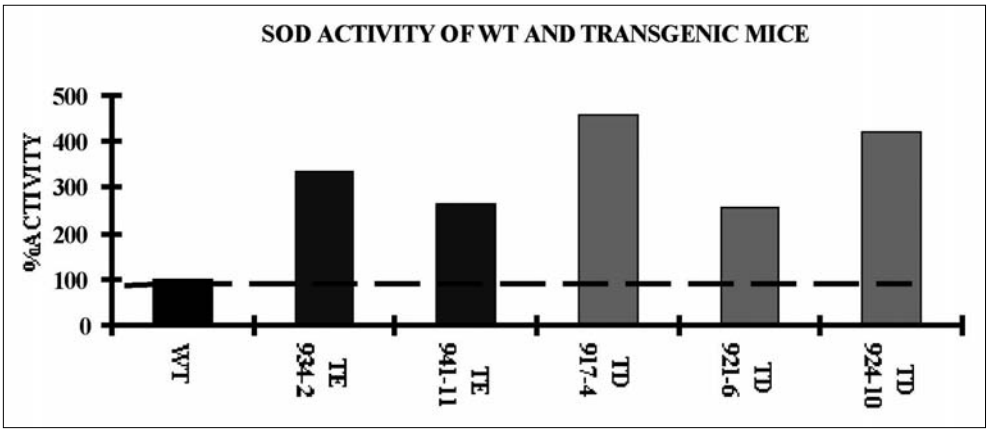


Figure 4. *Lysates from WT and transgenic mice were prepared and electrophoresed on 10% (CuZnSOD) or 12.5% (ECSOD) SDS-PAGE gels under reduced conditions and transferred to PVDF membranes. Membranes were blocked and probed with rat anti-HA high-affinity antibody (CuZnSOD) or rabbit anti-Flag antibody (ECSOD) followed by HRP-labeled second antibodies. Bands were detected using SupersignalWest Pico chemiluminescent substrate. CuZnSOD is a dimeric protein consisting of two 16-kDa subunits, while ECSOD is a tetrameric protein consisting of four 23-kDa subunits. Specific bands were seen for transgenic mice only.*



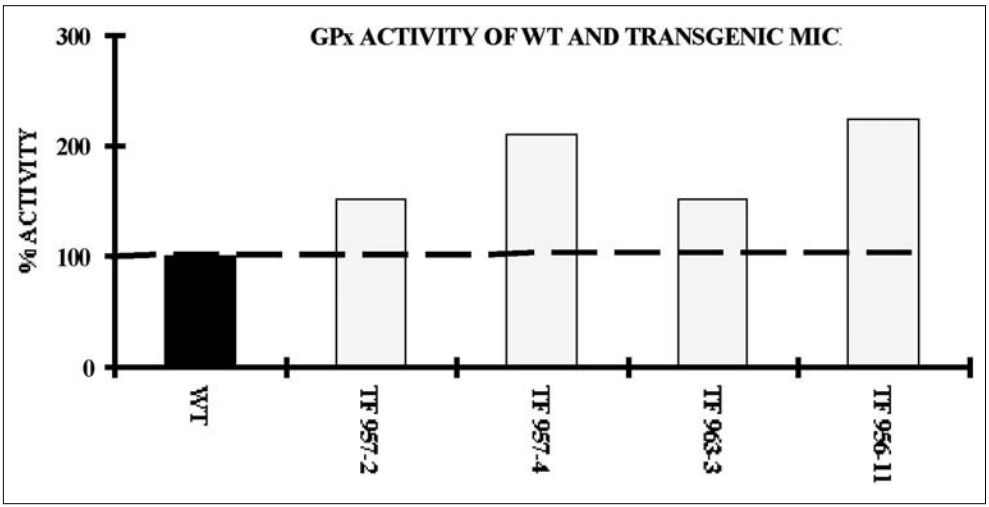


Figure 5. *SOD and GPx activity of lung lysates from transgenic mice. Results are expressed as the % activity compared with WT mice (100%). TD transgenic mice express human CuZnSOD, TE mice express GPI-linked ECSOD and TF express human GPx.*

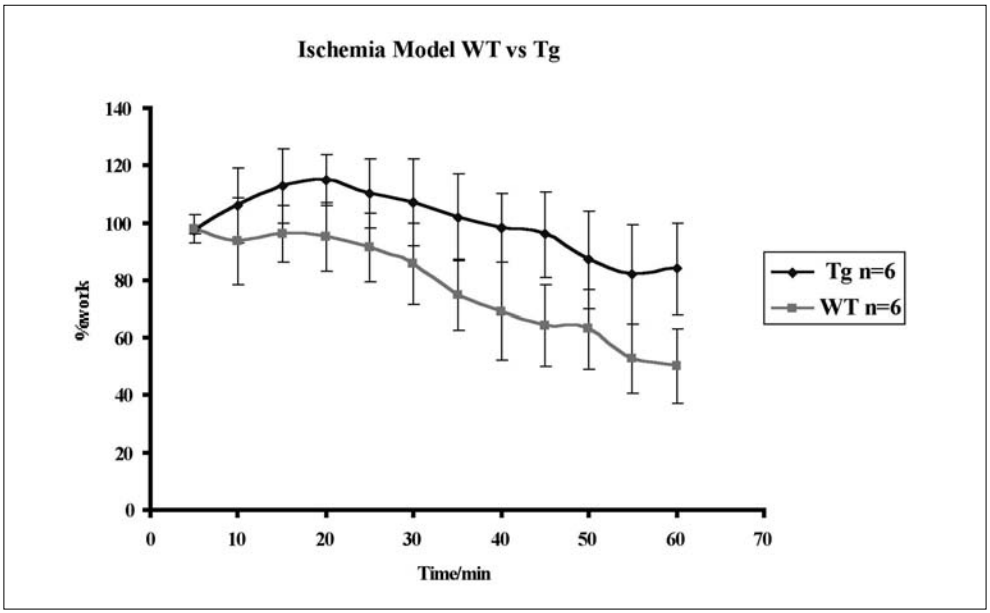


Figure 6. *Heart work (measured as the product of heart rate and force of contraction) of triple transgenic and non-transgenic (WT) mice. Hearts were subjected to cold ischemia for a period of 30 minutes followed by reperfusion for a period of 60 minutes. Points represent the mean ± SEM.*

Transgenic line	Number mice	Number transgenics	Number expressors
CuZnSOD-HA	76	10	7
ECSODGPI-FLAG	49	5	5
GPx-HIS	65	7	5
CuZnSOD-HA + ECSODGPI-FLAG + GPx-HIS	67	10	3 triple 3 double 1 single

Table 1. *Generation of transgenic mice by microinjection. Mice were screened for transgenesis by PCR analysis of tail tip DNA and for expression by RT-PCR.*

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Dr. David Witte, Collaborator

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Molecular Regulation of Liver Cell Transplantation

Introduction and Specific Aim

Hepatocyte transplantation is a central component of *ex vivo* gene therapy and is an emerging treatment modality for patients with liver failure or metabolic disease. In both settings, the ability of hepatocytes to engraft and undergo proliferation in the recipient liver is critical to meet metabolic demands. Although the factors regulating hepatocyte transplantation are largely unknown, the plasminogen family of proteases has been proposed to regulate engraftment and clonal expansion of transplanted cells via proteolysis of the adjoining microenvironment. This family of proteases is comprised by plasminogen, two activators (urokinase- and tissuetype plasminogen activators), and plasminogen activator inhibitors. The proteases control fibrinolysis during clotting and the reparative response of extra-hepatic tissues to an injury. Therefore, we proposed a series of experiments to define the regulatory role of these proteases on the biological fate of transplanted hepatocytes. The experiments were carried out during the three-year tenure of the ROTRF award. Our overall strategy was to determine the cellular proliferation and reorganization of the liver architecture following an acute toxic injury in mice, genetically engineered to lack one or more proteases simultaneously.

Specific Aim: To define the role of the plasminogen activator family of proteases on engraftment and clonal expansion of transplanted hepatocytes.

Plasminogen deficiency results in defective liver repair

In order to study the regulatory role of the members of the plasminogen family of proteases on hepatocyte proliferation and matrix re-structuring in the liver, we determined the reparative response of murine livers to an acute toxic insult. To this end, we used mice that carry the targeted inactivation of the gene coding for plasminogen in a well established experimental model of liver injury. In the experiments, we injected carbon tetrachloride (CCl_4), a liver-specific toxin, into mice lacking plasminogen (Plg^{o}) and control littermates (Plg^{+}). Two days after CCl_4 administration, livers of Plg^{+} and Plg^{o} mice displayed a similar diseased pale/lacy appearance, followed by restoration of normal appearance in Plg^{+} livers by day 7. In contrast, Plg^{o} livers remained diseased for as long as 2.5 months, with a diffuse pale/lacy appearance and a persistent damage to centrilobular hepatocytes. The persistent centrilob-

ular lesions were not a consequence of impaired proliferative response in Plg^{o} mice. Instead, the deposition of fibrin and other extracellular matrix substrates was a prominent feature in diseased centrilobular areas in Plg^{o} livers for at least 30 days after injury. However, removal of fibrin by the superimposed inactivation of the fibrinogen gene in Plg^{o} mice did not restore normal repair. In mice lacking both plasminogen and fibrinogen, the proteolytic clearance of necrotic cells remained impaired, and newly proliferated hepatocytes could not populate the centrilobular area and restore normal liver architecture.

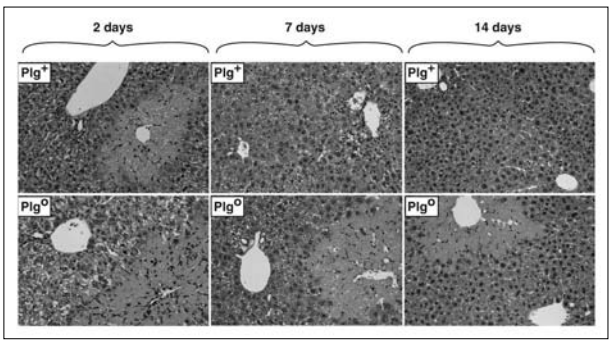


Figure 1. *Plg^{+} liver sections in the upper panel showing an injury to centrilobular hepatocytes 2 days after CCl_4, followed by normalization at day 7–14. In contrast, the lesion in Plg^{o} livers (lower panel) persists through day 14.*

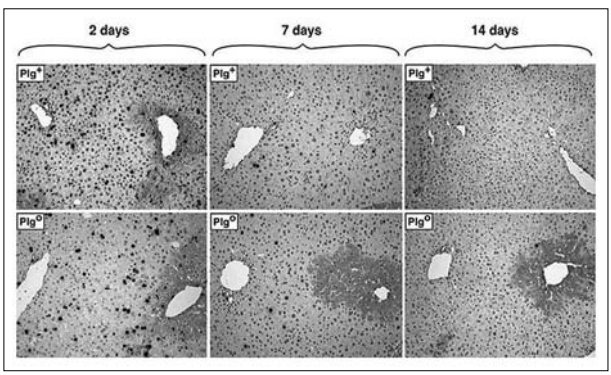
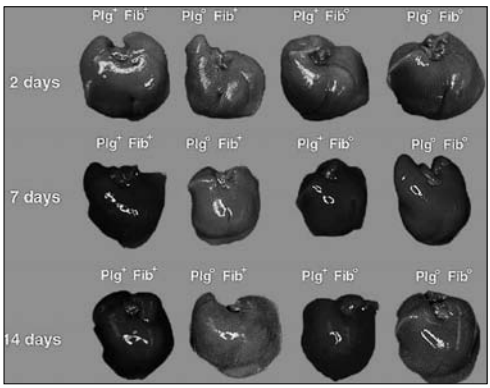


Figure 2. *BrdU-labeled hepatocytes (shown as black nuclei) are abundant and distributed uniformly throughout the non-injured lobular region 2 days after CCl_4 in Plg^{+} and Plg^{o} livers. Thereafter, labeled hepatocytes are infrequently found in Plg^{+} livers. Note that labeled hepatocytes are similarly inconspicuous in Plg^{o} livers, despite the persistent centrilobular injury at day 7–14.*

Figure 3. *The upper panel shows the diffuse diseased appearance in livers of mice of all genotypes. By day 7 (middle panel), wild-type and fibrinogen-sufficient (Fib^{+}) littermates restore normal appearance, suggesting that fibrinogen deficiency does not adversely alter the outcome of treated livers. In contrast, livers of mice that lack plasminogen (Plg^{o}) and both plasminogen and fibrinogen (Plg^{o}/Fib^{o}) continue to display an abnormal appearance up to 14 days after a toxic insult.*



Together, these data demonstrated that plasminogen deficiency results in defective repair following an acute liver injury. The accumulation of non-fibrin matrix substrates in Plg^{o} livers also points to a newly identified role of plasminogen in the proteolytic reorganization of the hepatic matrix, which occurs during a physiologic reparative response after an injury[1].

Plasminogen activators work synergistically to drive liver repair

The next logical step in defining how other members of the plasminogen system participate in the regulation of liver repair was to carefully determine the phenotype of mice lacking the tissue- (tPA) and urokinase-type (uPA) plasminogen activators following an acute hepatotoxic insult. Based on the findings of defective repair in mice lacking plasminogen, we used the same experimental protocol in mice carrying the targeted inactivation of the genes coding for uPA and/or tPA. We found that tPA-deficient mice displayed a mild defect in hepatic repair, while livers of uPA-deficient mice had a more substantial delay in repair, with injury of centrilobular hepatocytes persisting up to 14 days after CCl_4 administration. Notably, functional cooperativity between plasminogen activators was strongly inferred from the profound reparative defect in livers of mice lacking tPA and uPA simultaneously, with persistence of centrilobular injury for up to four weeks. The defective repair was not due to increased susceptibility of experimental mice to the toxin or to inadequate cellular proliferation. Instead, lack of plasminogen activators led to the accumulation of fibrin and fibronectin in injured areas and to poor removal of necrotic cells.

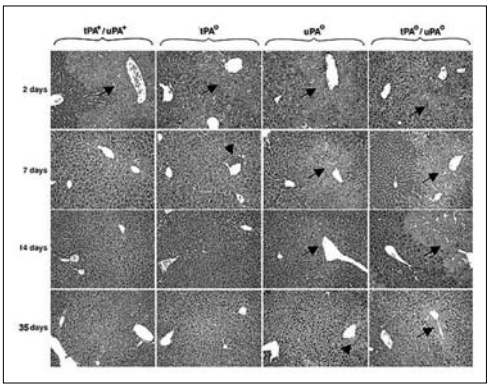


Figure 4. *Similar centrilobular injury in all genotypes 2 days after CCl_4. In control mice (uPA^{+}/tPA^{+}), repair occurs by day 7. In experimental livers, mild residual centrilobular lesions are still present in tPA^{-} livers, moderate in uPA^{-} livers, and severe in uPA^{-}/tPA^{-} livers 7 days after injury (arrows). Notably, defective repair in livers of mice lacking uPA or tPA/uPA persists as far out as 35 days after a single toxic insult.*

These data demonstrated that tPA and uPA play a critical role in hepatic repair via proteolysis of matrix elements and clearance of cellular debris from the field of injury[2].

Activation of soluble plasminogen regulates liver repair

Because data from other laboratories suggested that proteolysis in the immediate pericellular micro-environment of hepatocytes may play an important role in the migration of hepatocytes within the liver lobule and in the engraftment of transplanted cells into the

recipient liver, we next determined whether loss of the receptor for uPA (uPAR) is required for plasmin-mediated proteolysis during liver repair. In these experiments, we injected CCl_4 into mice with the targeted inactivation of the uPAR gene alone ($uPAR^{o}$) or in combination with uPA ($uPAR^{o}/uPA^{o}$) or tPA ($uPAR^{o}/tPA^{o}$). In contrast to the dramatic defect in repair in mice lacking specific proteases, the reparative response in the liver of $uPAR^{o}$ mice was not adversely affected. In these mice, removal of necrotic hepatocytes from the centrilobular area proceeded in a timely fashion, and components of the extracellular matrix underwent normal reorganization to restore the hepatic architecture. Within the same experimental setting, the defective repair observed in uPA^{o} mice was qualitatively and quantitatively similar to the phenotype of $uPAR^{o}/tPA^{o}$ mice. These results clearly demonstrated that binding of uPA to its hepatocyte receptor is not required for normal reparative response (manuscript in preparation).

Working model: Physiological synergism of the plasminogen system in liver repair

Based on the *in vivo* experiments summarized above, members of the plasminogen system of proteases that induce proteolysis (plasminogen, uPA, and tPA) work jointly to direct proteolytic clearance of necrotic hepatocytes and reorganize the extracellular matrix to restore normal liver architecture following an acute injury (Fig. 5). Notably, quantitative analysis of defective repair in uPA- and/or tPA-deficient mice points to a synergistic role of both proteases in the activation of plasminogen during liver repair. Moreover, the activation of plasminogen by soluble (not receptor-bound) uPA efficiently directs pericellular proteolysis.

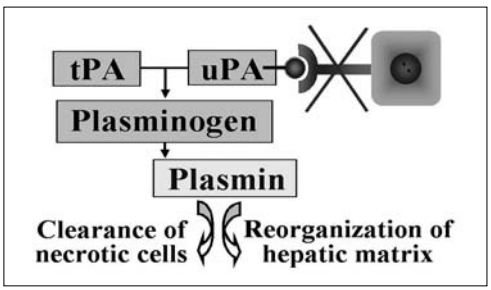


Figure 5. *The figure depicts the synergistic role of tPA and uPA to activate plasminogen. Once active, plasmin directs clearance of necrotic cells and proteolysis of components of the extracellular matrix during the reparative response of the liver to an injury. The successful completion of both processes results in the restoration of the normal lobular architecture and function of the liver. Binding of uPA to its receptor on the surface of hepatocytes is not required for efficient repair.*

Implications for liver cell transplantation

The use of unique mouse lines displaying the inactivation of specific components of the plasminogen family of proteases clearly demonstrates that plasmin-driven proteolysis is critical for cellular movement within the hepatic lobule. Therefore, plasminogen emerges as a potential agent to facilitate engraftment of hepatocytes into the liver lobule and to promote proliferative growth to repopulate diseased livers. The direct proof of this concept can be demonstrated in two ways. Firstly, mice lacking plasminogen can be used as recipients in transplantation experiments – a strategy suggested in the original application. Data from initial experiments, however, suggest that the accumulation of fibrin and fibrin-

unrelated components of the extracellular matrix in livers of plasminogen-deficient mice will form a physical barrier to the movement of hepatocytes within the liver lobule. Secondly, the role of plasminogen as a facilitator of engraftment and proliferative expansion of transplanted hepatocytes may be best determined in mice overexpressing the protease in the extracellular microenvironment. In these experiments, the working hypothesis is that the overproduction of plasminogen increases the re-population of diseased livers by transplanted hepatocytes. Additional experiments employing both experimental strategies will be pursued by our laboratory.

Publications

1. Bezerra JA, Bugge TH, Melin-Aldana H, Sabla G, Kombrinck KW, Witte DP, Degen JL. Plasminogen deficiency leads to impaired remodeling following a toxic injury to the liver. *Proc Natl Acad Sci USA* 1999;6:15143-15148.
2. Bezerra JA, Bugge TH, Melin-Aldana H, Sabla G, Kombrick KW, Witte DP, Degen JL. Plasminogen activators direct matrix remodeling following an acute liver injury. *Am J Pathol* 2001;158:921-927.

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Identification of Novel Porcine Immunoregulatory Molecules

Introduction

The use in transplantation of donor organs from pigs has the capacity to solve the major problem of a lack of available donors. The very recent generation of α-galactosyl transferase knockout pigs makes this an even more compelling issue. Donor organs from these animals lack the carbohydrate determinant that is the target for human natural antibodies, which are a major contributing factor in hyperacture rejection. Our earlier studies of human anti-porcine xenoresponses *in vitro* showed that the xenoresponse was significantly stronger than a human alloresponse. The explanation for this difference is primarily the constitutive expression of the costimulator CD86 on porcine endothelial cells (EC). In contrast, our *in vivo* studies using skin or vessel grafts in SCID mouse models have shown that the alloresponse is substantially stronger than the human anti-porcine xenoresponse.

The long-term survival of these grafts will depend on understanding the species differences in proteins that control the recognition of lymphocytes of the recipient with the graft, especially the endothelium. In addition, the induction of a variety of proteins in response to certain cytokines may differ with respect to expression and time course of induction. To that end, we have begun to characterize inducible proteins from porcine endothelial cells that are important immunologically.

Aims

The main aim of this project was to identify novel expressed genes that are potentially involved in regulating the response of human T cells to porcine endothelial cells in a xenograft setting. The core effort was to identify genes in interferon (IFN)-γ or tumor necrosis factor (TNF)-α-treated porcine aortic endothelial cells (PAEC) that are induced. We have initiated representation difference analysis (RDA) studies with RNA from untreated cells, and cells treated with porcine IFN-γ or human TNF-α.

A second aim of this work was to characterize the properties of genetically altered endothelium modified to be more resistant to cytolytic cells. Isolated PAEC or human umbilical vein endothelial cells (HUVEC) that have been cast in three-dimensional collagen-fibronectin gels that form an extensive network of microvessels within 24 hours. When implanted into SCID mice, extensive connections between mouse microvessels and porcine microvessels are made, resulting in perfusion of the vessels with mouse blood. Retroviral

transduction of cells with the anti-apoptotic Bcl-2 gene resulted in PAEC that showed recruitment of murine smooth muscle cells *in vivo*. Bcl-2 transduction protects HUVEC from CTL *in vitro*. Therefore, we are interested in performing RDA analyses on the transduced PAEC versus control vector-transduced PAEC to identify the important differences. Due to the availability of much more extensive human sequence data, we have first performed this analysis using HUVEC instead of PAEC.

Progress in Final Year

By the end of the second year we had optimized generation of the differentially expressed cDNAs and established a screening protocol with the radiolabeled difference product cDNA preparations. In the third year we added the subcloning of DpnII- and TspI-generated PCR products and generated four large populations of clones from TNF-α-treated PAEC and Bcl-2-transduced HUVEC. 496 PAEC-DpnII clones were analysed, and 358 clones had good sequence corresponding to a wide array of proteins. The DNA inserts from the clones were sequenced and grouped after a BLAST search of the entire DNA database. At a certain point we identified E-selectin and IκBα as common clones and eliminated redundancy in screening by making a probe for filter hybridizations to exclude them from subsequent DNA sequencing. The methodology is working very well in that genes known to be induced by TNF-α were found in significant numbers in the clone collection (e.g., MHC-I, E-selectin and IκBα). However, there are many more clones that were not previously known to be responsive to this cytokine treatment.

We have utilized quantitative RT-PCR to verify whether the genes are induced and to accurately determine the extent of induction. Table 2 shows the relative abundance expressed as a Ct value for cells culture in normal medium versus cells treated with TNF-α for 4 hours or, for comparison, porcine IFN-γ for 16 hours. The change in Ct shows a 2-fold increase over a medium control that is geometric, a change in Ct of 4 is actually a 16-fold increase in the level of RNA relative to the untreated sample. Thus, MCP-1 shows about 8-fold induction in response to TNF-α and only a 2-fold increase to IFN-γ. E-selectin is induced about 100-fold after TNF-α treatment. The small inducible cytokine/IFN-γ was induced over 200-fold at the transcriptional level by both cytokines. While this screening procedure shows an enormous enrichment for cytokine-induced genes, it clearly has some limitations in that some genes when analyzed by the quantitative RT-PCR do not show induction. These results will be repeated and additional genes that were isolated in multiple copies or genes that would be of special interest are on the list. The sensitivity of the quantitative RT-PCR is very high so characterization of transcriptional induction of even rare mRNAs is straightforward.

In addition to the 496 PAEC DpnII clones, 100 clones from TspI-digested TNF-α-treated PAEC have been sequenced. From the comparison of Bcl-2- vs EGFP-transduced HUVEC we have sequenced 324 DNA clones derived from DpnII difference products and 115 clones from TspI difference products. Again, novel cDNAs have been identified in these screens,

but verification by quantitative RT-PCR is essential. In addition, DNA microarray analysis is being performed on the HUVEC samples to identify differences in expression of DNA clones present on those microarrays. Approximately 20 000 genes were present on these arrays. The microarrays and RDA experiments are somewhat complementary in that RDA can identify new genes that are not on the arrays or in the databases at present.

Summary

This work has produced a series of cDNA clones that are differentially expressed in response to cytokine-treated PAEC as well as Bcl-2-transduced HUVEC. The methodology has been optimized for identification of these clones. The analysis of porcine gene expression patterns has verified that the cytokine responses are similar to those known for cytokine-treated HUVEC cells and revealed some new genes that can be used to examine the nature of the cytokine response. Analysis of the Bcl-2-transduced HUVEC cells also has identified candidate differentially expressed genes. Parallel experiments with human cells are being carried out using DNA microarrays. This work will be continued by further characterization of the expression of these identified cDNAs using quantitative RT-PCR. We plan to submit a manuscript for publication describing the induction of genes in PAEC in the near future once the quantitative RT-PCR analysis is complete.

Figure 1. *Real-time PCR analysis of PAEC-derived cDNAs*

Gene	Change in Ct in response to	
	IFN-γ	TNF-α
E-selectin	0.39	7.17
MCP-1	1.10	3.75
Small inducible cytokine/IFN-γ	10.81	10.80
Cytochrome P450	8.55	6.37
Clone 24707	3.02	2.28
Phosphatidic acid phosphatase	3.20	7.49
Aldehyde dehydrogenase	−2.69	1.74
Clone HS21C100	−1.88	2.45
Clone RP11-38N17	6.78	1.54
Ribosomal protein L6	0.18	0.86
Ribosomal protein L7a	5.08	7.46
Protein disulfide isomerase	0.84	1.32
Tenascin	2.40	0.550
Clone 5037	0.54	0.77
Fibronectin	−0.15	0.89
Capping protein	n.d.	n.d.
Myosin	4.66	−0.23
Dynactin	1.24	1.25
RNA-binding protein 1	2.61	−2.29
RNA-binding protein 2	0.29	1.55
Thrombospondin	−0.67	−1.27
Clone RP11-343K113	0.27	−3.54
Ubiquinol cytochrome reductase C	−16.09	−9.05
Proteosome	0.28	0.54
GAPDH	0.44	0.61

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Gene Therapy to Prolong Graft Survival

The fields of gene therapy, gene transfer and gene medicine are now increasingly focussed on methods for improving and regulating gene expression. Increasing attention has been given to the importance of non-specific, innate immune mechanisms that limit vector efficiency and persistence. These innate immune mechanisms include vector clearance by resident macrophages, and the release of cytokines and chemokines, which induce a profound inflammatory response of macrophages, neutrophils, and natural killer cells. This inflammatory response is not dependent on viral gene expression, and viral antigen specific immune recognition is also not required. This suggests that viral transduction of both parenchymal cells and leukocytes induces an innate, stereotyped response that inhibits viral function and gene expression. The results from the currently funded grant application confirm these notions. As noted in the previous two annual reports documenting the accomplished and preliminary data, Aims 1, 2 and 4 have been completed. These results showed us that our experimental efforts needed to be redirected to understanding the innate immune responses that are induced by gene transfer vectors, to define the cellular and molecular mechanisms of innate immunity, and to use this knowledge to devise strategies to inhibit the responses in order to improve gene transfer, gene expression, and the efficacy of the gene transfer vectors in transplantation.

As shown in previous reports, Specific Aims 1, 2 and 4 have been accomplished, specifically: 1) Demonstrate that vMIP-II and MC148 gene transfer inhibits alloimmunity and evaluate their effects on T cells, B cells, and APC components of the alloresponse; 2) Co-transfer vMIP-II or MC148 with other immunosuppressive cytokine genes, such as IL-10, or with conventional immunosuppressive agents to determine if there is a synergistic effect on prolongation of graft survival and inhibition of alloimmunity; 4) vMIP-II and MC148 constructs will be used to create adenoviral vectors to be evaluated in the allograft models.

Over the last year, additional attention has been directed toward understanding the interaction of gene transfer vectors with the parenchymal cells of organ allografts, and the interaction of gene transfer vectors with the innate immune system. Analyses have then been conducted to understand how these interactions affect innate immunity, adaptive immunity, gene expression, and the effects of transferring the immunosuppressive

molecules vMIP-II and MC148. In one study conducted this year using a cDNA array technique, we defined the chemokines and receptors induced by the transplant procedure, ischemia injury, alloantigen, and gene transfer vector administration in murine cardiac grafts. E1,E3-deleted AdRSVβgal was transferred into syngeneic allogeneic grafts at the time of transplantation, grafts were harvested after 1–14 days, total RNA isolated, reverse transcribed to cDNA, and cDNA hybridized to a gene array, which evaluated the levels of 67 chemokine and chemokine receptor genes. Real-time RT-PCR on selected genes was conducted to confirm the array results. Transplantation resulted in ischemia-reperfusion injury and induction of a number of similar genes in both syngeneic and allogeneic grafts, such as CXCL1 and CXCL5, which increased dramatically on day 1 and returned rapidly to baseline in syngeneic grafts. Alloantigens stimulated the adaptive immune response and induced the presence of more inflammatory genes within the grafts, particularly at later time points. The adenovirus vector induced a broader panel of genes, among them the potent inflammatory chemokines CXCL9 and CXCL10, which are induced earlier and more strongly compared with alloantigen stimulation alone. These data demonstrate that the inflammatory response to ischemia-reperfusion injury, alloantigen, and viral vectors in the setting of cardiac transplantation can be defined by time-dependent sets or cascades of molecules associated with distinct stimuli. Since alloantigen and adenovirus vector both induce similar sets of genes, targeting these molecules may not only inhibit alloimmunity, but also enhance the utility of the gene transfer vector.

This approach was extended to the gene transfer of adenoviral vectors encoding vMIP-II and MC148. While it would be expected that ischemia-reperfusion injury and vector transduction would result in similar induction of primary chemokines and chemokine receptors, the expression of vMIP-II or MC148 would be expected to decrease the continued expression of these genes and also prevent the expression of secondary chemokines and chemokine receptors. Indeed, it was found that MC148 or vMIP-II gene transduction both downregulated a wide variety of homeostatic and inflammatory chemokines and chemokine receptors. A manuscript describing these results is currently being prepared. The results show that the expression of inhibitory chemokines will not only prevent the effects of primarily induced chemokines, as shown in the reports for years 1 and 2, but also subsequently prevent the upregulation of secondary inflammatory chemokines and chemokine receptors.

In another series of studies we evaluated the ability of feline immunodeficiency virus (FIV) vectors to transduce allografts and express immunosuppressive cytokines. In addition, we also investigated the chemokines and chemokine receptors that were induced by this vector. FIV can integrate into the genomic DNA of non-dividing cells, resulting in definite transgene expression, making it a particularly attractive gene transfer vector. We hypothesized that FIV-mediated gene transfer could provide long-term gene expression, and improve allograft

survival. FIVvIL-10 and FIVβ-gal were produced using the FELIX vector system. After vector transfer to syngeneic cardiac grafts, β-galactoside reporter gene expression was noted as early as day 5, was strongly expressed at 10 and 20 days, and persisted for 50 days after transplantation. This is in contradistinction to gene transfer by plasmid-, retroviral-, or adenoviral-mediated gene transfer, where reporter gene expression was extinguished rapidly in less than 10 days. For allografts, FIVvIL-10 gene transfer more than doubled the mean survival time from 10.6 to 22.4 days. When combined with other immunosuppressants; such as anti-CD40L mAb, FTY720, or anti-CD3 mAb; the mean survival times were prolonged to 27, 27.7, and 45.5 days, respectively. Multiple chemokine and chemokine receptor genes were induced by ischemia-reperfusion injury in syngeneic grafts, and in allogeneic grafts more genes were induced and to a greater degree. In allogeneic grafts transduced with FIVvIL-10 a number of chemokine genes were suppressed. Therefore FIV-mediated vIL-10 gene transfer prolongs allograft survival, and in combination with other agents, produces an additive effect. Most important with regard to the ROTRF-funded aspect of this study is the fact that allogeneic stimulation, ischemia-reperfusion, and even FIVβ-gal viral transduction result in further upregulation of a number of chemokines and receptors, including CCR1, CCR7, CCL12, CCL19, CCL2, CCL28, CCL3, CXCL10, and CXCL11. Nonetheless, after vIL-10 gene transduction, many of these genes were further downregulated, proving that the innate immune system can be blocked at a number of different points using different modalities.

Another series of studies was initiated to investigate the interaction of vectors with pancreatic islets and a β-cell line. Freshly isolated murine islets or the murine cell line β-CT3 were transduced with adenovirus vector encoding the reporter gene β-galactosidase at a cellular multiplicity of infection of 100:1. The cells were infected for 4 hours, washed, and placed back into culture for 24 to 36 hours, harvested, RNA isolated and reverse transcribed to cDNA, and the cDNA used to probe a chemokine array hybridization membrane. The results in Figure 1A show the hybridization patterns of the membranes and demonstrate that many genes are upregulated as a result of the vector in both the cell line and the islets. These results are confirmed and quantitated in Figure 1B, which shows real-time RT-PCR performed on samples from the islets using primers specific for MIG, IP-10, MCP-1, or RANTES. The results clearly show that adenovirus vector transduction resulted in significant upregulation of the four chemokine genes. Furthermore, transduction of the adenovirus vector encoding vIL-10 resulted in significant inhibition of MIG and RANTES, but not IP-10 or MCP-1. Additional experiments with MC148 gene transduction and vMIP-II gene transduction are underway. These results confirm that the interaction of vector with islets or a β-cell line in the absence of lymphocytes induces several immunologically important chemokines that have significant roles in many inflammatory and immune responses. These results and conclusions were confirmed *in vivo.* Islets were isolated from C57BL/6 or BALB/c mice, uninfected or infected with adenovirus, and then transplanted to the renal capsule of

syngeneic or allogeneic C57BL/6 recipients. Four days after transplantation the islets were harvested, RNA isolated, cDNA reverse transcribed, and hybridized to the chemokine array. The results in Figure 1C show upregulation of MCP-1 mRNA as a result of vector transduction in both syngeneic and allogeneic islets.

In summary, our results and the evolution of our thought processes over the last three years show that the innate immune system has a major influence on the efficacy and safety of gene transfer vectors, including both viral and non-viral vectors. The interactions of vectors with parenchymal cells induce innate immune responses. The innate immune response is also induced by ischemia-reperfusion and surgical injury. The innate immune response itself interacts with multiple components of adaptive and autoimmunity to further amplify and focus their effects to the detriment of graft survival. The comprehensive understanding of the interactions among innate immunity, adaptive immunity, ischemia-reperfusion, parenchymal cells of vascularized and non-vascularized grafts, the influences of viral or non-viral gene transfer vectors, and the effect of the transferred immunosuppressive gene are necessary to understand fully the utility and value of gene transfer vectors and to be able to manipulate them for important therapeutic advantage.

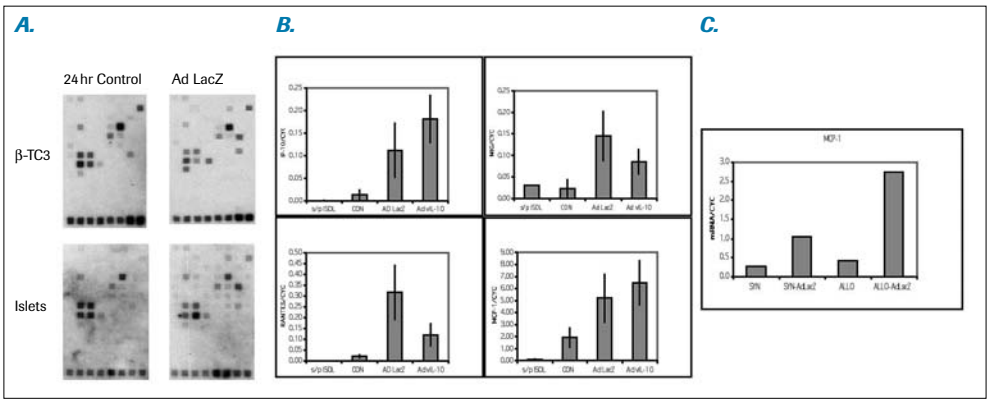

Figure 1. A. *Chemokine array analysis of b-TC3 or islets with or without AdLacZ transfection.*
B. *Quantitative comparison of IP-10, RANTES, MIG, and MCP-1 by real-time PCR in islets after isolation or transduction with AdLacZ or AdvIL-10.*
C. *In vivo expression of MCP-1 by real-time PCR in syngeneic and allogeneic islets transduced with AdLacZ.*

Publications

1. Fu S, Chen D, Mao X, Ding Y, Bromberg JS. Feline immunodeficiency virus mediated viral interleukin-10 gene transfer prolongs cardiac allograft survival. *Am J Transplant,* in press.
2. Chen D, Ding Y, Schroppel B, Zhang N, Fu S, Chen D, Zhang H, Bromberg JS. Ischemia-reperfusion, alloantigen, and adenovirus gene transfer result in differential expression of chemokine and chemokine receptor genes in cardiac grafts. *Gene Ther,* in press.
3. Fu S, Mao X, Chen D, Ding Y, Bromberg JS. FIV virus mediated viral IL-10 gene transfer prolongs allograft survival. *Am J Transplant* 2002;2 (Suppl.3):1224.

4. Chen D, Ding Y, Schröppel B, Chen D, Bromberg JS. Adenoviral vectors stimulate potent chemokine expression in transplanted hearts. *Mol Ther* 2002;5:S7.
5. Fu S, Mao X, Chen D, Ding Z, Bromberg JS. FIV virus mediated vIL-10 gene transfer prolongs cardiac allograft survival. *Mol Ther* 2002;5:S234.
6. Chen D, Ding Y, Fu S, Mao X, Bromberg JS. vIL-10 gene transfer inhibits expression of multiple proinflammatory genes in transplanted hearts. *Mol Ther* 2002;5:S297.
7. Zhang N, Schröppel B, Murphy B, Bromberg JS. Adenovirus transduction promotes the expression of multiple chemokines in β-TC3 cells and murine pancreatic islets. *Mol Ther* 2002;5:S346.
8. Zhang N, Schröppel B, Murphy B, Bromberg JS, Sung R. Adenovirus transduction impairs engraftment of murine pancreatic islet transplants. *Mol Ther* 2002;5:S350.

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Study of the Role of γδ T Lymphocytes in the Immune Response Directed against Human Cytomegalovirus

Introduction

A few years ago, we observed a dramatic and persistent increase of γδ T lymphocytes in the peripheral blood of renal transplant patients who developed a cytomegalovirus (CMV) infection[1]. While circulating γδ T cells mainly express the Vδ2/Vγ9 variable regions under normal conditions, only $V\delta2^{neg}$ cells are affected by the CMV-associated amplification (mainly Vδ1 which is the major $V\delta2^{neg}$ population in the body, but also Vδ3 and Vδ5 cells). Some patients display among their peripheral T cells up to 50% of $V\delta2^{neg}$ γδ T lymphocytes whereas these cells do not exceed 1% in healthy individuals. These values persist for years after infection and never return to basic levels. The $V\delta2^{neg}$ T cell repertoire is highly restricted (monoclonal in some patients) suggesting an antigen-driven selection of these cells during CMV infection. *In vitro* CMV-infected cell lysates induce γδ T cell proliferation among PBMC. These results indicate that $V\delta2^{neg}$ γδ T cells may recognize an antigen induced during cellular infection by CMV.

As γδ T cells had never been reported to be involved in the immune response directed against CMV, the aim of our project was to understand the role played by $V\delta2^{neg}$ γδ T cells in this pathological context, notably through the identification of the antigens recognized by these still enigmatic cells.

Results

Expansion of $V\delta2^{neg}$ γδ T cells in CMV seropositive healthy individuals

Further analysis of renal transplant patients showed that γδ T cell percentage determination is a reliable prognosis factor to predict the resolution of CMV infection, suggesting a protective role of $V\delta2^{neg}$ γδ T cells in the anti-viral host defense[2]. This prognosis might be broadened to patients who received other organ transplants because we demonstrated that $V\delta2^{neg}$ γδ T cells are also expanded during CMV infection in lung, heart, liver or intestinal transplantation.

To address the question of the role of immunosuppression in the CMV-mediated $V\delta2^{neg}$ γδ T cell expansion, healthy individuals were studied. Interestingly, the percentage of cells is

enhanced in CMV seropositive immunocompetent subjects, compared with seronegative ones. In addition, in normal individuals seropositivity for CMV (but not for any other herpesvirus) was associated with a clear restriction in the number of Vδ1^{+} γδ T cells but not of Vδ2^{+} γδ T cells. The high prevalence of CMV in the population might explain the small number of Vδ1^{+} cells frequently observed in the blood of healthy individuals. These results expand the potential role of γδ T cells in the immune response against CMV, and indicate that immunosuppression is not required for γδ T cell implication during CMV infection (submitted for publication).

In vitro studies of the anti-CMV reactivity of Vδ2^{neg} γδ T cells

The potential anti-viral role for γδ T cells was then investigated *in vitro*. A large panel of Vδ2^{neg} γδ T cell lines and clones isolated from CMV-infected transplant patients was generated. Some of the T cell lines and clones specifically reacted against U373 or MRC5 cells only when they were infected with CMV (Figure 1). Optimal reactivity of clones was obtained 72 hours after the viral infection. Clones produced TNF-α, IFN-γ and GM-CSF, and killed the infected cells. Furthermore, they inhibited efficiently CMV replication in the cell culture.

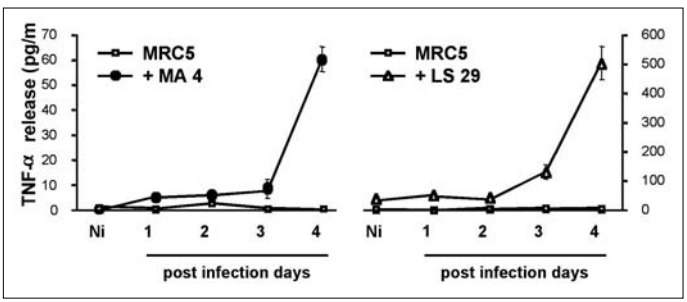

Figure 1. *Vδ2^{neg} γδ T cell clones (MA 4 and LS 29) are activated when cultured in contact with CMV infected MRC5 cells.*

By contrast, Vδ2^{neg} γδ T cell clones were not activated when MRC5 cells were infected by herpes simplex virus (HSV) or varicella zoster virus (VZV) (Figure 2) demonstrating that this immune response is specific to CMV and not to other herpes viruses.

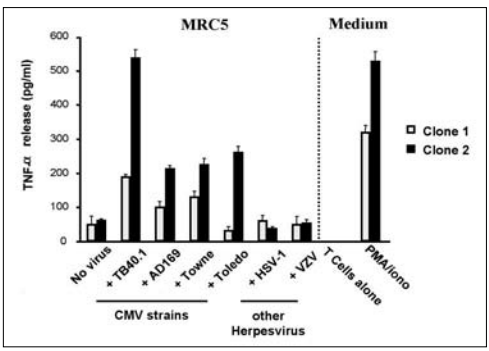

Figure 2. *Activation of Vδ2^{neg} γδ T cells by MRC5 cells infected with various clinical or laboratory CMV strains but not by HSV-1 or VZV infected MRC5 cells.*

Vδ2^{neg} γδ T cell reactivity was inhibited in the presence of an anti-TCR mAb and was associated with the internalization of γδ TCR, suggesting a direct involvement of TCR in recognition of CMV-infected cells (Figure 3).

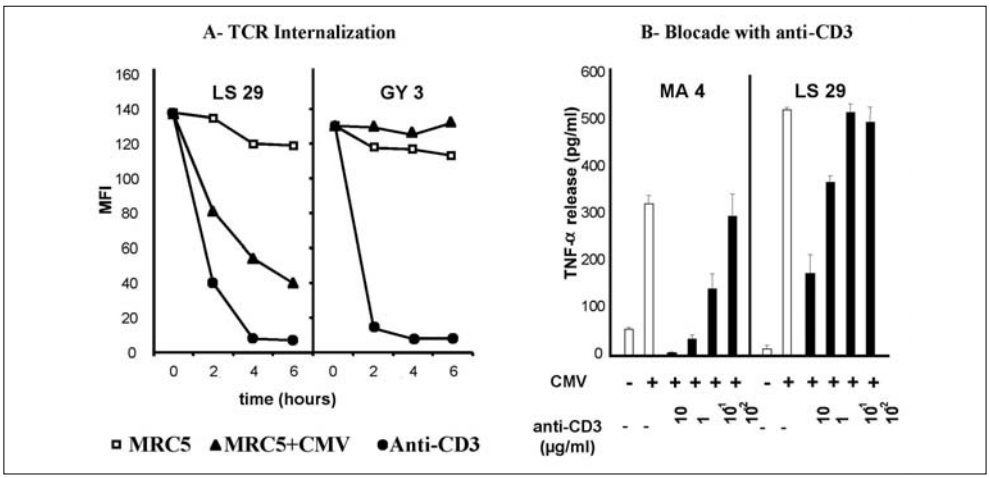

Figure 3. *Activation of Vδ2^{neg} γδ T cells by CMV-infected cells requires TCR engagement.*

As the few ligands of γδ TCR so far identified are widely expressed among eucaryotic and procaryotic cells, we tested whether the antigen recognized by our anti-CMV T cell clones could be expressed on other cell lines, particularly tumor cells. We observed a strong reactivity of Vδ2^{neg} γδ T cell clones against two intestinal epithelial tumor cell lines (HT29 and Caco2) and against the erythroblastoid cell line (K562) (Figure 4). Similarly, lung carcinoma primary cell lines were able to activate Vδ2^{neg} γδ T cell clones. No activation was obtained when a blocking anti-TCR mAb was added to the culture, or when using other cell lines such as epstein barr virus (EBV) transformed B cell lines (Daudi, P815, Jurkat).

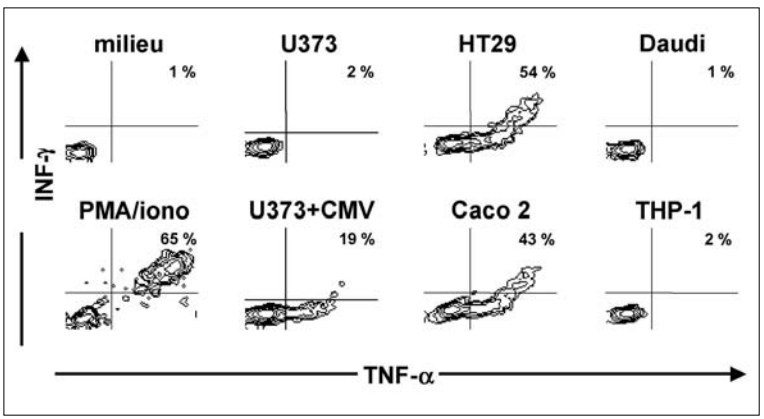

Figure 4. *Shared reactivity of Vδ2^{neg} γδ T cells toward CMV-infected cells and intestinal epithelial tumor cells (HT29 and Caco2).*

From these results, we hypothesized that the ligand of Vδ2^{neg} γδ TCR is most probably a cellular antigen, induced on the cell surface by either CMV infection or tumor transformation. We then focused our attention on the identification of this antigenic ligand. We immunized mice with either the HT29 cell line or the CMV-infected MRC5 cells. MAbs were screened for their ability to inhibit the reactivity of Vδ2^{neg} γδ T cell clones against HT29, K562 and CMV-infected cells (Figure 5). Out of 4 fusions, only two mAbs could be selected (2E9 and 7C2, both IgMs) suggesting that the ligand is poorly expressed on the surface of cells. This was confirmed by flow cytometry analysis showing a weak labeling of HT29 and CMV-infected MRC5 cells by 2E9 and 7C2 mAbs. This low expression probably also explains why only IgMs were selected in this functional screening. Despite a sustained effort, immunoprecipitation experiments performed with 2E9 mAb did not permit identification of a band corresponding to the ligand. However, treatment of HT29 cells with pronase abrogated labeling with 2E9 mAb, showing that the recognized antigen is a protein. We are currently trying to enhance ligand expression by activating HT29 cells in various conditions. This could improve the results of immunoprecipitation experiments and allow microsequencing of the antigen by mass spectrometry. Besides, we also plan to clone the ligand from an HT29 cDNA library through expression in COS cells.

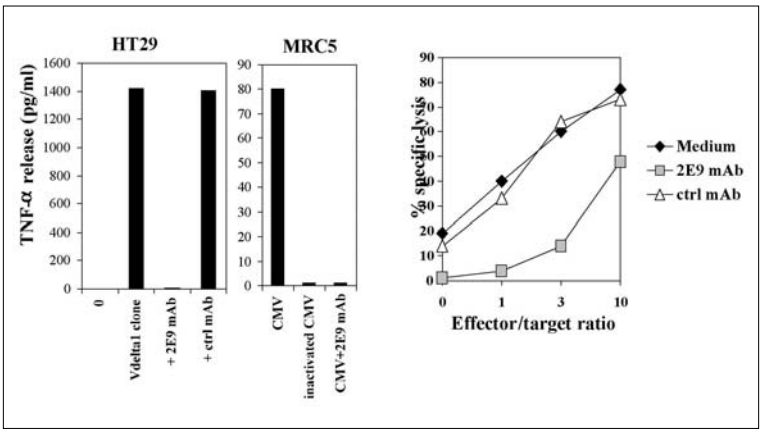

Figure 5.
The 2E9 mAb directed against HT29 cells abrogates the activation of Vδ2^{neg} γδ T cells cultured with HT29 or CMV-infected MRC5 cells, and inhibits their cytoxicity toward K562 cells.

NKR expression by Vδ2^{neg} γδ T cells

The Vδ2^{neg} γδ T cells expanding in CMV infection frequently expressed natural killer cell receptors (NKR) such as CD94, NKG2A, NKG2D, CD158b and CD85. The functionality of these receptors on Vδ2^{neg} γδ T cells is difficult to address because of the redundancy of activating and inhibitory receptors on the same cell. However, preliminary data eliminated a putative role of NKG2D in the recognition of CMV-infected cells by the clones.

The limited diversity of Vδ2^{neg} γδ T cells and their highly restricted number in CMV-infected patients allowed for the analysis of NKR expression on a monoclonal population of Vδ5^{+} γδ T cells, representing 20% of peripheral T cells in a CMV-infected lung transplant patient.

This study indicates that despite their monoclonality these cells display *in vivo* a heterogeneous NKR phenotype for Ig and lectin. This result definitively demonstrated that NKR are not clonally expressed *in vivo*. NKR expression could represent a functional diversity for memory T cells in addition to TCR rearrangements (manuscript submitted for publication).

Vδ2^{neg} γδ TCR transfer

We also studied the generation of Vδ2^{neg} γδ TCR transfected Jurkat cell lines, which could be very useful to avoid technical limitations of low proliferating Vδ2^{neg} γδ T cells, to prove the involvement of the TCR in the anti-CMV γδ T cell activation, and to screen other blocking anti-ligand mAbs. We chose the J.RT3-T3.5 Jurkat cell line, which does not express original Ab TCR nor CD3 on its surface, but can express a TCR when transfected with cDNA encoding γ and δ TCR chains. Vδ and Vγ cDNA (isolated from three Vδ2^{neg} γδ T cell clones from two CMV seropositive transplanted patients and one CMV seronegative control patient) were successfully cloned in pREP vectors, but no expression was achieved after electroporation of J.RT3-T3.5 cells. Cloning in lentiviral vectors is in progress and should considerably improve transfection efficiency (90% with a reporter vector in these cells).

Role of DC-SIGN in CMV infection

Among our efforts to understand the mechanisms underlying the interplay between the immune system and CMV, we recently focused our attention on another important immune cell type, the dendritic cell (DC). We investigated *in vitro* the role played by DCs in the capture and the transmission of CMV to target cells. DCs which display strong migratory capacities have also been reported to internalize pathogens such as HIV. We demonstrated that this also holds true for CMV through binding of the envelope glycoprotein B to DC-SIGN, a recently characterized C-type lectin receptor mainly expressed by DC, and allows for the transmission to permissive cells. Moreover, blocking DC-SIGN by specific antibodies inhibited DC infection by primary CMV isolates and expression of DC-SIGN or its homologue DC-SIGNR (77% amino acid homologies) rendered susceptible cells permissive to CMV infection. These results provide new insights into the molecular interactions contributing to cell infection by CMV and imply an involvement of DC-SIGN in virus propagation and immune evasion[3].

Publications

1. Merville P, Déchanet J, Bergé F, Bone-Mane G, Taupin JL, Michel P, Joly P, Bonneville M, Potaux L, Moreau JF. Cytomegalovirus infection in kidney allograft recipients is followed by a prolonged expansion of γδ T lymphocytes *Transplant Proc* 2000;32:357-359.
2. Lafarge X, Merville P, Cazin MC, Bergé F, Potaux L, Moreau JF, Déchanet-Merville J. CMV infection in transplanted patients resolves when circulating T lymphocytes expand, suggesting their protective antiviral role. *J Infec. Dis* 2001;184:533-41.
3. Halary F, Amara A, Lortat-Jacob H, Messerle M, Delaunay T, Houlès C, Fieschi F, Arenzana-Seisdedos F, Moreau JF, Déchanet-Merville J. Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity* 2002;17:653-64.

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Creating Artificial Immunoprivilege for Allograft Acceptance

This research proposal was aimed at using Fas ligand to induce artificial immunoprivilege for organ transplant acceptance. Since certain tissues are protected from the immune system because local Fas ligand (FasL) expression can induce apoptotic death of self-reactive T cells, forcing FasL expression in allografts should create artificial immuno-privileged sites in organ transplants. The experimental strategy consisted of producing chimeras comprised of the extracellular domain of FasL fused to single-chain antibodies (scFvs) recognising MHC class-I molecules specific to allografts. The ability of these molecules to induce deletion of alloreactive T cells was to be tested in a skin allograft rejection model. It has been reported that forced FasL expression in allografts could induce massive neutrophil infiltration leading to accelerated graft rejection, suggesting that FasL might be chemotactic towards neutrophils. In order to create artificial immunoprivilege, it is necessary to dissociate the apoptotic and chemotactic properties of FasL. We proposed that the molecular nature of FasL might be involved in distinguishing these two properties, with the assumption that membrane-bound trimeric FasL would be pro-apoptotic, while soluble monomeric FasL could be chemotactic. Through molecular modelling, mutants predicted to form either forced monomers or forced trimers were to be constructed by site-directed mutagenesis and tested for their apoptotic and chemotactic abilities. The initial research proposal consisted of 3 specific aims: 1. Dissociate the chemotactic properties of FasL from its apoptotic properties; 2. Generate specific allograft targeting molecules using single-chain antibodies; 3. Evaluate the ability of targeting molecules to induce artificial immunoprivilege *in vivo*. The ROTRF provided funding to investigate the first specific aim.

The proposed forced monomers and forced trimers were constructed and transfected in COS cells. Supernatants containing metalloproteinase-cleaved FasL were tested in apoptosis assays using T cells as targets and chemotaxis assays using purified human neutrophils. The forced trimer proved to be 5-fold more efficient in inducing T cell apoptosis compared with wild-type FasL, while the forced monomer was inactive, arguing that the molecular predictions made were accurate. Given that it is well documented in the literature that FasL trimers are required for efficient apoptosis, these results were not unexpected. When these molecules were used in chemotaxis assays, no neutrophil migration could be observed under any conditions tested, even with wild-type FasL. This prompted us to re-investigate

published reports showing that FasL was chemotactic towards purified neutrophils. Using Fas agonists (anti-Fas IgG, anti-Fas IgM antibody, cross-linked recombinant FasL), no migration could be observed, while response to chemoattractants, such as IL-8 or fMLP, was readily demonstrated. The functionality of agonists was evaluated in apoptosis assays and showed that cross-linked FasL and IgM were efficient while the dimeric IgG was ineffective, demonstrating that the reagents used were functional. Given the body of evidence for *in vivo* FasL-mediated neutrophil infiltration, the cause for such a discrepancy was investigated.

The neutrophils used in the chemotaxis assays were >99% pure, as assessed by CD15 staining. When we opted to use unfractionnated leukocytes in Boyden chamber assays, neutrophil migration was restored in response to Fas engagement, arguing that FasL is not directly chemotactic towards neutrophils and that contaminating leukocytes were responsible for the migration observed by other groups. Using magnetic cell sorting (MACS), highly purified leukocyte populations (>99%) were obtained (monocytes/macrophages, B cells, T cells, neutrophils and NK cells) and used in Boyden chamber reconstitution experiments. 250 000 highly purified neutrophils were placed in the upper chamber together with different numbers of other cell types, while Fas agonists were placed in the lower chamber. Following 2 hours of incubation, cells having migrated in the lower chamber were enumerated by FACS using cell surface markers. Again, purified neutrophils alone did not respond to Fas agonists. The addition of 0.1% to 10% of T cells, B cells or monocytes/ macrophages in the upper chamber did not restore migration. Surprisingly, the addition of 1% or 10% NK cells to the upper chamber completely restored migration. Since the different purified cell populations did not migrate in response to Fas engagement, we reasoned that NK cells that had fallen into the lower chamber during the experiment were involved. Indeed, putting 0.1% NK cells in the lower chamber gave higher migration indexes than any number of NK cells placed in the upper chamber. This argues that NK cells respond to Fas engagement by producing a neutrophil chemoattractant.

To understand the mechanisms involved, we used neutralising antibodies and caspase inhibitors. It has been suggested that, *in vivo,* local production of IL-1β by macrophages might be involved in neutrophil recruitment. While it is known that IL-1β is not a neutrophil chemoattractant, we investigated its contribution in our system. The addition of saturating amounts of a neutralising IL-1β antibody did not affect migration and, surprisingly, the addition of recombinant IL-1β to the upper chamber severely impaired migration. This is likely to be caused by IL-1β-mediated priming, as neutrophils sense high concentrations of pro-inflammatory cytokines as their final destination. Thus, *in vivo,* high local IL-1β concentrations might participate in neutrophil accumulation, but our results clearly show that this cytokine is not involved in neutrophil recruitment following Fas engagement. These results were confirmed by using a caspase-1 inhibitor, since caspase-1 converts pro-IL-1β

and pro-IL-18 to their active forms. The caspase-1 inhibitor was totally ineffective in suppressing neutrophil migration, but the caspase-8 inhibitor reduced migration by about 75%. This argues that NK cell chemokine release is partly caspase-dependent, which is not totally unexpected given that Fas engagement leads to caspase-8 activation.

One of the most potent neutrophil chemoattractants is IL-8, which is produced by several cell types, including neutrophils. We used a neutralising IL-8 antibody in the Boyden chamber assays and this severely reduced neutrophil migration. It has been demonstrated that neutrophils engaged in migration release IL-8 and we believe them to be the source in our system since the neutralising IL-8 antibody also severely impaired migration in response to fMLP, without any NK cells in the system.

In summary, Fas engagement leads to the caspase-8-mediated release of an unknown chemokine by NK cells and neutrophil-derived IL-8 participates in amplifying this response. We are currently using RNAse protection to identify the chemokines produced by NK cells following Fas cross-linking. The identification of soluble mediators involved will be critical to control the pro-inflammatory properties of FasL in artificial immunoprivilege induction.

We also evaluated apoptosis of neutrophils and NK cells following Fas engagement. No apoptosis was observed at the concentration of Fas agonists used in the migration assays. In fact, to achieve significant apoptosis, a 100-fold higher concentration of agonists was required and the incubation time had to be increased 6-fold. It should be noted these high doses were very poor at eliciting neutrophil migration. Furthermore, the dimeric IgG antibody was totally ineffective at apoptosis induction, while it was potent in migration assays. This argues that FasL-mediated chemotaxis and apoptosis can be dissociated and the main factor distinguishing these properties is concentration. These observations are consistent with reports showing that FasL transgene dosage leads to neutrophil recruitment at lower concentrations and graft acceptance at higher concentrations. To control local allograft FasL concentration, a reagent that can attain high organ accumulation and allows dosage control is required. Such a reagent would be the FasL-scFv chimera proposed in the original grant application.

The skin allograft rejection model used consists of mice transgenic for a TCR that recognises grafts through either the direct or indirect pathways. The MHC-I molecules expressed by donors are D^p and K^p for the direct pathway, D^b and K^b for the indirect pathway, while the recipient expresses the D^k and K^k haplotypes. An antibody recognising donor but not recipient molecules was obtained and its specificity was confirmed by FACS. This antibody was assembled as a single-chain antibody and further modified through linker length alteration to create diabodies and triabodies. Since trimeric FasL is known to be more efficient at apoptosis induction, we expect the triabody fusion will increase FasL potency.

An expression vector was constructed to secrete scFv-FasL fusion proteins and transfected into Neuro-2A cells. These three molecular constructions are currently being mass-produced in hollow-fiber bioreactors and will be tested for their chemotactic and apoptotic abilities. To that end, individual class-I molecules of donor and recipient were cloned by RT-PCR and transfected into HeLa cells; these transfectants will be used as a presentation platform for the FasL-scFv chimeras in Boyden chamber and T cell killing assays.

To sum up the 2-year funding period, the assumptions based on published literature have been challenged by experimental evidence obtained through ROTRF funding. The basic knowledge acquired through this research has allowed the identification of molecular handles to control the undesirable effects of FasL expression. We anticipate that the reagents under production will allow rapid testing of the feasibility of FasL for organ transplantation acceptance.

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Dendritic Cells Transduced with the FasL Gene as a Tool to Regulate Allograft Immunity

Introduction

CD95 (Fas)-mediated apoptosis of activated T lymphocytes is critically involved in the homeostasis of the T cell pool and the maintenance of peripheral tolerance to self antigens. Moreover, it has been proposed that the immune privilege status of particular anatomical sites could be related to local expression of CD95L and that expression of CD95L by tumor cells might protect them from immune attack. On this basis, is has been considered that expression of CD95L on allo- or xenografts might promote their acceptance by deleting host T cells specific for transplanted antigens. Indeed, CD95L expression on Sertoli cells was suggested to be directly responsible for testis allograft survival. It was then reported that implantation of syngeneic muscle cells transfected with CD95L together with allogeneic grafted pancreatic islets allowed long-term survival of the transplanted islets. More recently, CD95L overexpression on allogeneic endothelium was shown to inhibit transplant-associated intimal hyperplasia. However, several of these observations have been refuted so that the role of CD95L in confering immune privilege is currently a matter of controversy. Furthermore, chemoattraction of neutrophils leading to a massive inflammatory reaction has emerged as a major consequence of CD95L overexpression. Neutrophil infiltration leading to graft destruction was observed after implantation of pancreatic islets in which the CD95L gene was overexpressed. Likewise, CD95L transgenic islet β cells or heart allografts were shown to be rejected more rapidly than their wild-type counterparts, in association with a massive influx of neutrophils in the transplant.

In order to promote deletion of allospecific T cells without inducing inflammation at the graft level, it has been proposed to condition allograft recipients with antigen-presenting cells overexpressing CD95L prior to transplantation. Allogeneic macrophages transduced with murine CD95L induce profound alloantigen-specific T cell unresponsiveness. DC represent a suitable cell type for such an approach because injection of an ovalbumin-pulsed DC line transfected with murine CD95L induces antigen-specific T cell hyporesponsiveness. In the transplantation setting, Min et al. reported significant enhancement of heart allograft survival in mice repeatedly injected with high doses of donor-type BM-derived DC transfected with human CD95L.

Herein, DC genetically engineered to overexpress CD95L were derived from BM precursors of CD95-deficient lpr/lpr mice as rapid apoptosis was observed when wild-type mice were used as BM donors. We assessed the allostimulatory capacities of CD95L-DC in two models involving either a single MHC class I or MHC class II disparity. Unexpectedly, CD95L-DC were found to elicit stronger Th1-type and cytotoxic T cell (CTL) responses than control DC *in vivo*. These observations led us to investigate the role of neutrophils during the induction phase of alloreactive T cell responses triggered by CD95L-DC and to revisit the consequences of the injection of CD95L-DC on the fate of a subsequent tissue allograft.

Results

DC overexpressing CD95L function as killer DC *in vitro*

In a first set of experiments, DC were generated from WT C57BL/6 BM-progenitors during 10 day culture in the presence of mGM-CSF and were submitted to mFasL.2MFG retroviral transduction. This resulted in massive cell death; more than 90% of BM-cells were annexin V and propidium iodide positive 48 hours after the first CD95L transduction, compared with 5% after the control transduction. Suicidal or fratricidal death was probably involved, since more than 85% viability of CD95L-transduced DC was obtained at the end of the culture when CD95-deficient lpr/lpr mice were used as BM donors. After transduction of lpr/lpr BM-progenitors with either mFasL.2 or control retrovirus and culture in GM-CSF, around 85% of the cells were $CD11c^{pos}$ $GR1^{low}$ DC with an immature phenotype, as indicated by low expression of MHC class II, CD80, CD86 and CD40.

DC transduced with CD95L induced a dose-dependent lysis of $CD95^{pos}$ cells that was dependent on CD95-CD95L interaction since it was blocked by the addition of mFas-hFc fusion protein (Fig. 1). As expected from their immature phenotype, DC transduced with control vector induced only low T cell proliferation in mixed leukocyte culture and this was further reduced when CD95L-DC were used as stimulators. Whatever the retroviral vector used, immature DC did not induce significant production of IFN-γ. As expected, both the proliferative response and the production of IFN-γ elicited by CD95L-DC after maturation with LPS were lower than when control DC were used as stimulators.

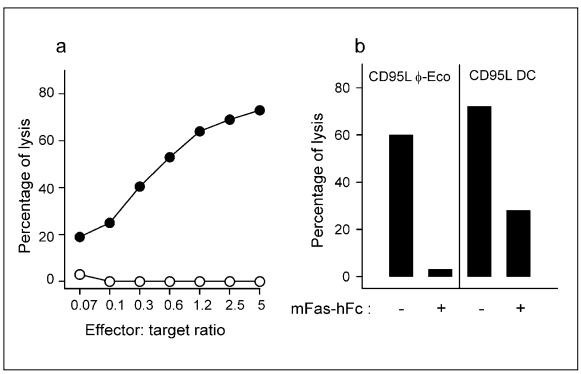
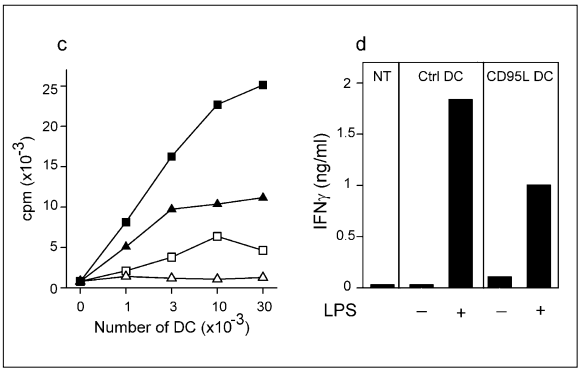


Figure 1.
CD95L-transduced DC are cytotoxic and down-regulate allogeneic MLC in vitro.
a) *mCD95-transfected P815 cells were incubated with CD95L-DC (solid circle) or control-DC (open circle). Data are representative of 15 experiments.*
b) *CD95-P815 cells were cocultured with CD95L-DC (right panel) or CD95L-transfected PhoenixECO cells (left panel) at an effector: target cell ratio of 2.5:1, in the presence or not of mFas-hFc. Percentages of lysis are representative of 2 experiments.*



(Figure 1.)
c) *Triplicate culture of 2×10^5 bm12 LN cells and lpr/lpr CD95L- (without LPS, open triangle; with LPS, solid triangle) or control-DC (without LPS, open square; with LPS, solid square) was incubated for 3 days. Results are representative of 3 experiments.*
d) *10^6 BALB/c LN cells were seeded with 3×10^5 irradiated C57BL/6 lpr/lpr CD95L- or control-DCs activated or not with LPS. Supernatants were collected after 72 hrs for IFN-γ quantification. Similar data were obtained in 3 experiments.*

DC overexpressing CD95L induce vigorous Th1 and CTL responses *in vivo* and prime for acute allograft rejection

The next series of experiments were designed to determine whether lpr/lpr CD95L-DC would inhibit alloreactive responses *in vivo*. We first observed that injection of CD95L-DC in the footpad of bm12 mice was followed by swelling of the draining popliteal lymph node (LN) with a significant increase in cellularity compared with LN draining of the site of injection of control DC (Fig. 2a). In parallel, we assessed the presence of DC in LN using a semi-quantitative PCR for the lpr/lpr mutation. Similar levels of donor-type DNA were found after injection of CD95L-DC or control DC (Fig. 2b), suggesting that CD95L overexpression did not influence DC migration.

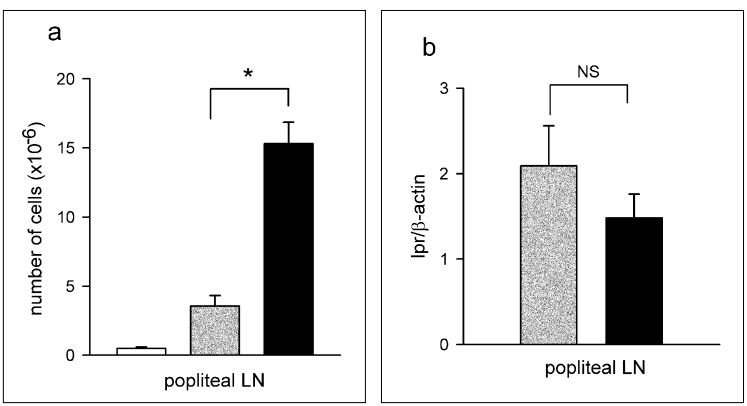


Figure 2.
CD95L-DC induce strong T cell proliferation in vivo.
a) *bm12 mice received one footpad injection of 10^6 lpr/lpr DCs transduced with CD95L (black bars) or control retrovirus (grey bars) or were untreated (white bars). Five days later, the total number of popliteal LN cells was determined. Results were expressed as a mean number of cells ± SEM (*$p<0{,}02$).*
b) *bm12 mice were injected with 10^6 lpr/lpr CD95L- (black bar) or control-DCs (grey bar). Five days later, DNA was extracted from popliteal LN and the level of lpr mutation was measured by PCR. Results are expressed as the mean of lpr versus β-actin signals ± SEM. Each group contains 4 individual mice.*

To characterize the T cell responses induced *in vivo* by C57BL/6 lpr/lpr CD95L-DC or control DC, MLC were prepared between responder LN T cells from bm12 (MHC class II mismatch) or bm13 (MHC class I mismatch) mice inoculated with the transduced DC and donor-type or third-party splenocytes as stimulators. Consistent with previous studies, the response elicited by control DC in MHC class II-incompatible mice was Th2 skewed, as indicated by a high production of IL-4 and IL-5 and a low production of IL-2 and IFN-γ (Fig. 3a). In contrast, the *in vivo* response to CD95L-DC injection in the same strain combination was characterized by a dominant induction of IL-2 and IFN-γ and a low production of IL-5 and IL-4 (Fig. 3a). This Th1-skewed response elicited by CD95L-DC was specific for the donor alloantigen, as it was not observed with third-party stimulators. The absence of Th1-type cytokines hyperproduction induced by CD95L-DC in CD95-deficient lpr/lpr bm12 recipients confirmed that this Th1-skewed response was dependent on CD95-CD95L interactions (Fig. 3b).

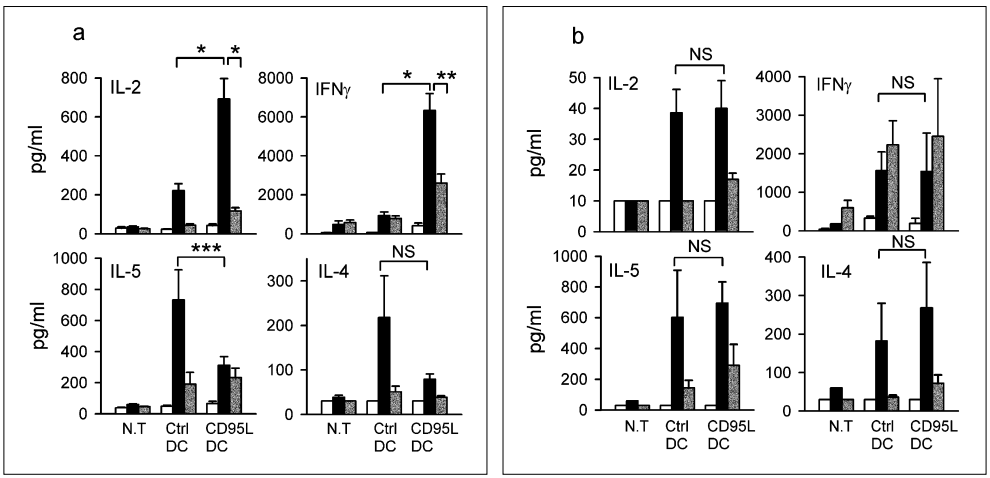


Figure 3.
CD95L-DC promote a Th1 response in MHC class II disparate mice.
CD95L-DC exacerbated Th1 cytokines production in bm12 but not in lpr/lpr bm12 mice. 5 days after the subcutaneous footpad injection of 3×10^5 CD95L- or control-DC, draining popliteal and inguinal LN cells from bm12 ***a)*** *or lpr/lpr bm12 mice* ***b)*** *were cultivated with bm12 (syngeneic, white bars), C57BL/6 lpr/lpr (allogeneic, black bars) or BALB/c (third-party, grey bars) spleen cells for 3 days. Supernatants were collected after 24 hrs for IL-2 measurement and 72 hrs for IL-4, IL-5 and IFN-γ quantification. Results were expressed as mean ± SEM of 11 to 21 mice per groups of bm12 mice (*p≤ 0,0001; **p=0,0015; ***p=0,03) and 3 mice per groups of lpr/lpr bm12 mice (NS: not significant with a two-tailed Student's test).*

When similar experiments were repeated in MHC class I-incompatible bm13 mice as recipients, we found that injection of CD95L-DC significantly enhanced the production of IFN-γ by donor-reactive T cells compared with injection of control DC. In this strain combination, neither IL-4 nor IL-5 were detectable in the MLC supernatants whatever the

DC injected. The injection of CD95L-DC but not control DC significantly increased the donor-specific CTL activity generated by LN T cells in MLC (Fig. 4). As control, we found that the anti-third party CTL response was not modified by the injection of CD95L-DC. As the single MHC class I incompatibility in the (C57Bl/6 → bm13) strain combination is not sufficient to trigger skin allograft rejection, this strain combination was adequate to assess the priming effect of CD95L-DC in a transplantation setting. A single footpad inoculation of as few as 3×10^5 C57Bl/6 lpr/lpr CD95L-DC was sufficient to prime bm13 mice for acute rejection of C57Bl/6 skin allograft (Fig. 5), whereas DC transduced with a control vector did not do so.

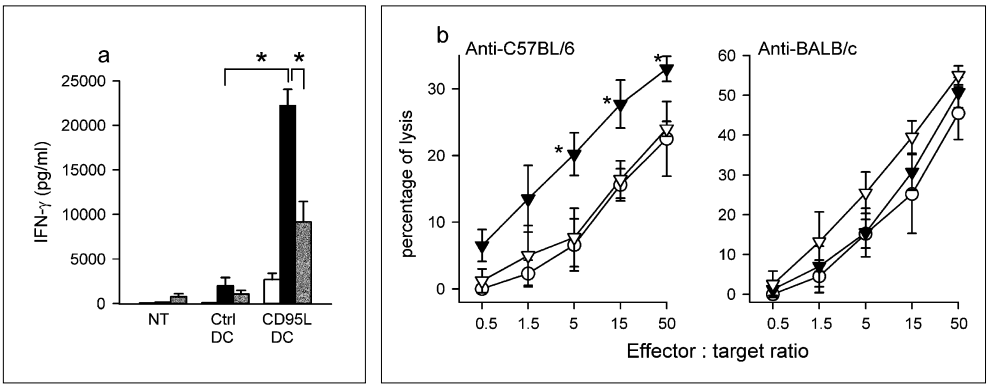


Figure 4.
CD95L-DC induced increased allospecific Th1 type response and cyctotoxic activity in a MHC class I disparate model.
a) *4 to 6 bm13 mice received 3×10^5 lpr/lpr control- or CD95L-DC into the footpad. MLC were performed 5 days later with drained LN cells and either syngeneic (bm13, white bars), allogeneic (lpr/lpr, black bars) or third-party (BALB/c, grey bars) spleen cells. Results were expressed as mean ± SEM (*p<0,03). Similar results were obtained in two experiments.*
b) *Donor-type (left panel) or third-party (right panel) specific CTL activity was evaluated after the same control- (open circle) or CD95L- (close triangle) DC treatment. Open triangles represent CD95L-DC with RB6-8C5 (anti-Gr1) mAbs treatment. Results are mean percentage of lysis ± SEM of 3 to 4 individual mice per group (*p<0,02 compared with the ctrl-DC group and *p<0,004 compared with anti-GR1 treated group with the two-tailed Student's test). Similar results were obtained in three separate experiments.*

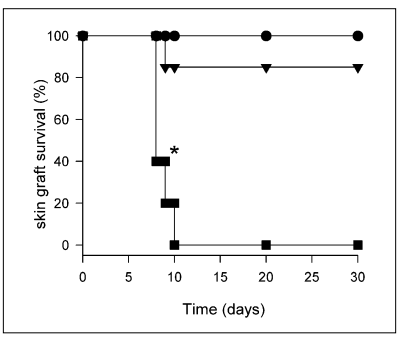


Figure 5.
CD95L-DC induce skin allograft rejection.
*10 bm13 mice were inoculated subcutaneously into the footpad with either 3×10^5 control- (solid triangle), CD95L- (solid square) DC or were untreated (solid circle). After five days, C57BL/6 skin allografts were performed and graft survival was monitored daily (*p<0,001 for CD95L-DC treated mice compared with ctrl DC-injected or untreated mice).*

Neutrophil infiltrate sites of CD95L-DC injection

CD95L was reported as a potent chemoattractant of neutrophils *in vitro* and *in vivo*. Histological examination of footpads 5 days after injection of CD95L-DC revealed a major thickening of the dermis, which was massively infiltrated with neutrophils. A highly invasive neutrophil infiltration was also observed in the underneath muscle cells layer. The injection of control DC did not modify the skin structure and resulted only in a minor mononuclear cell infiltration in the dermis. A dominant neutrophil recruitment was also observed in peritoneal cavities of bm12 mice injected 16 h before with lpr/lpr CD95L-DC, as revealed by flow cytometry and May-Grünwald Giemsa staining. In mice injected with CD95L-DC, 67 ± 10.7% (mean ± SEM, $n = 8$) of the peritoneal exudate cells were $Gr1^{pos}$ $CD11b^{pos}$ neutrophils with characteristic nuclear morphology, whereas only 2.5 ± 2.1% of these cells were found in mice injected with control DC ($n = 6$). The neutrophil recruitment induced by CD95L-DC was strictly dependent on CD95-CD95L interactions as it was not observed after injection in CD95-deficient lpr/lpr bm12 mice. In agreement with previous reports, we found that the neutrophil influx triggered by injection of CD95L-DC in footpad was dramatically reduced in $IL\text{-}1R^{-/-}$ mice. In those animals, the only change in the dermis consisted of a moderate infiltrate of mononuclear cells and eosinophils. In parallel, we observed that the influx of $GR1^{pos}$ $CD11b^{pos}$ neutrophils after intraperitoneal injection of CD95L-DC was drastically decreased in $IL1\text{-}R^{-/-}$ mice (mean ± SEM of $GR1^{pos}$ $CD11b^{pos}$ cells: 22 ± 7% in $IL1R^{-/-}$ mice vs 67 ± 10% in C57BL/6 WT mice).

Involvement of neutrophils in the induction of allospecific Th1 and CTL responses by CD95L-DC

To investigate the role of neutrophils in the allospecific T cell responses induced by CD95L-DC, we depleted neutrophils by injection of the RB6-8C5 mAb specific for Gr1. Mice treated with RB6-8C5 mAb were indeed free of $Gr1^{pos}$ $CD11b^{pos}$ neutrophils in the peritoneal cavity after *i.p.* injection of CD95L-DC. We then analyzed the helper T cell responses induced in bm12 mice by *i.p.* inoculation of CD95L-DC after injection of either 250 µg RB6-8C5 or the same amount of control rat IgG mAb given on days -1, 0, 1, 2 and 3. MLC were performed with mesenteric T cells 5 days after DC injection. The hyperproduction of IFN-γ elicited by the injection of CD95L-DC was abolished by previous neutrophil depletion but not by injection of the control rat mAb (Fig. 6). This was not related to impaired DC migration as the levels of lpr mutation detected in the mesenteric LN after CD95L-DC injection were not affected by neutrophil depletion. We also evaluated the role played by neutrophils in the increased MHC class I-specific cytotoxic activity elicited by C57BL/6 lpr/lpr CD95L-DC in bm13 mice. Neutrophil depletion reduced the cytotoxic activity to the level observed after injection of control DC (Fig. 4b). Furthermore, when we analyzed the cytokine profile of donor-reactive T cells in draining LN after subcutaneous injection of lpr/lpr bm12 DC into C57BL/6 mice, we found an increased IFN-γ/IL-5 production ratio in wild-type but not in $IL1\text{-}R^{-/-}$ mice, consistent with the involvement of neutrophils in the Th1 skewing of the response induced by CD95L-DC (Fig. 7).

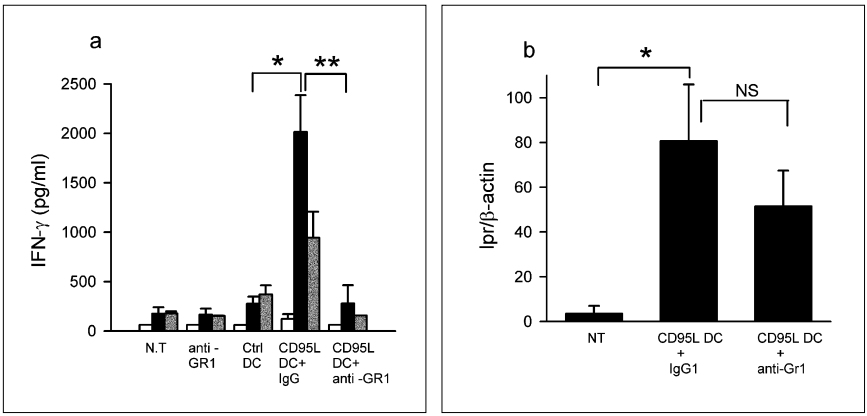


Figure 6.

Involvement of neutrophils in the allospecific Th1 response in MHC class II disparate mice.

a) *The anti-Gr1 neutrophil depletion inhibits the CD95L-dependent allospecific Th1 response. Bm12 mice were intraperitoneally injected with 5×10^5 lpr/lpr control- or CD95L-DC and treated or not with control IgG or RB6-8C5 mAbs. After 5 days mesenteric LN cells were cultivated with syngeneic bm12 (white bars), allogeneic lpr/lpr (black bars) or third-party BALB/c (grey bars) spleen cells for 3 days. Supernatants were collected after 72hrs for IFN-γ quantification. Each group contains 3 to 7 individual mice. Results were expressed as mean ± SEM (*p=0,0025; **p=0,036).*

b) *anti-GR1 mAbs treatment does not deplete the injected DC. DNA of mesenteric LN was extracted and the lpr/lpr mutation was detected by semi quantitative PCR. Results are means of lpr versus β-actin signals ± SEM of 4 mice/group (*p=0,02).*

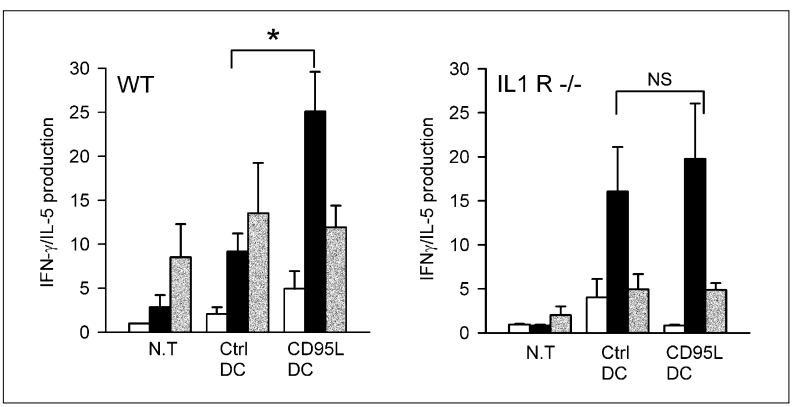


Figure 7.

Th1 versus Th2 response is not increased by CD95L-DC in IL1R$^{-/-}$ mice.

*3×10^5 lpr/lpr bm12 CD95L- or control-DC were inoculated subcutaneously into the footpad of C57BL/6 wild type (WT) or C57BL/6 IL-1R$^{-/-}$ mice. Five days later, drained LN cells were cocultured with syngeneic (C57BL/6, white bars), allogeneic (lpr/lpr bm12, black bars) or third-party (BALB/c, grey bars) spleen cells for 3 days. Supernatans were collected after 72hrs for IFN-γ and IL-5 measurements. Results are expressed as mean of IFN-γ/IL-5 production ± SEM of 6 to 8 mice for the WT group and 4 to 7 mice for the IL1R$^{-/-}$ group (*p<0,02).*

Discussion

The data presented indicate that, when resistant to CD95 engagement, DC that overexpress CD95L induce vigorous Th1-type and cytotoxic T cell responses, which depend on neutrophil recruitment *in vivo.* In the transplantation setting this property of CD95L-DC results in priming for allograft rejection instead of the anticipated tolerogenic effect.

Our observations contrast with previous reports providing evidence that such CD95L-DC are immunosuppressive. In the earlier studies, DC transduced with the CD95L gene coexpressed a functional CD95 that might predispose them to CD95-mediated apoptosis. Although unmanipulated DC or transformed DC were found to be resistant to CD95 engagement, we found that the overexpression of CD95L obtained by gene transfer at an early stage of DC differentiation promotes their apoptosis. The downregulation of the T cell responses observed in previous studies could therefore be related to indirect presentation of alloantigens derived from apoptotic cells by host immature DC. This possible drawback was circumvented in our experiments by the use of CD95-deficient DC. The first cells to be considered as targets for the injected CD95L-DC are host T lymphocytes, but the consequences of CD95 engagement on T cells are not univocal. Depending on the activation status and on the naive vs memory phenotype of the T cells, they either undergo apoptosis or receive costimulatory and proliferation signals. CD95-mediated costimulation was reported to be effective in both $CD4^{pos}$ and $CD8^{pos}$ naive T cells and could therefore be involved in the capacity of CD95L-DC to prime both helper T cell responses against MHC class II and CTL responses against MHC class I alloantigens. However, CD95L overexpression did not enhance and actually inhibited the capacity of DC to activate alloreactive T cells *in vitro,* suggesting that the allostimulatory potential of CD95L-DC *in vivo* might depend on their action on other cell types than T cells.

Neutrophils are known to be recruited at sites of CD95L overexpression and to contribute to destruction of tumors and allografts overexpressing CD95L. It was demonstrated that the soluble form of CD95L has chemotactic ability for neutrophils *in vitro* but the full-length transmembrane CD95L form appears as the predominant neutrophil chemoattractant *in vivo.* Caspase activation elicited by CD95 engagement was shown to be involved in the proinflammatory properties of CD95L by promoting the processing and secretion of IL-1β. We observed that CD95L-DC indeed induced a major neutrophil influx at the site of their injection, either in the footpad or in the peritoneal cavity, and we confirmed that IL-1 mediates this phenomenon as it was dramatically reduced in IL-1 receptor-deficient mice. Furthermore, the reduced production of IFN-γ elicited by CD95L-DC in the latter animals and in mice depleted of neutrophils revealed that neutrophil recruitment is critically involved in the Th1 alloimmune response induced *in vivo* by DC overexpressing CD95L. Neutrophils were found previously to promote Th1 polarization of $CD4^{pos}$ T cell responses in experimental models of infection with *Legionella pneumophilia* or *Toxoplasma gondii.* Herein, we found that neutrophils also participate in the induction of $CD8^{pos}$ cytotoxic T cell responses against MHC class I alloantigen expressed on CD95L-DC. The influence of neutrophils on $CD4^{pos}$

and $CD8^{pos}$ T cell responses might be related to their release of Th1 polarizing factors such as IL-12, IFN-γ, and chemokines active on Th1-type cells such as MIG and MIP-1α. It is also possible that neutrophils enhance T cell responses *in vivo* by promoting maturation of the injected DC via production of TNF-α and IL-1β. Host DC might also be activated by these mediators and by CD95 engagement and could contribute to the alloimmune response via the indirect pathway of antigen presentation. Thus, neutrophil influx appears as a critical factor governing the ultimate consequences of CD95L-CD95 interactions on T cell responses. The expression of CD95L in immune privilege sites is often associated with the production of inhibitors of neutrophilic inflammation such as TGF-β. Likewise, Chen et al. demonstrated that cotransfection of the TGF-β gene in tumor cells overexpressing CD95L was sufficient to facilitate tumor growth, whereas single transfectants expressing only CD95L were readily destroyed. The suppressive versus immunogenic properties of cells overexpressing CD95L might depend on a number of factors, including the tissue microenvironment, the release of soluble CD95L, and the level of expression of the transgene.

Because of their potent immunostimulatory properties *in vivo,* it is unlikely that CD95L-DC will find applications in transplantation as initially proposed. We rather suggest to consider CD95L-DC as a possible tool to prime anti-tumor responses in cancer immunotherapy.

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Manabu Yanagita, Research Associate

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The Role of Plasmacytoid Dendritic Cells in the Development of Tolerance

The murine "paucity of lymph node T cell" (*plt*) mutation is a deletion of genes encoding the CCR7 ligand chemokines CCL19 and CCL21, leading to defects in the migration and localization of T cells and dendritic cells (DC) in secondary lymphoid organs. *plt* mice display increased and prolonged immune responses to protein antigens, a defect in the development of oral tolerance, and a failure to develop tolerance to allogenic transplants when the recipients are treated with co-stimulatory blockade and donor specific transfusion. Studies exploring the mechanism of these defects led to our discovery of murine plasmacytoid DC (pDC), which we hypothesized to be a tolerogenic DC population within murine LN. With funding from the ROTRF, we explored the mechanisms by which pDC may stimulate tolerance and the defect in pDC that may lead to a failure in the development of tolerance.

Effects of pDC on T cell proliferation

In initial experiments, the ability of pDC to stimulate T cell proliferation was compared with classic myeloid DC (mDC) under various conditions. Unstimulated immature pDC do not stimulate T cell proliferation (Fig. 1). pDC activated by CpG stimulate robust T cell proliferation that is equal to that seen with mDC. Treatment with LPS, CD40 stimulation, or influenza virus infection of pDC does not stimulate T cell proliferation above that seen with immature pDC. In contrast, mDC stimulate robust T cell proliferation when cultured in GM-CSF, and this activity is not significantly enhanced by any of the agents tested.

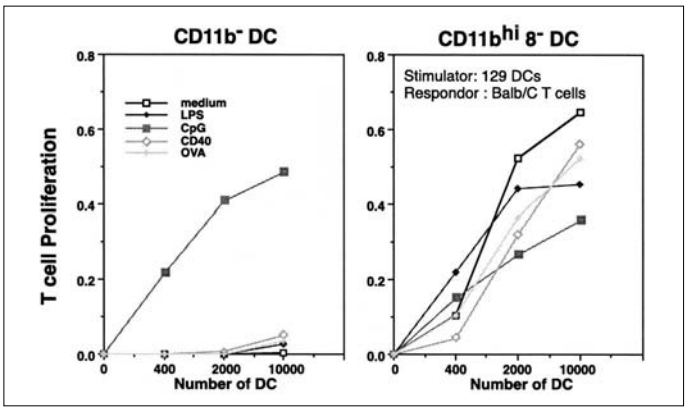

Figure 1.
T cell proliferation induced by CD11b⁺ pDC and CD11b^hi mDC after in vitro stimulation.

Cytokine production by activated pDC

pDC produce large amounts of interferon (IFN)-α and some interleukin (IL)-12 in response to virus stimulation (Fig. 2). In response to CpG stimulation, pDC express lower but significant amounts of IFN-α and similar amounts of IL-12. Unlike viral stimulation, CpG stimulation of pDC leads to significant amounts of IL-10 production.

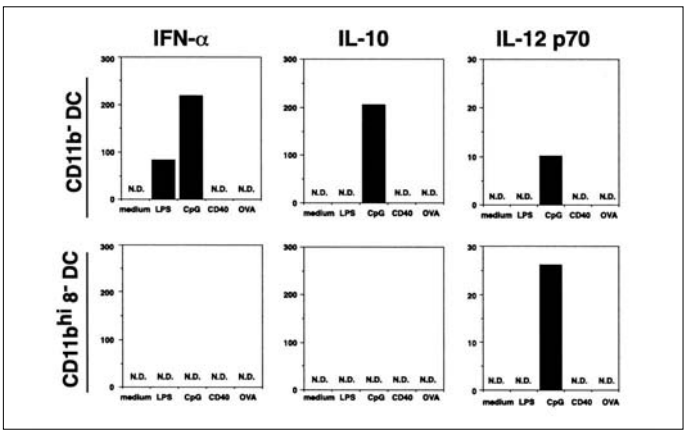

Figure 2.
Cytokine production by pDC and mDC after in vitro stimulation.

Cytokine production by pDC-stimulated T cells

T cells cultured with immature pDC produce high levels of IL-10 with little, if any, production of IL-4 or IFN-γ. Importantly, this is the phenotype of IL-10-producing T regulatory (Tr-1) cells. T cells stimulated with CpG-activated pDC continue to produce IL-10, although at lower levels than immature pDC-stimulated T cells, and display markedly increased levels of IFN-γ production. Consistent with previously reported results, T cells cultured with mDC produce IL-4 and IL-10 but no IFN-γ . Stimulation of pDC *in vivo* leads to a similar pattern of T cell cytokine production. These findings suggest that pDC may promote the development of tolerance via the production of Tr cells.

pDC-stimulated T cells suppress T cell proliferation

pDC-stimulated T cells proliferate poorly upon mDC restimulation compared with naive T cells (Fig 3A). Importantly, the addition of pDC-stimulated T cells to a naive T cell culture totally inhibits the proliferation of the latter cell type. Similar results were obtained when T cells were restimulated with CD3 and CD28 (data not shown). To examine the effect of pDC-stimulated T cells on the proliferation of previously activated T cells, an increasing number of pDC-stimulated T cells was added to a constant number of mDC-stimulated T cells, this mixture restimulated with mDC, and their proliferation assessed. pDC-stimulated T cells suppress the proliferation of T cells previously activated by mDC in a dose-dependent manner (Fig. 3B). Thus, T cells initially primed with immature pDC demonstrate a suppressive activity toward both naive and previously activated T cells.

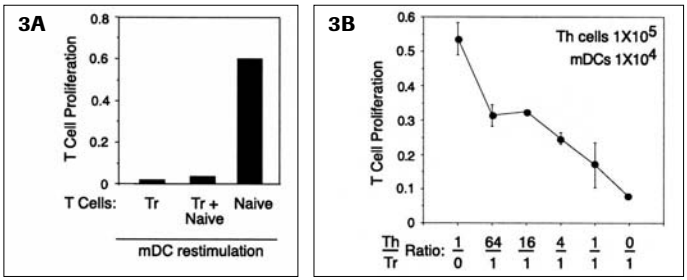

Figure 3.
T regulatory activity of pDC-stimulated T cells.

DC-subset-specific defects in *plt* mice

The above results suggest that the pDC-mediated production of Tr cells may play a role in tolerance induction and that *plt* mice may have a defect in the migration, localization, or function of pDC. We have shown previously that *plt* mice have a 50% decrease in the number of LN DC at baseline. Here we examine the frequency of DC in *plt* LN on a subset-specific basis (Fig. 4). Surprisingly, the number of mDC ($CD11b^{+}CD8^{-}$) in *plt* LN is normal while the number of "lymphoid" $CD11b^{int}CD8^{+}$ DC is severely reduced. The number of LN pDC is reduced about 50% in *plt* mice. We also examined changes in LN DC numbers after immunization (OVA/CFA SQ) (Fig. 5). *plt* mice display no defect in the accumulation of $CD11b^{+}CD8^{-}$ mDC. In contrast, they display an almost total absence of $CD11b^{int}CD8^{+}$ "lymphoid" DC accumulation and a partial (~60%) defect in the accumulation of pDC.

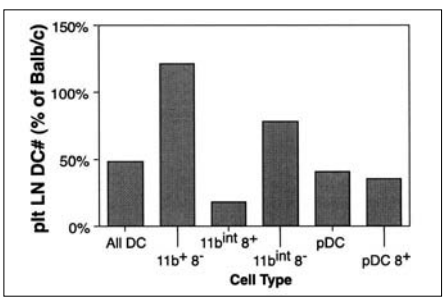

Figure 4.
Frequency of individual DC subsets in plt LN relative to WT mice.

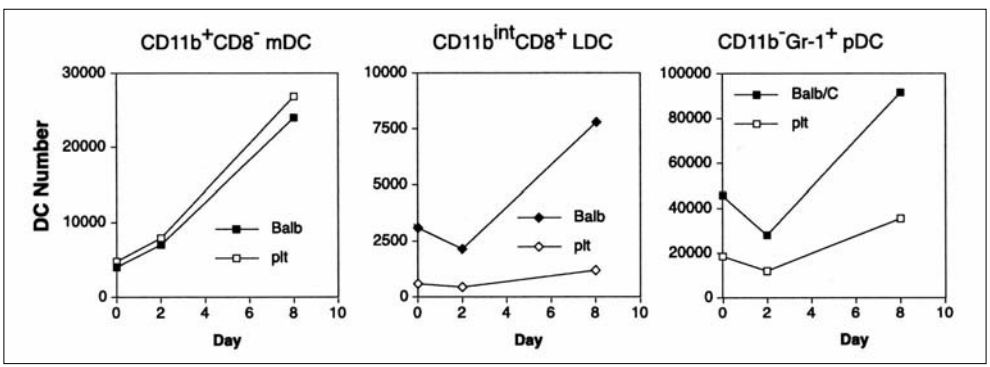

Figure 5. *LN DC accumulation after immunization in WT vs plt mice.*

Abnormalities in cytokine production in *plt* mice

As shown above, pDC stimulate the production of Tr cells. In several recent studies, $CD11b^{int}CD8^{+}$ "lymphoid" DC have been implicated in the peripheral deletion of self-reactive T cells. Thus, *plt* mice display defects in two DC populations that have been implicated in the inhibition of immune response and the development of tolerance while displaying no defect in the accumulation of the most immunostimulatory DC population ($CD11b^{+}CD8^{-}$ mDC). To determine if pDC display a functional abnormality in *plt* mice, we examined cytokine production in the LN of these animals after immunization. Three days after immunization with OVA/CFA, cytokines were examined in total LN preps by ELISA. In this assay, *plt* mice display a 3-fold increase in IL-2 production and an 8-fold increase in IFN-γ production after immunization (Fig. 6). Because IL-10 protein was not detectable by ELISA, we examined IL-10 mRNA expression in T cells. Two days after immunization *plt* mice display an 8-fold decrease in IL-10 production by draining LN T cells. These results are consistent with a defect in the pDC-mediated production of IL-10 producing Tr cells in *plt* mice. This would explain, in part, the increased T cell immune responses in these animals and their failure to develop tolerance. It is possible that *plt* mice will also demonstrate a defect in the peripheral deletion of activated Ag-specific T cells, due perhaps to their failure to accumulate $CD11b^{int}CD8^{+}$ "lymphoid" DC. Studies to address this point are in progress.

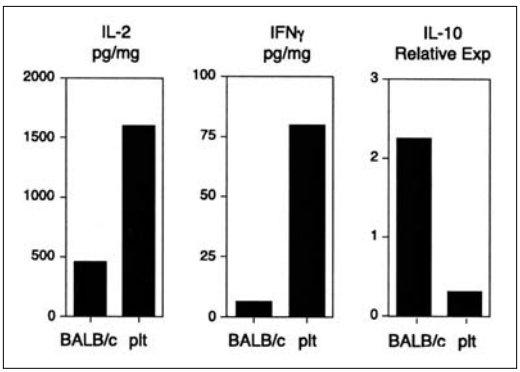

Figure 6.
Cytokines produced in LN after immunization in WT vs plt mice.

We attempted to demonstrate more directly the role of pDC in tolerance induction by depleting these cells *in vivo* using anti-Gr-1 Abs. While a loss of tolerance induction was seen, administration of both Gr-1 and control Abs led to a marked increase in inflammatory response. Thus, the results of these experiments are inconclusive. We are currently exploring alternative means to deplete or inhibit pDC *in vivo.*

Publications

1. Gunn et al. Immature Plasmacytoid Dendritic Cells from Mouse Secondary Lymphoid Organs Induce the Development of IL-10 Producing CD4 T Regulatory Cells, submitted for publication.

Dr. Jörg Koglin, Principal Investigator
Prof. Mary E. Russell, Consultant

University of Munich, Munich, Germany

Role of Innate Human Recognition as Initiator of Chronic Rejection after Cardiac Transplantation

Building upon the original proposal, we hypothesized that innate immune mechanisms, mediated by activation of human Toll-like receptors (hTLR) in monocytes, might play a crucial role in the activation of adaptive immune mechanisms by inducing co-stimulatory signaling and by triggering the differentiation of Th1 effector cells.
We characterized the expression of hTLR2 and hTLR4 in monocytes from transplant recipients and correlated the expression patterns with the regulation of the co-stimulatory molecule B7–1 and the release of Th1 inducer cytokine IL-12 as typical downstream effects of hTLR activation. To distinguish between transplant recipients with and without ongoing chronic rejection, we used endothelial dysfunction as a clinical surrogate marker. Endothelial function testing was performed in the cardiac catheter laboratory using an intracoronary Doppler wire to measure flow responses to acetylcholine. Endothelial dysfunction was defined as a relative flow reserve less than 2.0. As an additional control, expression of hTLR, B7–1 and IL-12 was assessed in healthy controls.
To characterize hTLR2 and hTLR4 expression on circulating $CD14^+$ monocytes, we used two-color flow cytometry in 30 cardiac transplant recipients and 10 healthy controls. B7–1 expression was also assessed by flow cytometry. IL-12 expression of these monocytes was evaluated using an ELISA technique in monocyte supernatants after *ex vivo* stimulation.
For immunohistochemistry, an endomyocardial biopsy from each transplant recipient was taken and snap frozen from the right ventricle for immunhistochemistry.

Results

Endothelial function

In the cascade culminating in angiographically visible coronary stenosis, endothelial dysfunction is considered to be the earliest clinically detectable sign of vessel injury. Clinically, endothelial dysfunction can be diagnosed by demonstration of a compromised coronary flow reserve to an endothelial-dependent vasodilator such as acetylcholine given intracoronarily. The coronary flow reserve was calculated as the epicardial cross-sectional area times the coronary flow velocity during acetylcholine administration divided by the epicardial cross-sectional area times the coronary flow velocity at rest. Endothelial function testing was performed in the proximal left anterior descending coronary artery (LAD). The

epicardial cross-sectional area was calculated using quantitative coronary analysis of the proximal LAD. The coronary flow velocity was measured as average peak velocity using an intracoronary Doppler wire. Using this technique, endothelial dysfunction was detected in 8 out of 32 transplant recipients. 24 patients showed normal endothelial function with acetylcholine administration. Both groups showed no significant differences in time after transplantation, age, gender, cholesterol levels, cytomegalovirus (CMV) status or immunosuppressive regimen.

Immunhistochemistry

Qualitative analysis of immunhistochemistry on frozen sections from endomyocardial biopsies revealed a significant rise in expression of TLR2 and TLR4 antigen in perivascular spaces co-localizing with infiltrating immune cells in patients with endothelial dysfunction, as compared with patients with normal endothelial function. An attempt to quantitate these differences in frozen sections and to expand the number of biopsies evaluated by this technique is ongoing. Large variantions in tissue preservation and staining quality in these snap frozen sections are currently still limiting this goal.

FACS analysis of TLR expression

Two-color flow cytometry was used to characterize and compare TLR2 and TLR4 expression on the surface of $CD14^+$ monocytes. Surface staining of PBMCs was performed with FITC-labeled $CD14^+$ antibodies. HTLR staining was performed with commercially available polyclonal goat antibodies for hTLR2 and hTLR4 at room temperature for 15 minutes. Toll receptor density was detected by adding a secondary PE-labeled rabbit-anti-goat antibody for 30 minutes at room temperature. Intra-assay variability was assessed by independently activating, staining and analysing three aliquots of the same blood sample.

Taken together cardiac transplant recipients showed a significantly increased expansion of both CD14/hTLR2 (37.7 ± 23.4 vs 12.9 ± 5.4; $p < 0.005$) and CD14/hTLR4 monocytes (28.6 ± 21.0 vs 9.9 ± 5.5; $p < 0.02$) when compared with normal controls. Surprisingly we could not detect any significant difference in CD14/hTLR2and $CD14^+$/$hTLR4^+$ monocytes between recipients with and without endothelial dysfunction (TLR2: $p = 0.15$; TLR4: $p = 0.89$).

FACS analysis of B 7–1 expression

Considering this significant up-regulation of Toll-like receptors we were interested whether this might translate into changes in the B 7–1 expression on circulating monocytes. Two-color flow cytometry was used to characterize and compare the extracellular B 7–1 expression on CD14 monocytes. Therefore, PBMCs were incubated for 20 hours *ex vivo* with 100 U/ml INF-α at 37 °C. After surface staining with a FITC-labeled $CD14^+$ antibody, surface staining with PE-labeled B 7–1 antibody was performed. Up-regulation of hTLR2 and hTLR4 on circulating monocytes after transplantation was associated with increased expression of B 7–1 (38.9 ± 17.2 vs 24.5 ± 7.9, $p < 0.02$). Again we could not demonstrate any significant

differences in B7–1 expression on peripheral circulating CD14 monocytes between recipients with and without development of endothelial dysfunction ($p=0.13$).

ELISA for detection of secretion of IL-12 by monocytes

In a next step, we were interested if the enhanced Toll expansion is associated with elevated secretion of the Th1 inducer cytokine IL-12 by monocytes. After *ex vivo* stimulation of CD14 monocytes with 0.1 µg/ml LPS and 100 U/ml INF-α at 37 °C for 20 hours, the analysis of ELISA of *ex vivo* stimulated monocytes revealed that circulating monocytes exhibit significantly increased secretion of IL-12 after cardiac transplantation. 7.9 pg/ml ± 4.3 pg/ml IL-12 in the supernatant in controls was compared with 14.7 pg/ml ± 9.7 pg/ml in cardiac transplant recipients ($p<0.05$). Again, there was no significant difference in IL-12 secretion by monocytes between the cardiac transplant recipients with or without endothelial dysfunction ($p=0.21$).

Conclusion

After recruitment of 30 cardiac transplant recipients we are able to further back up our hypothesis that innate immune recognition of allograft-specific molecular patterns might be responsible for induction of adaptive immune mechanisms, which orchestrate the known immunological events cumulating in the development of cardiac allograft vasculopathy.

With this first set of data, we can show that receptor signaling pathways and downstream effects of the innate immune system are up-regulated in monocytes from cardiac transplant recipients. These findings provide first evidence that activation of Toll-like receptors inducing co-stimulatory molecules (e.g. B7–1) and expression of signaling cytokines (e.g. IL-12) might be part of the cascade, that activates adaptive immune responses, such as T cell receptor activation and Th1 differentiation, known to be essential for the development of the allo-immune responses to the transplanted organ.

Taking these results into consideration we would like to define possible endogenous and exogenous ligands for the two examined human Toll-like receptors. Moreover, our research is focusing on why we could describe an activation of innate immune mechanisms in all cardiac transplant recipients and what causes the development of an endothelial dysfunction in only one quarter of them.

Publications

1. Methe H, Zimmer E, Meiser B, Koglin J. Innate immune mechanisms as part of the allo-immune response after cardiac transplantation. *J Heart Lung Transplant* 2002;21:78.
2. Methe H, Zimmer E, Meiser B, Koglin J. Bedeutung humaner Toll Rezeptoren bei Abstoßung des transplantierten Herzens. *Z Kardiol* 2002;91 (Suppl. 1):133.

Dr. David H. Sachs, Principal Investigator
Dr. Kazuhiko Yamada, Co-Investigator

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Thymic Transplantation to Achieve Tolerance

Introduction

Our thymokidney protocol has been limited solely to renal transplantation, and as such, the full potential of the vascularized thymic transplantation technique will only be realized if the scope of its applicability can be broadened. The creation of other thymic-composite organs has proven technically difficult; however, the ability to perform thymic transplantation as a vascularized thymic lobe (VTL) allograft permits thymic facilitation to occur with any solid organ or tissue transplanted simultaneously. The technical challenges of VTL transplantation have limited its widespread application. VTL transplantation was first performed in the rat model and was later extended to a mouse model; however, no report has been published in any large animal models. With numerous experiences with porcine thymectomies in our laboratory, we recently established the procedure of VTL transplantation in miniature swine. Our data have demonstrated in a fully allogeneic miniature swine model that 1) VTL transplantation is technically feasible in a large animal model; 2) VTL allografts are resistant to ischemic and structural damage following transplantation; 3) VTL allografts support thymopoiesis of recipient-type cells; and 4) CD4 and CD8 single-positive thymocyte development occurs simultaneously in the allografts. VTL transplantation has the potential for broad applications for many different organ and tissue transplantations. The model permitting transplantation of vascularized thymic tissue with any solid organ would clearly expand the scope of this protocol.

In year 3, we extended this study into xenogeneic baboons using the same regimen used for recipients of the thymokidneys in this study. The specific accomplishments made in the year 2001–2002 are detailed in the following report.

Specific Aim 3: Test the effect of porcine thymus transplantation on the induction of xenograft tolerance.

We have performed six VTL xenotransplantations using transgenic hDAF swine as donors and baboons as recipients. These animals also had additional thymic tissue implanted into the omentum at the time of transplantation. VTL in baboons B and C were irradiated (700 rad) on day –2. All baboons, except baboon A, were thymectomized 2 weeks prior to VTL transplantation. In addition, two baboons (baboons G and H) were used as controls:

one received a sham operation, and the other received 5×10^8/kg porcine thymocytes on day 0 instead of vascularized thymic lobe transplantation. The treatment protocol was similar to that used for thymokidney xenotransplantation. However, we reduced the dose of cyclophosphamide to avoid thymic damage and administered FK506 for 28 days due to success in our allogeneic VTL study with FK506. Cyclophosphamide was given at days −6, −5 and −2, and tacrolimus was used at 0.15 mg/kg/day for 28 days.

We unfortunately lost the first three animals due to non-immunologic causes at early time points (unknown-cause GI bleeding, catheter trouble and respiratory arrest during sedation). However, the rest of the baboons survived longer than 30 days with xenografts. After the initial three cases, the remaining baboons maintained stable clinical courses without any evidence of clinical xenograft rejection, which typically manifests by disseminated intravascular coagulation. One baboon was sacrificed on POD 38 due to a serious documented systemic infection from a central venous line.

Baboon thymocyte progenitors migrated into non-irradiated and irradiated porcine grafts: evidence for early thymopoiesis.

The xenogeneic grafts were biopsied for gross and histological analysis, and evaluation by flow cytometry. Porcine or baboon-specific antibodies were used for FACS analysis. Biopsies of non-irradiated thymic grafts one month following transplantation, and from the irradiated thymic grafts on PODs 15 and 21, demonstrated the presence of immature thymocytes which were CD44/CD25 double positive (DP), and CD44/CD4 low DP. These data suggested that progenitor thymocytes were capable of migrating and establishing residence in xenogeneic grafts following transplantation, providing evidence that these grafts were capable of early thymopoiesis across xenogeneic barriers. Neither baboon CD3-positive cells (graft infiltrating cells) nor pig cells (depleted by irradiation) were detected by FACS analysis of biopsy specimens taken up to one month following transplantation.

FACS analysis of a POD 49 graft biopsy of one baboon and a POD 113 biopsy of another demonstrated a small population of CD4/CD8 double-positive cells. However, the majority of mononuclear cells were either CD4 or CD8 single-positive cells. Persistence of CD4/CD8 double-positive cells suggested that thymopoiesis occurred in the graft. However, since the number of double-positive cells was much smaller than single-positive cells, some of the CD4 or CD8 single-positive cells could have been graft-infiltrating cells.

The presence of porcine thymic stromal cells in baboons receiving grafts.

Cytokeratin staining of grafts was performed to determine if thymic stroma cells persisted in the grafts following xenotransplantation. A large number of porcine thymic epithelial cells persisted in the non-irradiated grafts on POD 30 biopsies. Although cytokeratin-positive cells were observed in the previous study in the thymokidney, their numbers were much greater in the vascularized thymic lobe grafts. However, graft biopsies on POD 49 and POD 77 in two of these animals showed few cytokeratin positive cells, and demonstrated a focal

polynuclear cell infiltrate, suggestive of humoral rejection having occurred within the graft. Anti-Gal type 6 antibodies were also found in their serum.

Baboon recipients of non-irradiated porcine grafts demonstrated donor specific unresponsiveness *in vitro.*

In vitro responses to donor (porcine) and allogeneic (baboon) stimulators was measured by *in vitro* MLR analysis 8 days after stopping immunosuppression. Baboon recipients of non-irradiated grafts surviving longer than 3 months demonstrated donor-specific unresponsiveness on POD 36, while maintaining anti-allogeneic responses to third-party baboon stimulators. MLR analysis demonstrated that anti-pig responses were restored by POD 49 in one of these animals. However, the second animal, which maintained persistent low levels of α-Gal antibodies by extended GAS 914 administration, maintained donor-specific unresponsiveness until POD 77 and then restored anti-pig responses on POD 113. The return of responses to the donor pig stimulators correlated with histological evidence of rejection with returning Nab.

Porcine grafts are capable of reconstituting baboon peripheral T cells following transplantation.

Reconstitution of T cells peripherally following graft transplantation was also assessed by detection of $CD45RA^{high}$/CD4 positive cells by FACS analysis of baboon blood. Two recipients of non-irradiated grafts reconstituted T cells by evidence of an increase in $CD45RA^{high}$/CD4 positive cells starting on POD 35. One of these baboons demonstrated a cessation of increasing levels in this T cell population after POD 45, presumably due to rejection of the graft, suggesting that the reconstituted $CD45RA^{high}$/CD4 population originated from the porcine graft. In order to confirm that the CD45RA/CD4 population was composed of new thymic emigrants, control animals treated with the same immunosuppressive regimen, pre-operative conditioning, and a sham thymic lobe transplantation (without porcine thymic tissue transplantation in one animal and with thymocyte infusion in the other) were performed. Neither control demonstrated an increase in the $CD45RA^{high}$/CD4 double-positive population of T cells. In addition, a control animal did maintain an anti-pig response on POD 35.

These data indicate that only the transplantation of porcine thymic grafts was capable of reconstituting peripheral T cells and inducing donor specific T cell unresponsiveness across a large animal model of xenotransplantation.

Conclusion

We believe that the strategy of vascularized thymic lobe plus omental thymic tissue xenotransplants may provide a successful method for the induction of xenogeneic donor-specific tolerance with any co-transplanted organ in a large animal model. In addition, we believe that the technique of vascularized thymic lobe transplantation offers a promising

strategy to support long-term thymopoiesis across xenogeneic barriers. Unfortunately, the immunologic response generated by the circulating natural antibodies (α-Gal) remains a formidable obstacle in maintaining graft survival. Recent reports on the generation of α-1,3-galactosyltransferase knockout pigs could eventually allow for the successful induction of xenogeneic thymopoiesis and tolerance utilizing an α-1,3-Gal-deficient porcine grafts. The concomitant co-transplantation of an organ with the graft could possibly be a solution for the severe shortage of organs seen in clinical transplantation.

Dr. Peter Terness, Principal Investigator

University of Heidelberg, Heidelberg, Germany

Prevention of Allograft Rejection by Local Expression of the IDO Gene

Nature has developed a basic model of immunological tolerance against allogeneic fetal tissues during pregnancy. Several mechanisms have been suggested to mediate this tolerance. Recently, a novel way of tolerance induction has been identified based on expression of the immunosuppressive IDO gene (indoleamine 2,3-dioxygenase) by placental trophoblasts and macrophages. The precise mechanism of suppression has not been elucidated but it is known that IDO catabolizes tryptophan and that locally reduced tryptophan concentrations preclude T cell proliferation. We wanted to apply to an allogeneic organ transplant model what nature has developed for suppression of fetal rejection during pregnancy. In a first series of experiments we cloned the IDO gene into a replication-defective adeno-5 virus and expressed it in human dendritic cells (IDO-DCs). Transgenic DCs decreased the concentration of tryptophan, increased the concentration of kynurenine – the main tryptophan metabolite – and inhibited allogeneic T cell proliferation *in vitro.* Suppressed T cells could not be restimulated. These findings show that the IDO mechanism also works in humans and that it efficiently eliminates the allogeneic T cell response. It has been speculated that the T cell suppressive action of IDO is due to destruction of tryptophan – an essential amino acid required for cell proliferation. We verified this hypothesis by culturing T cells in medium without tryptophan. Although reduced, T cell proliferation was not completely abolished – a finding which could not explain the total inhibition induced by IDO-producing DCs. Therefore, we addressed the question whether tryptophan metabolites (kynurenine, 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid, quinolinic acid) are responsible for T cell suppression. Our findings showed that kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, but not other IDO-induced tryptophan metabolites, strongly suppress the T cell response at concentrations which can be expected at local sites of IDO production *in vivo.* Interestingly, the suppressive effects of active metabolites were additive. As shown by our studies, T cells, once stopped in their proliferation by the action of tryptophan metabolites, can not be restimulated. This effect may result either from T cell activity or cell death. In comprehensive FACS analyses we demonstrated that tryptophan metabolites induce T cell death and that this action preferentially affects activated T cells. Another finding was that the cytotoxic action of metabolites increase gradually with exposure time.

IDO production by DCs is a natural phenomenon induced by certain cytokines and other biomolecules. Therefore, our studies delineate a novel mechanism by which DCs suppress the immune response. This mechanism might have far-reaching consequences for immunoregulation and for the pathogenesis of certain diseases. As already mentioned, IDO is involved in suppression of fetal rejection and possibly in control of autoreactive T cells. According to our observations, this suppression is mediated by tryptophan metabolites, which result from increased IDO activity. Consequently, high levels of such metabolites in the fetal-maternal interface may constitute a "gateway to inferno" which sentences to death all T cells which pass through. Interestingly, our findings show that not only T cells but also B and NK cells are affected. In contrast, DCs – the cells that produce IDO – are resistant to tryptophan metabolite cytotoxicity.

If the immune system "uses" IDO-producing DCs for suppression of "unwanted" immune reactions, the same strategy could be attempted for induction of tolerance in a transplant setting. It has already been shown that donor DC progenitors, when injected into the prospective transplant recipient, prolong graft survival, conceivably by inactivating donor-reactive T cells. Our study points out a way for deliberately generating "suppressive" donor DCs that can be used for tolerance induction in transplant recipients. Conceivable alternatives would be the direct expression of the IDO gene in the graft or the use of tryptophan metabolites as immunosuppressive agents. The latter therapeutic strategy is particularly interesting in the light of our observation that tryptophan metabolites preferentially kill activated T cells, the cells that mediate graft rejection.

Publications

1. Terness P, Bauer T, Röse L, Dufter C, Watzlik A, Simon H, Opelz G. Inhibition of allogeneic T-cell proliferation by IDO-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* 2002;196:447.

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Studies of a Novel Dendritic Cell (DC) Population in Organ Allograft Survival

The aims of this study were to further characterize a recently identified dendritic cell (DC) subset in the mouse and to evaluate its role and therapeutic potential in the outcome of organ transplantation. Early *in vitro* work had suggested that this subset ("lymphoid-related" DC), distinguished from classic myeloid DC by surface expression of CD8α homodimer, could regulate peripheral T cell proliferative responses, but its influence on alloimmune reactivity *in vivo* had not been examined. $CD8\alpha^+$ DC were identified as rare, tissue-resident leukocytes in lymphoid (spleen) and non-lymphoid tissue (liver). Freshly isolated $CD8\alpha^+$ DC were phenotypically and functionally immature, but underwent maturation equivalent to that observed for $CD8\alpha^-$ DC following overnight culture in GM-CSF. These mature $CD8\alpha^+$ and $CD8\alpha^-$ DC were strong and efficient stimulators of naive allogeneic T cells[1]. Immunohistochemical and genomic DNA analysis revealed that, *in vivo,* highly purified allogeneic $CD8\alpha^+$ DC migrated from peripheral sites to T cell areas of secondary lymphoid tissue[1]. Compared with $CD8\alpha^-$ DC, these mature $CD8\alpha^+$ DC were equally efficient at allogeneic T cell priming *in vivo*. Analysis of signal cytokine production (interferon-γ [IFN-γ], IL-4 and IL-10) revealed potent stimulatory activity for T helper (h) 1 (mainly) and Th2 cells, and the induction of IFN-γ–secreting T cell clusters *in vivo*[2]. In addition to lymphoid and non-lymphoid tissues, circulating $CD8\alpha^+$ DC were also identified for the first time in both normal and hematopoietic growth factor (flt3 ligand)-treated mouse blood[3].

Our studies have shown that mobilization of $CD8\alpha^+$ and $CD8\alpha^-$ DC in donor hematopoietic (spleen) cell infusions can be used to induce skin graft tolerance across full MHC barriers, accompanied by specific deletion of donor-reactive T cells[4]. Tolerance is associated with blood macrochimerism and confirmed by second set skin grafting, 100 days after the first graft.

Comparative studies on $CD8\alpha^+$ and $CD8\alpha^-$ DC have provided further insights into their immunobiology and their influence on organ allograft survival. Thus, in contrast to spleen $CD8\alpha^+$ and $CD8\alpha^-$ DC, which show strong differences in their respective Th1 cell-driving cytokine (IL-12 p70)-producing capacities, $CD8\alpha^+$ and $CD8\alpha^-$ DC isolated from a non-lymphoid organ (the liver) produce IL-12 p70 efficiently in amounts comparable to splenic

CD8α$^+$ DC[5]. These observations are consistent with the equivalent ability of both liver mature CD8α$^+$ and CD8α$^-$ DC to induce Th1-type cytokines in naive allogeneic T cells[2]. The data also indicate that low IL-12 p70-producing capacity is not an intrinsic property of the murine CD8α$^-$ DC subset and supports the view that the site of DC development and maturation plays a dominant role in defining DC function.

An important additional aim of this study was the direct assessment of the influence of CD8α$^+$ DC on organ allograft survival. This was tested in the fully MHC-mismatched B10 (H2b) to C3H (H2k) mouse strain combination. Whereas immature donor CD8α$^+$ and CD8α$^-$ DC infused systemically 1 week before vascularized organ (heart) transplant significantly prolonged graft survival in non-immunosuppressed recipients, only mature CD8α$^+$ donor DC retained this therapeutic effect[6]. Marked prolongation of graft survival was associated with specific impairment of *ex vivo* anti-donor T cell responses. Analyses of possible underlying mechanisms indicated that neither immune deviation nor induction of regulatory cells was a significant contributing factor. These findings demonstrate the innate capacity of CD8α$^+$ DC to regulate alloimmune reactivity and transplant survival, independent of their maturational status. Mobilization of such a DC subset, with ability to modulate anti-donor immunity may have significant implications for the therapy of allograft rejection.

Publications

1. O'Connell PJ, Morelli AE, Logar AJ, Thomson AW. Phenotypic and functional characterization of mouse hepatic CD8α$^+$ lymphoid-related dendritic cells. *J Immunol* 2000;165:795-803.
2. Morelli AE, O'Connell PJ, Khanna A, Logar AJ, Lu L, Thomson AW. Preferential induction of Th1 responses by functionally mature hepatic (CD8α$^-$ and CD8α$^+$) dendritic cells: Association with conversion from liver transplant tolerance to acute rejection. *Transplantation* 2000;69:2647-2657.
3. Donnenberg VS, O'Connell PJ, Logar AJ, Zeevi A, Thomson AW, Donnenberg AD. Rare event analysis of circulating human dendritic cell subsets and their presumptive mouse counterparts. *Transplantation* 2001;72:1946-1951.
4. Eto M, Hackstein H, Kaneko K, Nomoto K and Thomson AW. Promotion of skin graft tolerance across MHC barriers by mobilization of dendritic cells in donor hemopoietic cell infusions. *J Immunol* 2002;169:2390-2396.
5. O'Connell PJ, Son Y-I, Giermasz A, Logar AJ, Thomson AW, Kalinski P. Type-1 polarized nature of mouse liver CD8α$^-$ and CD8α$^-$ dendritic cells: tissue-dependent differences offset CD8α-related DC heterogeneity. Submitted for publication.
6. O'Connell PJ, Li W, Wang Z, Specht SM, Logar AJ, Thomson AW. Immature and mature CD8α$^+$ dendritic cells prolong the survival of vascularized cardiac allografts. *J Immunol* 2002;168:143-154.

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Gene Expression of Tolerance-Mediating Allospecific Cells

Introduction

Recently, we showed that the non-depleting anti-rat CD4 mAb, RIB5/2, is a powerful inductor of tolerance to allografts. The tolerance is adoptively transferable by spleen or graft-infiltrating cells in an infectious manner[1,2]. In order to characterise these cells in more detail, we performed differential display RT-PCR and suppression subtractive hybridisation with graft-infiltrating cells from anti-CD4 and control mAb treated rat allograft recipients. We defined several gene fragments that were specifically up- or down-regulated in graft-infiltrating cells from anti-CD4 mAb-treated rats. The full-length cDNA of one of these fragments showed strong homology to the anti-apoptotic gene Bag-1, which was first identified in the mouse. *In vitro* allo-stimulation in the presence of anti-CD4 mAb strongly up-regulates Bag-1 expression in responder T cells. The associated relative resistance to antigen-induced apoptosis may explain the long-lasting survival of regulatory T cells *in vivo* despite the persistence of alloantigen.

The ROTRF-funded project focused on the following topics

i) **Bag-1:** In order to prove the hypothesis that Bag-1 is essential for stable tolerance, we wanted to study whether Bag-1 k.o. mice (in development) are able to develop tolerance-mediating T cells. Additionally, we wanted to investigate whether *ex vivo* down-regulation of Bag-1 by antisense technology in T cells derived from anti-CD4 mAb-treated rats abolishes their tolerance-mediating properties.

ii) **New genes:** We further characterised other gene fragments specifically up- or down-regulated in our system. Therefore, we concentrated our work on expression kinetics studies of all isolated fragments in various transplantation tolerance models using real-time PCR in order to identify reliable tolerance-associated markers for post-transplant monitoring. Furthermore, we started to characterise some isolated gene fragments in more detail.

Results

Further investigation of the importance of anti-apoptotic proteins (e.g. Bag-1) for the generation of long-living regulatory T cells and the maintenance of allograft tolerance.
Within the project we could already demonstrate that inhibition of Bag-1 expression *in vitro*

leads to increased sensitivity against spontaneous and activation-induced apoptosis of the cells derived from anti-CD4 mAb-treated allogeneic mixed lymphocyte reactions[1-4]. Further investigations showed that anti-CD4 mAb-treated allo-reactive T cells are able to inhibit the proliferation of naive responder T cells in an allo-specific manner. Interestingly, inhibition of Bag-1 protein expression in anti-CD4 mAb-treated allo-reactive T cells diminished their ability to regulate the proliferation naive responder T cells (Fig. 1, manuscript in preparation). Currently we are trying to inhibit Bag-1 expression in tolerance-mediating T cells from anti-CD4 mAb-treated kidney and heart recipients before adoptive transfer. That may show whether an enhanced Bag-1 expression is needed for the induction and maintenance of transplantation tolerance.

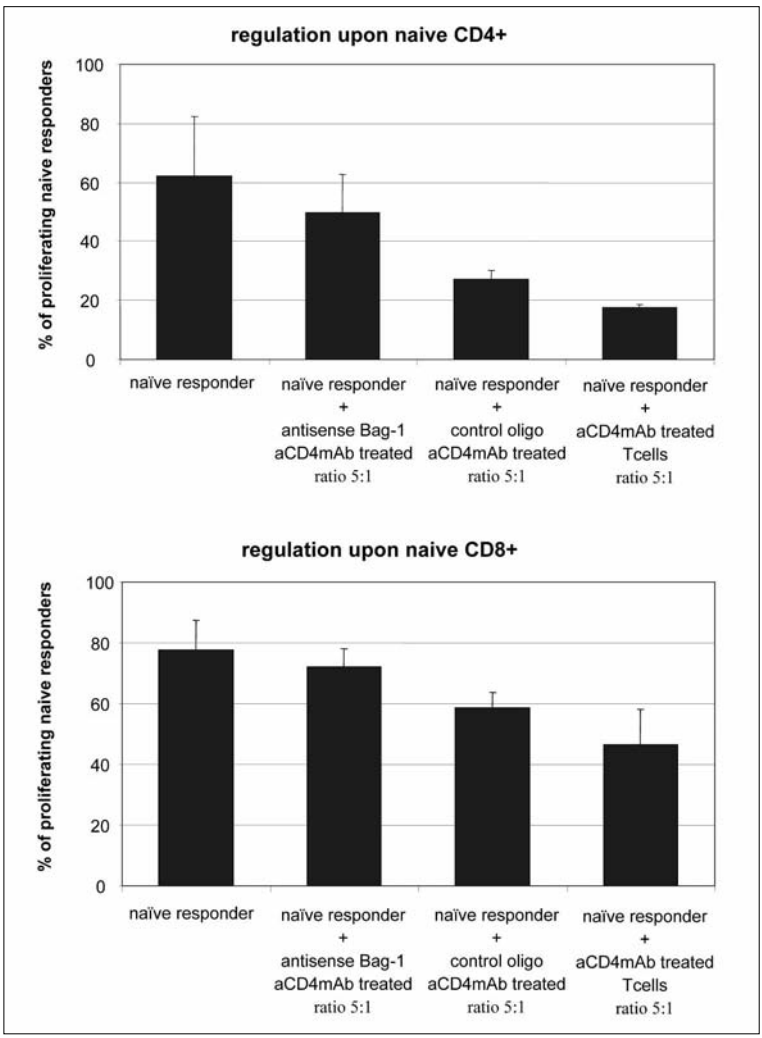


Figure 1.
Influence of Bag-1 protein expression in anti-CD4 allo-reactive T cells on their ability to inhibit the proliferation of naive responder T cells.

We demonstrated that enhanced protection against apoptosis in anti-CD4 mAb-treated T cells is not due to differences of Bcl-xl and Bax expression between anti-CD4 mAb-treated and non-treated allogeneic cultures or diminished FasL expression after restimulation of responder T cells[1-4]. Currently, we are analysing the expression of different anti- and pro-apoptotic proteins *in vivo* after transplantation (see also III below).

We isolated and sequenced the genomic clone of the mouse Bag-1 gene. The complete genomic clone consists of 9708 bp and contains 7 exons (Fig 2). We have chosen exon 2 as a target to generate a T cell-specific inducible k.o. mouse. Currently we are establishing a transplant tolerance model using C57/BL6 mice as recipients. This will enable us to investigate our hypothesis that Bag-1 expression is essential for stable tolerance.

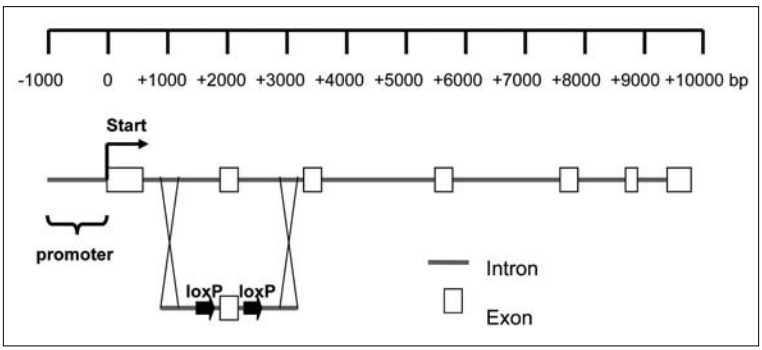


Figure 2.
Bag-1 mouse genomic sequence/Generation of T cell specific k.o. mice.

I. Characterisation of other genes differentially expressed in the renal transplantation model

We had chosen 3 gene fragments termed ACD4-2, ACD4-3 and ALLO-5 to further characterise in regard to their expression and function during T cell activation. ACD4-2 and ACD4-3 were originally isolated from GIC of anti-CD4 antibody-treated recipients, whereas ALLO-5 was preferentially expressed in GIC of control antibody-treated recipients. ACD4-2 is identical to rat phosphatidylinositol transfer protein (PITP) and ACD4-3 and ALLO-5 have no homology to any known gene.

We generated a primer/probe panel for use in real-time RT-PCR for all 3 gene fragments. The panels were used to analyse the expression of ACD4-2, ACD4-3 and ALLO-5 during an allogeneic mixed lymphocyte reaction (DA to Lewis) comparing anti-CD4 mAb-treated and non-treated cultures. We could not detect any up- or downregulation of ACD4-2 or ACD4-3 mRNA expression during allospecific T cell activation. Furthermore, anti-CD4 antibody treatment of allogeneic cultures did not affect the expression of ACD4-2 and ACD-3. We also determined their expression at different time points after transplantation in several transplantation models (see also III). Again, there was no consistent up- or down-regulation of their expression in the grafts of tolerance-developing recipients (Fig. 4). Thus, it is unlikely that ACD4-2 or ACD4-3 play an important role during T cell activation or inhibition of T cell

activation. However, ALLO-5 mRNA expression during allogeneic MLR increases shortly after the onset of culture (Fig. 3). This up-regulation of ALLO-5 transcription could be prevented by anti-CD4 mAb treatment. We also investigated ALLO-5 mRNA expression at different time points in several transplantation models (Fig. 5). In all analysed models ALLO-5 expression was increased during rejection (10–50 fold), whereas the upregulation in tolerance-developing recipients (anti-CD4 antibody-treated kidney recipients or spontaneous acceptance of liver grafts) was only minor and transient.

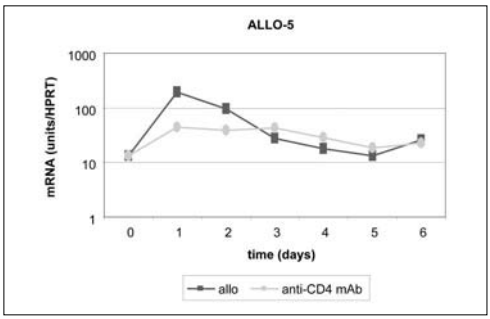
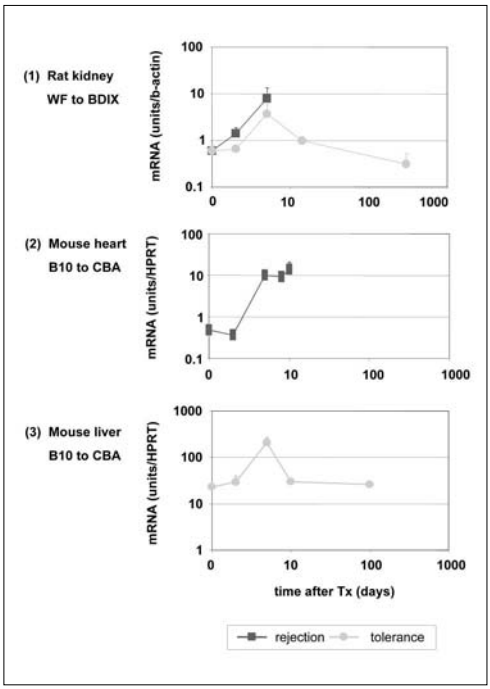


Figure 3.
ALLO-5 mRNA expression during an allogeneic MLR. Shown are the data of one representative of three experiments.



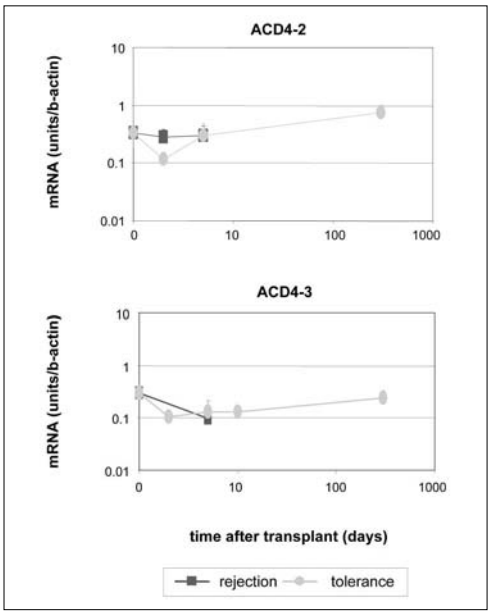


Figure 4.
Expression of ACD4-2 and ACD4-3 in rat kidney grafts (WF to BDIX). Data are shown as mean of expression (n=3).

Figure 5.
Expression of ALLO-5 in different transplant models. Data are shown as mean of expression (n=3).

II. Expression studies in other transplantation models

So far we recruited samples from four transplantation tolerance models which we used to analyse the expression kinetics of all isolated cDNA fragments.

A. In the first model, we used the non-depleting anti-CD4 mAb RIB5/2 (10×20 mg/kg body weight) to induce donor-specific tolerance against renal allografts in two strain combinations: Wistar Furth to BDIX and DA to Lewis[5,6]. The expression of the isolated cDNA fragments in anti-CD4 mAb-treated recipients was analysed at day 2, 5, 10, 14, 25 and >100 and compared with the expression at day 2 and 5 in grafts from control mAb-treated control animals.

B. The "adoptive tolerance" kidney transplant model was established by Tullius et al. A Fisher allograft is transplanted into Lew recipients leaving one of its own kidneys inside. The allograft then undergoes chronic rejection. Four weeks later both kidneys are removed and a second Fisher allograft is transplanted. This second renal allograft is permanently accepted without any treatment. Samples from both the first allograft (as control) and the second allograft 2, 4, 8, 12 and 16 weeks after transplantation will be analysed for expression of the isolated cDNA fragments.

We also recruited samples from two mouse tolerance models, which were used to compare the expression kinetics of the isolated cDNA fragments after transplantation between different donor grafts and in different species (rat and mouse).

C. Donor alloantigen is administered in combination with anti-CD4 mAb to CBA mice before transplantation of a B10 cardiac allograft. This leads to the induction of specific unresponsiveness to donor alloantigens at the time of transplantation[7,8]. The expression pattern of the isolated gene fragments was analysed at days 0, 1, 2, 5, 7 and 8 for the non-treated control mice and at days 0, 1, 2, 5, 7, 8, 10, 14, 25, 40 and 100 for the treated mice.

D. B10 liver grafts are spontaneously accepted when transplanted into CBA recipients. To analyse the expression pattern of all isolated cDNA fragments after liver transplantation, grafts were harvested at days 0, 1, 5, 10 and 100. We generated primer panels for all isolated cDNA fragments, which were used to quantify their expression using the Sybrgreen application of the TaqMan Cycler.

So far we have analysed the time-dependent expression pattern of all isolated fragments in 3 of the 4 mentioned transplant models [A: rat kidney, Wistar Furth to BDIX; C: mouse heart, B10 to CBA; D: mouse liver, B10 to CBA]. The results for some cDNA fragments are attached to this report (Fig. 5, 6). As mentioned earlier, analysis of ACD4-2 expression in kidney grafts did not reveal any differences between anti-CD4 mAb-treated recipients and control mAb-treated recipients. Similar results were obtained for ACD4-3. As mRNA expression of both cDNA fragments was not regulated in kidney grafts, we did not determine their expression in the other transplant models. In contrast, ALLO-5 mRNA expression became strongly up-regulated in rejecting recipients shortly after transplantation in all analysed transplant models (Fig. 6). We also detected a small increase in ALLO-5 expression in grafts of tolerance-developing recipients, but levels of ALLO-5 expression

decreased again at later time points. This makes ALLO-5 a good candidate for a post-transplant monitoring in order to predict rejection. In Fig. 6, the expression patterns of other isolated cDNA fragments T4 (kallikrein), T5 (adenylate kinase 4), T8 (unknown) and T10 (unknown) are shown. Their expression is highly up-regulated in long-term-surviving kidney grafts. When we analysed their expression after mouse heart transplantation, only T8 expression was regulated and showed again a strong association with permanent acceptance. Thus, the expression of T8 within the graft could be used to determine whether a tolerance induction protocol has been successful (manuscript in preparation).

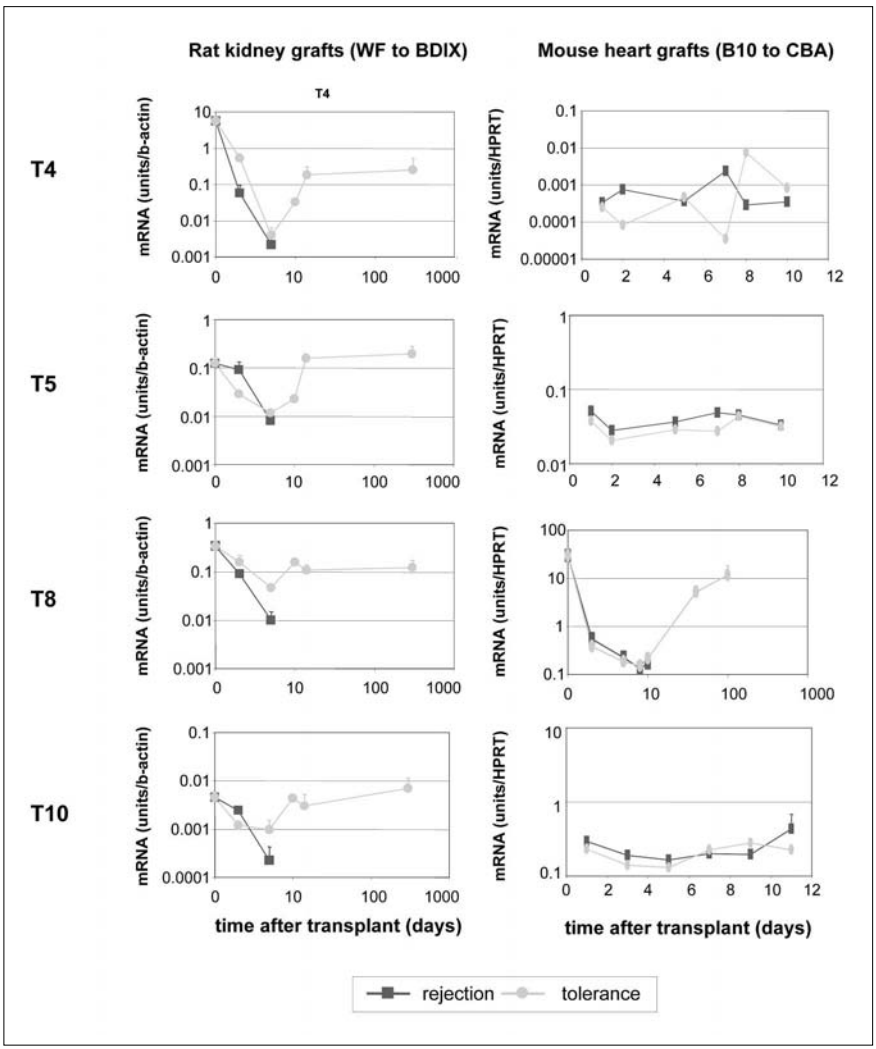


Figure 6. *Expression of T4, T5, T8 and T10 in different transplant models. Data are shown as mean of expression (n=3).*

Concluding Remarks

Within this project, we could demonstrate that the up-regulation of the anti-apoptotic protein Bag-1 is essential for the survival of anti-CD4 mAb-treated allo-reactive T cells. Furthermore, Bag-1 is also important for their regulatory capacity towards naive responder T cells *in vitro*. Whether it is also essential for the induction and maintenance of unresponsiveness *in vivo* after transplantation remains to be investigated. We could also show that up-regulation of Bag-1 expression in anti-CD4 mAb-treated allo-reactive T cells was specific for Bag-1 and has not been observed for any other anti-apoptotic protein.

Further expression studies of other differentially expressed cDNA fragments in different transplant models led to the identification of putative diagnostic markers for a post-transplant monitoring. Their expression could be used to predict rejection episodes and to evaluate the success of tolerance induction.

References and Publications

1. Sawitzki B, Lehmann M, Ritter T, Graser E, Kupiec-Weglinski J, Volk HD. Regulatory Tolerance-Mediating T cells in Transplantation Tolerance. *Transplant Proc* 2001;33:2092-93.
2. Sawitzki B, Lehmann M, Vogt K, Risch K, Brock J, Kupiec-Weglinski J, Volk HD. Bag-1 up-regulation in anti-CD4 mAb-treated allo-activated T cells confers resistance to apoptosis. *Eur J Immunol* 2002;32:800-809.
3. Sawitzki B, Lehmann M, Vogt K, Seifert M, Risch K, Brock J, Kupiec-Weglinski J, Volk HD. Bag-1 up-regulation in anti-CD4 mAb-treated allo-activated T cells confers resistance to activation-induced cell death (AICD). *Transplant Immunology* 2002;9:89-91.
4. Sawitzki B, Amersi F, Ritter T, Fisser M, Shen X, Ke B, Busuttil R, Volk HD, Kupiec-Weglinski JW. Upregulation of Bag-1 by *ex vivo* gene transfer protects rat livers from ischemia/reperfusion injury. *Hum Gene Ther* 2002;13:1495-1504.
5. Lehmann M, Kuttler B, Siegling A, Gordella A, Riedel H, Lacha J, Hahn HJ, Brock J, H.-D. Volk. Characterization of the anti-CD4-induced permanent acceptance of rat renal allografts. *Transplant Proc* 1994;26:3155.
6. Lehmann M, Graser E, Risch K, Hancock WW, Muller A, Kuttler B, Hahn HJ, Kupiec-Weglinski JW, Brock J and Volk HD. Anti-CD4 monoclonal antibody-induced allograft tolerance in rats despite persistence of donor-reactive T cells. *Transplantation* 1997;64:1181-1187.
7. Niimi M, Witzke O, Bushell A, Hara M, Morris PJ, Wood KJ. Nondepleting anti-CD4 monoclonal antibody enhances the ability of oral alloantigen delivery to induce indefinite survival of cardiac allografts: oral tolerance to alloantigen. *Transplantation* 2000;70:1524-1528.
8. 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;168:1080-1086.

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Use of Proteosome Inhibitors to Control Rejection and to Induce Long-Term Tolerance of Organ Grafts

Background

The proteasome, a large protease complex in cells, is the major machinery for protein degradation. It was previously considered a humble garbage collector, performing housekeeping duties to remove misfolded or spent proteins. Until recently, the interests of immunologists in proteasomes were focused largely on its role in antigen processing. Its real importance in cell biology and immunology has only been revealed recently due to the availability of relatively specific inhibitors. It has now become increasingly clear that many aspects of immune responses depend highly on proper proteasome activity. Consequently, proteasome inhibitors should be effective in treating immunological disorders including allograft rejection.

Results

With the financial support from the ROTRF grant, we have explored the mechanism of action of proteasomes in regulating immune responses, and possible application of proteasome inhibitors as immunosuppressants in organ transplantation. We have reported that proteasome inhibitors can suppress T cell aggregation during mitogen activation, and such suppression is, at least partly, due to inhibited expression of adhesion molecules on T cells[1]. Since T cells need to interact with antigen-presenting cells and other T cells to be fully activated in an immune response, the inhibited lymphocyte aggregation is likely one of the mechanisms for the observed immunosuppressive effect of proteasome inhibitors. We have also documented that proteasome inhibition leads to apoptosis of activated and cycling lymphocytes[2]. Mechanistically, such apoptosis is caused, in part, by reduced degradation of a pro-apoptotic Bcl-2 family member Bik. Proteasome inhibition also results in compromised electron transport in mitochondria, and leakage of cytochrome c from mitochondria into cytoplasm. The leaked cytochrome c, in turn, participates in activation of the apoptosis pathway. We have demonstrated that a proteasome inhibitor, dipeptide boronic acid (DPBA), effectively prevents heart allograft rejection in a mouse model[3]. With 16-day i.p. administration of DPBA (1.0 mg/kg/day for 4 days, followed by 0.5 mg/kg/day for 12 days), the survival of heart allografts can reach 36.2 + 10.4 days (mean + SD), compared with 7.3 days in the control group. DPBA can curb ongoing graft rejection: delayed administration of

DPBA for mere 4 days starting from day 3 post operation can prolong the graft survival to 19.8 + 7.8 days. DPBA-induced apoptosis of activated alloantigen-specific T cells might contribute to such effectiveness. It is conceivable that administration of proteasome inhibitors once rejection is ongoing can result in clonal reduction of antigen-specific T cells in the host. Indeed, we have proved *in vitro* that this is the case, using L^d-specific transgenic T cells as markers. Currently, we are verifying whether such clonal deduction can also be achieved *in vivo.* At the effective dosages of DPBA in transplantation, there are no obvious acute side effects in mice, according to serum biochemistry. However, we could not find an effective and safe dose window in a rat renal allograft model. It suggests that different species have different sensitivity to proteasome inhibition; the rat is not a suitable model to study proteasome inhibitors as immunosuppressants for transplantation; mice, on the other hand, seem more closely related to humans in terms of tolerance to proteasome inhibition, according to our data and according to information available from DPBA clinical trials. The mouse is therefore the model of choice to test additional novel proteasome inhibitors that are under further development by our group, which are based on competitive inhibition of the five protease activities of the proteasome.

Significance

Proteasome inhibition represents a new frontier in immunosuppressant development[4]. Concerning the side effects of proteasome inhibitors, phase II and III clinical trials of the inhibitors in treating various tumors have shown that the side effects of the inhibitor are tolerable. Since proteasome inhibition is a totally different mechanism from that employed by all the current front line immunosuppressants, drug combination including proteasome inhibitors will probably be beneficial in organ transplantation. The ability of proteasome inhibitors to control ongoing rejection and to reduce alloantigen-specific T cell clones are added advantages for using such inhibitors in controlling acute or chronic graft rejection.

Additional results

With the support from the ROTRF grant, we have investigated other possible ways to modulate allograft rejection in organ transplantation. DcR3/TR6 is a secreted protein belonging to the TNF receptor family. We have discovered that it can inhibit cytotoxic T cell activity *in vitro,* and repress lymphokine production *in vivo* by alloantigen-stimulated T cells. Administration of TR6 *in vivo* can significantly prolong heart allograft survival in our mouse model[5]. Mechanistically, we have shown that a TNF member, LIGHT, and a TNF receptor family member, HVEM/TR2, can bi-directionally costimulate T cells, and soluble TR6 blocks such costimulation[6,7]. These data suggest that TR6 has potential applications in combating both acute and chronic graft rejection. We have also discovered that a receptor tyrosine kinase, EphB6, mediates costimulation in T cells[8,9]. The consequence of blocking such costimulation in organ transplantation is currently under investigation.

Publications

1. Kanaan N, Luo H, Wu J. Proteasome activity is required in T lymphocyte aggregation after mitogen activation. *J Cell Biochem* 2001;81:347-356.
2. Marshansky V, Wang X, Bertrand R, Luo H, Duguid W, Chinnadurai G, Kanaan K, Vu MD, Wu J. Proteasome modulates balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J Immunol* 2001;166:3130-3142.
3. Luo H, Wu Y, Qi S, Wu L, Wan X, Chen H, Wu J. A proteasome inhibitor effectively prevents mouse heart allograft rejection. *Transplantation* 2001;72:196-202.
4. Wu J. On the Role of Proteasomes in Immune Responses and Proteasome Inhibition as a Novel Frontier in the Development of Immunosuppressants. *Am J Transplant* 2002;2:904-912.
5. Zhang J, Salcedo T, Wan X, Ullrich S, Hu B, Gregorio T, Feng P, Qi S, Chen H, Cho Y, Li Y, Moore P, Wu J. Modulation of T Cell Responses to alloantigens by DcR3/TR6. *J Clin Invest* 2001;107:1459-1468.
6. Shi Q, Luo H, Wan X, Salcedo TW, Zhang J, Wu J. Mouse T cells receive costimulatory signals from LIGHT, a TNF family member. *Blood* 2001;100:3279-3286.
7. Wan X, Zhang J, Luo H, Shi G, Kapnik E, Kanakaraj P, Kin S, Wu J. A TNF family member reversely transduces costimulatory signals into human T cells. *J Immunol* 2002;169:12.
8. Luo L, Wan, Wu Y, Wu J. Crosslinking of EphB6 resulting in signal transduction and apoptosis in Jurkat cells. *J Immunol* 2001;167:1362-1370.
9. Luo H, Yu G, Wu Y, Wu J. EphB6 cross-linking results in co-stimulation of human T cells. *J Clin Invest* 2002;110:1141-1150.

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Induction of Transplant Tolerance by an Antibody against CD45RB

Introduction

We previously demonstrated that antibody targeting of a cell surface molecule, CD45RB, can predictably induce tolerance in animal transplant models, and we believe that this approach will have an important role in clinical transplantation. Therefore, the purpose of this project is to characterize the mechanisms of tolerance induced by CD45RB therapy in order to allow its rational use in clinical transplantation. We have made the following progress.

1. Interaction of anti-CD45RB mAb with cyclosporine (CsA) and rapamycin (Rap)

New immunosuppressive agents are usually assessed in combination with traditional agents during early phase clinical trials. Therefore, it is of great importance to determine whether established immunosuppressive agents can augment or antagonize the effect of anti-CD45RB mAb in pre-clinical studies. The following data demonstrate the importance of pre-clinical evaluation of drug combinations.

Table 1. *Murine cardiac allograft survival using adjunctive immunosuppressive therapy*

Group	Treatment	Dose	Survival (days)	Median survival ±SE (days)
1.	Control	none	8 (3), 9 (6), 11 (3), 14	9.6 +/– 0.4
2.	Anti-CD45RB mAb	3 mg/kg iv days –1 to 7	34, 42, 43, 57, 63, 113, 120, 124	74.5 +/– 13.4
3.	Anti-CD45RB mAb + Cyclosporine	3 mg/kg iv days –1 to 7 5 mg/kg iv days 0 to 6	19, 20, 28, 29 (2)	25.0 +/– 2.3*
4.	Anti-CD45RB mAb + Rapamycin	3 mg/kg iv days –1 to 7 4 mg/kg iv days 0 to 13	>100 (8)	>100**

* $p < 0.05$ groups 3 vs 2 ** $p < 0.05$ groups 4 vs 3

These data indicate that CsA inhibits tolerance induced by anti-CD45RB mAb, while rapamycin has a synergistic effect with anti-CD45RB mAb. The precise mechanism of this phenomenon is currently being investigated.

2. Mechanisms of tolerance induction by anti-CD45RB mAb

In the past, it was hypothesized that anti-CD45RB mAb inhibits major histocompatibility complex (MHC)-T cell receptor (TcR) interactions through steric hindrance, since CD45RB had been shown to be physically cross-linked to the TcR. However, our recent work has shown that direct MHC class II–TcR interactions are critical for tolerance using anti-CD45RB mAb therapy. Treatment of recipients of renal allografts from MHC class II knock-out mice with anti-CD45RB mAb leads to long-term engraftment but is associated with chronic allograft nephropathy in all mice, whereas long-term recipient wild-type control kidneys remain free from injury at 100 days. Furthermore, splenocytes from long-term recipients with renal allografts from MHC class II knock-out mice given anti-CD45RB mAb are unable to transfer tolerance, in contrast to wild-type controls, demonstrating that direct engagement with donor MHC class II is required for tolerance induction.

A likely potential mechanism involves T cell subset shifts. T cell subsets with differing CD45RB isoform expression have significantly different functional capacities. T cells that heavily express CD45RB are considered $CD45RB^{hi}$ (bright), and T cells with lower CD45RB cell surface expression are considered $CD45RB^{lo}$ (dim). The $CD45RB^{hi}$ T cells produce greater amounts of interleukin-2 (IL-2) and interferon gamma (IFN-γ), while the $CD45RB^{lo}$ cells preferentially make IL-4. Rothstein showed that anti-CD45RB mAb therapy dramatically reduces CD45RB expression in graft-infiltrating cells and a $CD45RB^{hi} \rightarrow CD45RB^{lo}$ isoform shift occurred. Taken together, they hypothesized that anti-CD45RB mAb therapy induces an isoform shift from $CD45RB^{hi}$ to $CD45RB^{lo}$ cells, and that the presence of the 'regulatory' $CD45RB^{lo}$ graft-infiltrating cells may be associated with the $Th_1 \rightarrow Th_2$ subset shift. We have recently found that the CD45RB isoform shift also occurred in renal allografts from anti-CD45RB mAb-treated tolerant animals.

Is there a role for apoptosis in CD45RB-induced tolerance? The most stringent model of tolerance is believed to be derived via deletion or apoptosis of allo-reactive T cells. Although a connection between the anti-CD45RB mAb-derived tolerance and apoptosis had never been exhaustively studied, there is evidence to suggest that apoptosis is associated with CD45RB mAb therapy. Klaus et al. showed that isoform non-specific CD45 ligation is able to induce programmed cell death in T and B cells. We have shown that anti-CD45RB mAb markedly reduces leukocyte peripheral blood counts *in vivo*. However, by day 14, the peripheral blood leukocyte counts return to normal levels, despite the absence of graft rejection at this time point. Hence, the immunoregulatory property of anti-CD45RB mAb does not appear to be related to overwhelming depletion of T cells, but may be related to depletion of a specific T cell subset, with subsequent reconstitution of a different, tolerogenic subset. Using annexin V staining, we have shown that anti-CD45RB mAb increases both $CD4^+$ and $CD8^+$ T cell apoptosis. T cell death also occurred in association with a reduction in mitochondrial transmembrane potential, but without evidence of nucluear

condensation and cell shrinkage. Additionally, the CD45RB^{hi} cells were found to be more profoundly affected by anti-CD45RB mAb than the CD45RB^{lo} cells, and as a result, there was a shift in the total CD45RB^{lo}:CD45RB^{hi} cell ratio. While it is possible that this selective deletional mechanism contributes to the effector CD45RB^{hi} → regulatory CD45RB^{lo} subset shift and thus tolerance, the data from our experiments using MHC null donor kidneys strongly suggests that apoptosis is not the sole element responsible for anti-CD45RB mAb-mediated tolerance. However, in the presence of antigen-specific activation of T cells, the preferential deletion of CD45RB^{hi} cells might facilitate tolerance induction by a relative overexpression of CD45RB^{lo} regulatory cells, which are antigen specific, and have the capacity to inhibit T cell-mediated rejection. This is currently being studied in *in vivo* models.

The generation of regulatory or suppressor T cells has been implicated as a mechanism for tolerance. However, the surface phenotype of these regulatory cells appears to differ according to the method of tolerance induction. We transplanted C57BL/6 renal allografts into BALB/c mice that were treated with a short course of anti-CD45RB mAb. Splenocytes were harvested from animals surviving >100 days, and either bulk splenocytes, CD4$^+$ cells, CD8$^+$ cells or B cells were adoptively transferred to naive BALB/c animals. Indefinite graft survival was achieved in each of 3 animals (all >60 days) transfused with purified CD4$^+$ cells, and each of 5 animals (all >60 days) transfused with splenocytes. Adoptive transfer of CD8$^+$ cells led to prolonged survival in only 1 of 3 animals (56 days), while transfer of B cells was unable to prolong graft survival compared with untreated animals. These data suggest that anti-CD45RB mAb therapy generates immunoregulatory CD4$^+$ T cells that have the ability to tolerize naive T cells. Whether the primary regulatory CD4$^+$ T cell has a CD45RB^{lo} phenotype is unknown. We have recently demonstrated that adoptive transfer of CD45RB^{lo} cells from tolerant CD45RB mAb-treated animals is able to induce tolerance.

3. Synergistic effects of anti-CD45RB mAb and LF 15-0195

We have recently demonstrated that anti-CD45RB mAb and LF 15-0195, a new analog of deoxyspergualin (DSG) have synergistic effects in induction of tolerance in a mouse cardiac transplant model. Table 2 indicates that neither low-dose LF therapy (2 mg/kg for 1 wk) nor anti-CD45RB mAb (3 mg/kg for 1 wk) was able to induce tolerance in a murine heart transplant model. However, the combination of low-dose anti-CD45RB mAb and LF induced indefinite survival of cardiac allografts (Fig. 1). Furthermore, long-term survivors permanently accepted donor-specific skin grafts, while rejecting third party skin, indicating the establishment of donor-specific tolerance (Table 2).

Table 2. *Synergistic effects of anti-CD45RB mAb and LF in C57B6 BALB/c heart transplant model*

Groups	Survival (days)	Mean survival (days)
mAb 3 mg/kg D0-7	18, 25, 26, 32, 34, 53	34 +/– 5
LF 2 mg/kg D0-7	12, 13, 14, 15, 16	14 +/– 1
mAb 3 mg/kg + LF 2 mg/kg	>100 (8)	>100

* Skin graft survival: C3 H: 11 C57/BL6: >100 days

We have further demonstrated that the tolerance is induced by generation of regulatory T cells by anti-CD45RB mAb, generation of a tolerogenic subset of dendritic cells (DC) by LF, and interaction of T regulatory and tolerogenic DC. We are currently testing this hypothesis in a non-human primate model.

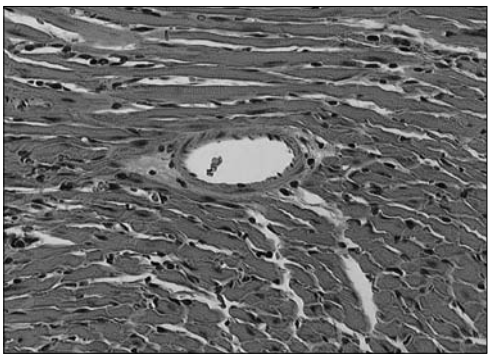

Figure 1. *C57/BL6 allograft treated with LF + mAb on day 100, showing a normal histology.*

Publications

1. Luke P, O'Brien C, Jevnikar A, Zhong R. Anti-CD45 monoclonal antibody-mediated transplantation tolerance. *Curr Mol Med* 2001;1:521-53.
2. O'Brien C, Luke P, Jiang J, Garcia B, Zhong R, Jevnikar A. Donor MHC class II required for tolerance induction by CD45RB mAb in mouse renal allografts. *Am J Transplant* 2001;1 (Sup 1):224.
3. Min W, Zhou D, Visser L, Lian D, Kanai N, Huang X, Garcia B, Zhong R. Synergistic effect of anti-CD45RB AND LF 15-0195 on tolerance is due to interaction of regulatory T cells and tolerogenic dendritic cells. *Am J Transplant* 2002;1 (Sup 3):389.
4. Luke P, O'Brien C, Zhong R, Jevnikar A. CD45RB monoclonal antibody induces $CD45RB^{hi}$ to $CD45RB^{lo}$ T cell subset shift through cell death. *Am J Transplant* 2002;2 (Sup 3):441.
5. Luke P, O'Brien C, Zhong R, Jevnikar A. CD45RB monoclonal antibody mediated cell death: implications to alteration in $CD45RB^{hi}$ cell:$CD45RB^{lo}$ T cell subset, manuscript submitted.
6. Min W, Zhou D, Xia X, Yang J, Zhang X, Strejan G, Ichim T, Hill J, Huang X, Garcia B, White D, Dutartre P, Jevnikar A, Zhong R. Inhibitory feedback loop between tolerogenic DC and T regulatory cells in transplant tolerance, *in press*.

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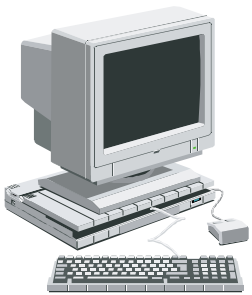

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