13th Annual
Great Lakes Transplant Immunology Forum

Wednesday & Thursday, October 10–11, 2012

UNIVERSITY OF WISCONSIN • UNION SOUTH
1308 WEST DAYTON STREET • MADISON, WISCONSIN

University of Wisconsin
Department of Surgery
The regional transplantation immunology meeting known as the Great Lakes Transplantation Immunology Forum (GLTIF) was inaugurated in Ann Arbor, Michigan in 2000. Subsequent annual meetings were held in Columbus, Cleveland, Chicago, Indianapolis, Atlanta, and Pittsburgh. The meeting has evolved as a forum for principal investigators, but also promising young investigators, actively engaged in research to present and openly discuss their work, to promote awareness of transplantation immunology research in the region, and to encourage collaborative links.

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2001 - Ohio State University
2002 - Cleveland Clinic Foundation
2003 - University of Chicago
2004 - Indiana University
2005 - University of Pittsburgh
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2009 - Cleveland Clinic
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XIII Great Lakes Transplant Immunology Forum

Welcome From Dixon Kaufman
Chair of the Division of Transplantation and
Director of the Transplant Service Line
University of Wisconsin–Madison

The University of Wisconsin Transplant Program welcomes you to Madison for this year’s Great Lakes Transplant Immunology Forum. This is the 13th annual event that commenced in 2000, an inspiration of Charles Orosz, Keith Bishop and Rob Fairchild. This important scientific meeting will provide a terrific arena for an in-depth exploration of potential breakthroughs in transplant immunology. The meeting will be highly successful with your enthusiastic participation on the podium, as an audience participant asking questions, and during the posters engaging in face-to-face discussions. We hope you take advantage of this wonderful venue to share your scientific views, reacquaint with friends, and most importantly, make new connections with your colleagues. If there is anything we can do to make your visit more productive, please let any one of our UW faculty hosts know. We are delighted with the privilege of hosting this unique event and wish you an enjoyable forum.

Warm regards,

Dixon

Dixon B. Kaufman, MD, PhD
Professor and Chief
Division of Transplantation
keynote speaker

Charles G. Orosz Memorial Lecture
11:15 AM
Thursday, October 11, 2012

Keynote Speaker
J. Lee Nelson, MD
Fred Hutchinson Cancer Research Center

Biological Diversity Within the Self: Microchimerism

J. Lee Nelson, MD is a Member of the Fred Hutchinson Cancer Research Center and Professor of Rheumatology at the University of Washington. She heads a research group that has an overall goal of elucidating the beneficial and detrimental effects of maternal-fetal cell exchange during and subsequent to pregnancy. Dr. Nelson began her work investigating immunological changes that occur during pregnancy and described fetal-maternal HLA-disparity in the pregnancy-induced amelioration of rheumatoid arthritis. She later spearheaded a new area of research examining the long-term consequences of naturally acquired microchimerism. Microchimerism refers to harboring a small number of cells (or DNA) that originated in a genetically disparate individual. The most common sources of microchimerism are maternal cells in her progeny and cells of fetal origin in women who have been pregnant. Studies of microchimerism are primarily focused on systemic sclerosis and rheumatoid arthritis. The research team she leads is interdisciplinary and also investigates microchimerism in fetal origins of adult disease, complications of pregnancy, and in cancer.
Wednesday, October 10, 2012

AM
10–11:30 Pre-Meeting Conference – PIs Only (Governance Room)
Registration (Northwoods Conference Room)
Northwoods Conference Room

11:30 Lunch/Welcome Remarks
Dixon Kaufman, MD, PhD, FACS
Chief of Transplant Surgery
University of Wisconsin–Madison

Session I  B Cells, Complement and Graft Rejection
William Baldwin, MD, PhD
Cleveland Clinic

PM
12:00 Novel Granulocytes in Tissue Inflammation
Rob Fairchild, PhD
Cleveland Clinic

12:30 Tracing the Fate of Endogenous Alloreactive B Cells
Anita Chong, PhD
University of Chicago

1:00 Role of B Cells in Alloimmunity
Geetha Chalasani, MD
University of Pittsburgh

1:30 Effects of Hypoxic Airway Epithelium on Complement Regulatory Proteins
Pankita Pandya
Indiana University

2:00 Th17 Cells Are Not Required for Cellular Rejection and Fibrosis in a Mouse Model of Orthotopic Lung Transplant
Rebecca Shilling, MD
Indiana University

2:30 Break
Northwoods Conference Room

Session II  Control of Auto- and Alloimmunity
David Wilkes, MD
Indiana University School of Medicine

3:00  Making Treg "Stop and Smell the Roses"
David Rothstein, MD
University of Pittsburgh

3:30  Harnessing the Tolerogenic Properties of Human Invariant Natural Killer T Cells
Jenny Gumperz, PhD
University of Wisconsin

4:00  Pathogenesis of Autoimmune Islet Injury
Ronald Gill, PhD
University of Colorado

4:30  Role of CD103 in Initiation of Autoimmune Diabetes
Gregg Hadley, PhD
Ohio State University

Varsity Hall II

5:00  Poster Session/Wine and Cheese
–6:30
6:45–  Dinner at Wisconsin Institutes for Discovery

Thursday, October 11, 2012

AM

8:00  Breakfast (Varsity Hall II)

Northwoods Conference Room

Session III  Mechanisms of Tolerance, Resistance and Susceptibility
Anita Chong, PhD
University of Chicago

9:00  Targeting Donor-Reactive Memory T Cells in Transplantation
Mandy Ford, PhD
Emory University

9:30  Anti-IL-12/23 Synergizes with Costimulatory Blockade to Prolong Transplant Survival in Mice and Non-Human Primates
Andrew Adams, MD, PhD
Emory University

10:00  Memory T Cells and Lymphoablation in Allograft Recipients
Anna Valujskikh, PhD
Cleveland Clinic

10:30  A Proposed Mechanism for Microchimerism-Based Allograft Tolerance
Will Burlingham, PhD
University of Wisconsin

11:00  Coffee Break
Charles G. Orosz Memorial Lecture:
J. Lee Nelson, MD
Fred Hutchinson Cancer Research Center
Seattle, WA

PM
Varsity Hall II
12:15 Lunch
–1:15

Northwoods Conference Room
Session IV Mini Session – New Topics Forum
Marisa Alegre, MD, PhD
University of Chicago
1:30 The Aryl Hydrocarbon Receptor: A Novel Target for Immunomodulation in Organ Transplantation
Josh Mezrich, MD
University of Wisconsin
2:00 Personalized Medicine in Transplantation
David Perkins, MD, PhD
University of Chicago
2:30 Closing Remarks and Adjourn
–3:00
Poster Presentation Abstracts

1. Effects of Inhibiting C3 Convertase of Complement cascade on Alloantibody-Mediated Rejection; Hsiao-Hsuan Kuo

2. Tracing the fate of alloreactive B cells using MHC Class I Tetramers; Jianjun Chen

3. CD8+ T cells reduce alloantibody production by eliminating allogreprimed B cells through cytoxic mechanism; Jason Zimmerer

4. Role of MCP1 and macrophages in antibody-mediated rejection; Toyofumi Abe

5. A Split in the Loss of Established Allograft Tolerance: Maintaining B cell Tolerance During Acute Rejection; Melvin Daniels

6. Mice engrafted with human hematopoietic stem cells and thymic tissue develop pathology resembling chronic GVHD; Jennifer L. Lockridge

7. STAT1-deficient bone marrow prevents GVHD through induction of STAT3 in plasmacytoid DCs; Christian M. Capitini

8. Infusion of haploidentical NK cells expanded with 4-1BBL causes GVHD; Paul D. Bates

9. Acquisition of Allogeneic MHC-I Antigen is Related to Induction of Anergy of CD4+ T Cells in Mice; Bracamonte-Baran William

10. Human Prostate Tumor Antigen-Specific CD8+ Suppressor T Cells Mediate Infectious Tolerance via CTLA-4 and IL-35; Brian Olson

11. Inducible Costimulator (ICOS) Expression Promotes Proliferation but not Survival of Regulatory T Cells; G.J. Gardiner

12. Tolerance-promoting regimens permit extensive T cell activation but alter differentiation in vivo; Szu-I Wang

13. IL-33 Facilitates ST2+ T Regulatory Cell Expansion Directly and Indirectly Through Actions on Myeloid Dendritic Cells; Benjamin M. Matta

14. Th17 cells are not required for cellular rejection and fibrosis in a mouse model of orthotopic lung transplant; R.A. Shilling

15. Making Treg “Stop and Smell the Roses”; David Rothstein

16. Harnessing the tolerogenic properties of human invariant Natural Killer T cells; Jenny E. Gumperz

17. Pathogenesis of Autoimmune Islet Injury: A Riddle for the GLTIF; Ronald G. Gill

18. Role of CD103 in initiation of autoimmune diabetes; G.A. Hadley

19. Targeting Donor-Reactive Memory T Cells in Transplantation; Mandy Ford

20. Anti-IL-12/23 Synergizes with Costimulatory Blockade to Prolong Transplant Survival in Mice and Non-Human Primates; Andrew Adams

21. Memory T Cells and Lymphoablation in Allograft Recipients; Anna Valujskikh

22. A proposed mechanism for microchimerism-based allograft tolerance; William J. Burlingham

23. Biological Diversity Within The Self: Microchimerism; J. Lee Nelson, MD

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abstracts
FOR ORAL PRESENTATIONS
Novel Granulocytes in Tissue Inflammation

Robert Fairchild

Cleveland Clinic
Notes for Fairchild Presentation
Tracing the fate of endogenous alloreactive B cells

Jianjun Chen, Hao Yin, Jing Xu, Qiang Wang, Roger Sciammas and Anita Chong

Section of Transplantation, Department of Surgery
The University of Chicago, Chicago, IL60660

Alloantibodies mediate acute antibody-mediated rejection as well as chronic allograft rejection in clinic transplantation. We reason that the tracing the fate of alloreactive B cell in the secondary lymphoid organs upon alloantigen exposure will provide insight into how antibody responses are orchestrated. We used a modified staining approach with MHC Class I tetramers (K\textsuperscript{d}) to identify alloreactive B cells. By day 7-8 post-exposure to alloantigen there was a 1.5-3.2-fold increase in the total numbers of K\textsuperscript{d}-binding B cells. Within this K\textsuperscript{d}-binding B cell population, approximately half down-regulated IgD and upregulated expression of MHC Class II and CD86. Also 30-45 % of the K\textsuperscript{d}-binding B cells expressed a germinal center (Fas\textsuperscript{+}GL7\textsuperscript{+}) phenotype and 3-12 % were IRF-4\textsuperscript{hi} plasma cells; phenotypic changes consistent with the alloantibody response being T cell-dependent. With this ability to specifically track in vivo the fate endogenous, Class I-specific B cells, we are proposing to study the fate of alloreactive B cells during a recall response and established states of allograft tolerance.
Role of B cells in alloimmunity

Geetha Chalasani

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

Antibodies from B cells are recognized as effectors of graft injury leading to acute and chronic allograft rejection. However, the role of B cells in alloimmune responses in addition to antibody production is less clear. We have shown that B cell help for alloreactive T cells drives their differentiation into memory T cells resulting in robust recall responses. B cells also cause obliterative vasculopathy and chronic rejection by driving alloreactive T cell responses independent of antibody production. Additionally, antigen presentation by B cells to CD4 T cells is essential for optimal CD4 effector and memory T cell responses, and for CD4 help to CD8 T cell memory. Since B cells express TLRs that can recognize endogenous ligands elicited during transplantation, we explored the role of MyD88-mediated innate activation of B cells in alloimmune responses.

Methods and Results: Syngeneic bone marrow chimeras were created in irradiated μMT recipients by transplanting μMT, MyD88-/- and wt bone marrow cells. In μMT+MyD88-/- chimera, MyD88 was deficient in B cells but not in other APCs and T cells suggesting that MyD88-mediated innate activation is disrupted only in B cells. BALB/c allograft rejection was comparable between μMT+MyD88-/- and μMT+wt chimera. μMT+MyD88-/- chimera showed deficient IgG2a alloantibody responses with intact IgM, IgG1 and IgG2b alloantibody responses. Alloreactive effector T cell responses in μMT+MyD88-/- chimera were exaggerated with increased differentiation to short lived effector cells (SLEC, KLRG1+) and decreased regulatory T cells compared to that in μMT+wt chimera. Analyses of cytokine responses showed a paucity of B cell IL-10 in μMT+MyD88-/- chimera along with increased pro-inflammatory cytokines such as IL-6 that could be contributing to increased effector T cell differentiation in these mice. These findings suggest that MyD88-mediated innate activation in B cells is essential for B cell IL-10 production and dampening the pro-inflammatory cytokine milieu in alloimmune responses.

Conclusions: Antibody-independent mechanisms of B cells play an important role in modulating alloreactive T cell responses. B cells require MyD88-mediated innate activation for IL-10 production and controlling the pro-inflammatory cytokine milieu driving effector T cell differentiation. These findings underscore the multiple functions of B cells in alloimmunity.
Hypoxia Induces Down-regulation of Complement Regulatory Proteins on Airway Epithelium

Pandya PH1,2,3, Suzuki H2,3, Mickler EA2,3, Brown KM2,3, Wilkes DS1,2,3

1Department of Microbiology/Immunology Department, 2Center of Immunobiology, 3Indiana University School of Medicine

Introduction: Lung transplant is a definitive therapeutic solution for several end-stage pulmonary diseases. However, 20-30% of recipients suffer from ischemia reperfusion injury (IRI) which triggers early immunological responses such as complement activation that may contribute to lung rejection. These complement proteins can induce differentiation of Th17 cells or secretion of IL-17 which plays a role in the initiation of a chronic form of lung rejection known as Obliterative Bronchiolitis (OB). To regulate the activity of these complement proteins, Complement Regulatory Proteins (CRPs) such as CD55, CD46, and CRRY (murine homologue of CD46) bind to complement proteins and prevent their complex formation or inhibit their proteolytic cleavage necessary for complement activation. Interestingly, histology from OB mice suggested that these CRPs were down-regulated at protein and message levels. IRI is also able to up-regulate Matrix Metalloproteinases (MMPs), in particular MMP-2 and MMP-9. These zinc dependent endopeptidases are capable of degrading extracellular matrix proteins and may be able to cleave CRPs from the membranes resulting in the observed CRP protein down-regulation. We hypothesize that hypoxia of the airway epithelium during IRI in the transplant procedure contributes to down-regulation of these CRPs, thereby increasing complement activation and allowing the transplanted lung to be more susceptible to chronic rejection such as OB.

Methods: Lung isografts were obtained by performing lung transplant between a donor male C57BL/6 into recipient male C57BL/6 mice. Native and transplant lungs were harvested 1day, 7day, 14day, and 21 day post-transplant. RT-PCR and immunohistochemistry (IHC) was performed on these lungs for CD55 and CRRY expression. To mimic lung IRI in vitro, Small Airway Epithelial Cells (SAECs) were cultured and underwent hypoxia (1% O2) for following time points: 0, 1, 3, 6, 8, 12, and 16 hours. RNA lysates and protein lysates were collected to assess for CD55 and CD46 message and protein expression, respectively. Supernatants from these hypoxic SAECs were collected to analyze MMP-2 and MMP-9 protein expression. MMP-2 and MMP-9 message expression was assessed from the hypoxic SAECs RNA lysates.

Results: Compared to the native lung, CD55 and CD46 mRNA levels were down-regulated nearly 50% in isografts one day post transplantation. Immunostaining revealed CD55 and CRRY protein expression was down-regulated at 21 days post transplants in comparison to the native lungs. CD46 transcript down-regulation was observed as early as 3 hours in hypoxic SAECs, whereas CD55 transcription was decreased within 6 hours in the hypoxic SAECs. Decrease CD46 and CD55 protein expression is observed at 3 hours in hypoxic SAECs. MMP-2 and MMP-9 transcript levels were observed in hypoxic SAECs at 12 hours, but latent MMP-9 protein expression is observed in gelatin zymography in hypoxic SAECs supernatant at 3 hours.

Conclusion: Down-regulation of CD55 and CD46/CRRY due to IRI during lung transplantation, may occur by MMPs which can proteolytically cleave these CRPs from the airway epithelium surface. Thus, predisposing the transplanted lung to chronic rejection such as OB, due to an increase in complement activation mediated inflammation.
Notes
Th17 cells are not required for cellular rejection and fibrosis in a mouse model of orthotopic lung transplant


Indiana University School of Medicine, Indianapolis, IN, US, 46202.

The survival rate for lung transplant patients is much lower compared to other solid organs due in large part to chronic rejection and obliterative bronchiolitis (OB) in the lungs. We have used a mouse model of minor MHC mismatch orthotopic lung transplantation, which develops reproducible obliteration of the airways similar to the lesions found in humans. Previous studies found that IL-17 blockade reduced cellular rejection and fibrotic airway obliteration in allografts in our model. The hypothesis of the current studies is that CD4+ Th17 cells (Th17) are necessary to induce rejection and OB in the mouse model. The left lung from C57Bl/10 mice was transplanted orthotopically into C57Bl/6 mice and the lungs, draining lymph node and spleen cells were evaluated by flow cytometry for intracellular cytokine production at different time-points after lung transplant. Early after lung transplant the immune response in allografted lungs is characterized by the production of Interferon-γ from both CD4+ and CD8+ T cells. By Day 14 after lung transplant, IL-17A is being produced by αβ+CD4+ T cells and γδ+ T cells. The IL-17A response from CD4+ T cells and γδ T cells continues to increase in allografts compared to syngeneic grafts on Days 21 and 28. The development of an IL-17A response in the lungs correlates with the degree of airway fibrosis in the lung allograft. To determine the requirement for CD4+ T cells as the source of IL-17A, B10 allografts were transplanted into CD4.Cre.STAT3fl/fl mice and littermate controls. We found that CD4.Cre. STAT3fl/fl mice developed cellular rejection and fibrosis similar to littermate controls. Our data suggest CD4+ T cells producing IL-17A are not the major inducers of chronic rejection in a minor mismatch model of lung transplant and other sources of IL-17A, such as γδ T cells, may play a role in the development of OB.
Notes
Making Treg “Stop and Smell the Roses”

David Rothstein
University of Pittsburgh

CD4+ Foxp3+ regulatory T cells (Treg) play a critical role in modulating immunity. Expanding Tregs in vivo for therapeutic application would provide an advantage over ex vivo expansion and cell transfer. However, basic aspects of Treg peripheral homeostasis remain incompletely understood. Anti-CD45RB, is a potent tolerogenic agent with activity in both rodent and NHP transplant models. Anti-CD45RB acutely induces Tregs in wild-type mice, even in absence of exogenous antigen. This occurs through a striking augmentation in homeostatic proliferation of pre-existing Treg, as well as conversion of Foxp3- to Foxp3+ CD4 cells. Anti-CD45RB also specifically increases Treg proliferation to cognate antigen. Live-cell imaging shows that CD45 ligation preferentially reduces LFA-1-dependent motility in Treg and enhances Treg:DC interactions. Increased conjugate formation, in turn, augments NFAT nuclear translocation and Treg proliferation. These results demonstrate that Treg peripheral homeostasis can be modulated in vivo to promote Treg expansion and tolerance by specific enhancement of “stop signaling” via CD45.
Notes
Harnessing the tolerogenic properties of human invariant Natural Killer T cells

Jenny E. Gumperz, Ph.D., Associate Professor of Medical Microbiology and Immunology, UW-Madison School of Medicine and Public Health.

Graft-vs-host disease (GVHD) is the major complication of hematopoietic stem cell (HSC) transplantation protocols that are currently used to treat hematological malignancies, and is associated with very high rates of morbidity and mortality. Thus, the development of new approaches to prevent or ameliorate this pathology would be of great clinical value. My laboratory has been investigating mechanisms of immunological tolerance mediated by human invariant Natural Killer T (iNKT) cells, a population of innate T lymphocytes that has been shown to have potent immunoregulatory functions. iNKT cells are a comparatively radiation-resistant population that is thought to contribute to successful engraftment of transplanted HSCs and that may influence subsequent development of GVHD. We have found that human iNKT cells produce factors that specifically induce monocytes to differentiate into dendritic cells (DCs) that have a markedly tolerogenic impact on MHC-restricted T cells, and we believe that induction of these DCs may be an important route by which NKT cells mediate tolerogenic functions in vivo. This presentation will discuss our findings on this unique immunoregulatory pathway and how it may be harnessed for therapeutic benefit in the context of HSC transplantation.
Pathogenesis of Autoimmune Islet Injury: A Riddle for the GLTIF

Ronald G. Gill
University of Colorado Denver, Colorado Center for Transplantation Care, Research and Education

Several known barriers to inducing transplantation tolerance are well appreciated, such as the impact of infection/inflammation, immunological memory, and intrinsic genetic resistance to peripheral tolerance. Autoimmunity represents a related major barrier to allograft tolerance as represented by pancreatic islet transplantation in Type 1 diabetic (T1D) recipients. We have an ongoing interest in the nature of T cell-dependent islet allograft rejection and use the spontaneously autoimmune non-obese diabetic (NOD) mouse model to study the contribution of underlying disease pathogenesis in islet transplant injury. Results to date illustrate a current conundrum regarding how the islet-reactive disease process occurs, especially regarding islet allograft destruction as follows:

- Syngeneic NODrag-/- islets transplants undergo acute destruction in diabetic NOD mice in approximately 9-10 days with a selective destruction of graft islet beta cells, suggesting an actual recurrence of disease. Such NOD islets have indefinite survival in young, chemically-induced (streptozotocin, SZ) diabetic NOD mice, indicating that the destruction of syngeneic islets is related to the active disease status of the NOD recipient.
- Acute disease recurrence of NOD islets requires the expression of donor MHC molecules by the NOD islet graft; MHC-deficient (b2m/C2ta-double deficient donors) have greatly prolonged to indefinite survival in diabetic NOD mice. This result suggests that the disease pathogenesis requires a ‘cognate’, or direct MHC (H-2^d)-restricted interaction between autoreactive T cells and the target islet cells. Other studies show that donor MHC class I expression is more important than MHC class II expression on the NOD islet for disease recurrence.

These results above would suggest that MHC-mismatched islets should show a degree of protection in diabetic NOD mice. However, this is not the case as illustrated by the following results:
- Islet allografts show clearly accelerated destruction relative to NOD islet isografts in spontaneously diabetic NOD mice; MHC-disparate islets (e.g. B6, BALB/c, B10.BR) are all destroyed in 5-7. Thus, although the ‘memory’ response in diabetic NOD mice is largely islet-specific, there is nevertheless a more vigorous response to allogeneic islets. The tempo of islet allograft rejection in NOD mice is also related to the disease state; islet allografts show significantly accelerated rejection in diseased NOD mice relative to the response in pre-diabetic, SZ-induced diabetic animals.
- Islet allograft destruction in spontaneously NOD mice does not require islet donor MHC expression; B6 islets deficient in both MHC class I and II (b2m^-/-I-Ab^-/-) are acutely rejection in diabetic NOD mice, but are permanently accepted in non-autoimmune-prone BALB/c recipients.
- Islet specific BDC2.5scidTcR transgenic CD4 T cells are sufficient to destroy NOD or allogeneic islet grafts in vivo, including B6 MHC class II-deficient (I-Ab^-/-) islets. Such results suggest an ‘indirect’ mechanism of CD4 T cell-mediated islet injury.
- Autoreactive BDC2.5scid T cells can acutely trigger disease in allogeneic B6rag1^-/- recipients if these animals are grafted with NODscid bone marrow. Thus, disease does not require the NOD I-A^d expression either on the target islet or vascular endothelium in order to trigger disease. Again, this result strongly suggests an ‘indirect’ CD4 T cell-dependent mechanism of injury.
- Finally, acute rejection of either islet isografts or allografts appears to be CD4 T cell dependent and CD8 T cell independent based on T cell depletion studies.

Taken together, these results indicate that the requirements for actual disease recurrence differ from those for allograft recognition. Importantly, while disease recurrence clearly requires donor MHC expression, NOD mice demonstrate accelerated islet rejection without such a requirement for donor MHC. So, how can these differences be reconciled?
Role of CD103 in initiation of autoimmune diabetes

E. Stofko, C. Adin, Q-S. Mi, and G.A. Hadley

Ohio State University

Currently, less than 10% of pancreatic islet transplant recipients remain insulin independent after five years (Ryan, 2005) despite being on a continuous regimen of immunosuppressive drugs. The mechanisms that lead to such destruction remain poorly defined, hindering progress in the design of more effective immunosuppressive strategies. One possible reason for the abysmal success rate of pancreatic islet allografts is that unlike most other allografts they are potentially subject to both alloimmunity and recurrent autoimmunity, as islet allografts are nearly always transplanted into individuals with Type I (or autoimmune) diabetes. Indeed, there is compelling evidence that autoreactive CD8 T cells play a central role in precipitating the development of autoimmune diabetes (T1D) in NODshiLTJ (NOD) mice. We have previously reported that the integrin CD103 plays a key role in promoting destruction of the graft epithelium by alloreactive CD8 T cells, and we herein provide evidence that CD103 is expressed at high levels by CD8 T cells that infiltrate pancreatic islets in NOD mice both before and after diabetes development. Given that CD103 recognizes an islet-restricted ligand (E-cadherin) and plays a key role in destruction of epithelial allografts, we postulated that CD103 expression is required for initiation of disease. To test this hypothesis, we developed a strain of NOD mice with targeted disruption of the CD103 gene and compared the incidence of diabetes to that of wild type NOD mice. Our data indicate that the development of T1D in CD103 deficient NOD mice is significantly delayed compared to wild type NODs at early but not late time points, consistent with the possibility that CD103-expressing cells are critical for the initiation of T1D. To test this hypothesis, we are currently depleting CD103 expressing cells from wild type NOD mice at weeks 2-5 vs. 12-15 weeks of age and examining the impact on the incidence of T1D. For this, we are collaborating with scientists at Biogen Idec, who have provided us with a recombinant CD103 depleting reagent, in which the mouse constant regions in a non-depleting rat anti-mouse mAb (M290, rIgG1) are replaced with mouse sequences and a mouse IgG2a Fc portion spliced in to render it depleting in the mouse system. Our data indicate that this reagent is highly effective at depleting CD103+CD8+ cells in vivo, and effectively prevents GVHD mortality when used in combination with CD4 blockade. These data support the use of depleting CD103 mAbs for therapeutic intervention in T1D and islet allograft rejection.
Targeting Donor-Reactive Memory T Cells in Transplantation

Mandy Ford

Emory University

One major obstacle to successful prevention of transplant rejection using costimulation blockade-based therapy is the presence of donor-reactive memory T cells prior to transplantation that precipitate acute rejection. Thus, the identification of strategies to overcome these obstacles is of critical importance in order to optimize the use of belatacept in the clinic. Here, we found that CTLA-4 Ig-resistant CD8+ breakthrough responses can be inhibited by selective CD28 blockade using novel CD28 domain antibody (dAb) technology, which leaves intact CTLA-4 negative signals. Using powerful transgenic murine systems in which we can specifically identify, track, and characterize both CD4+ and CD8+ donor-reactive T cells following transplantation, we have identified a coinhibitory molecule (2B4, SLAMf4) that is specifically expressed on donor-reactive CD8+ T cells following selective CD28 blockade. 2B4 has been shown to play an important role in autoimmunity and in chronic viral infections in both mice and humans, yet has never before been studied in transplantation. Interestingly, 2B4 is expressed at increased levels on secondary CD8+ effectors relative to primary effectors, and a recent study demonstrated that the expression of 2B4 plays a functional role in inhibiting CD8+ secondary effectors during recall responses. In a skin graft model, we showed that CD28dAbs induced upregulation of 2B4, synergized with integrin antagonism in inhibiting memory T cell mediated graft rejection, and inhibited expansion and accumulation of donor-reactive CD8+ memory T cells. Experiments to interrogate whether the graft survival prolongation observed in the presence of CD28dAbs is dependent on 2B4 signaling are ongoing.
Anti-IL-12/23 Synergizes with Costimulatory Blockade to Prolong 
Transplant Survival in Mice and Non-Human Primates

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Introduction: Belatacept is the first clinically approved immunosuppressant for use in transplant based on costimulatory blockade (CoB). Kidney transplant recipients treated with belatacept had improved long-term renal function, albeit with increased rates of acute rejection. This finding has renewed focus on T cell subsets (such as Th17 T cells) with diminished costimulatory requirements that might mediate this resistance to CoB.

Methods: To study the role of Th17 cells in CoB-resistant rejection, we blocked cytokines required for Th1 and Th17 T cell development, using anti-IL-12/23 as an adjunct immunosuppressant in MHC-mismatched murine skin graft and heterotopic cardiac transplant systems. Mechanistic studies employed FoxP3 staining, multiplex cytometric bead assays and quantitative real-time PCR. We also validated this regimen in a non-human primate kidney transplant model.

Results: We found that combined CoB + αIL12/23 substantially prolonged skin graft survival compared to αIL12/23 or CoB alone (MST= 67 vs. 20 days, p<0.0001). Combined blockade also prolonged cardiac allograft survival compared to CoB alone (MST= 141 vs. 63 days, p=0.01). Experiments using selective IL-12 or IL-23 blockade showed that CoB synergized predominantly with IL-23 blockade, potentially highlighting a role for Th17 cells. Combined CoB and αIL12/23 suppressed alloreactive T cell proliferation, augmented the accumulation of allospecific FoxP3+ regulatory T cells and caused immunodeviation, with a significant drop in pro-inflammatory IFNγ and IL-17 production by alloreactive T cells compared to treatment with CoB alone. Intriguingly, IL-17 production was augmented in recipients treated with CoB alone compared to untreated recipients, suggesting that CoB may amplify Th17 alloresponses, perhaps due to its ability to suppress Th1 cytokines (e.g. IFNγ), which antagonize Th17 development. Ongoing kidney transplant experiments in rhesus macaques have validated our murine findings, showing prolonged allograft survival with combined ustekinumab (a humanized anti-IL-12/23) + belatacept (158, 149, 145, 51, and 51 days) compared to belatacept (8, 12 days) or ustekinumab (7, 7 days) monotherapy.

Conclusions: Given that humanized αIL12/23 (ustekinumab) is clinically approved to treat psoriasis, these findings could have significant translational potential for future clinical trials utilizing ustekinumab as an adjunct immunosuppressant to augment the clinical efficacy of belatacept.
Notes
Memory T Cells and Lymphoablation in Allograft Recipients

Anna Valujskikh lab

Our laboratory has focused on memory T cell functions in alloimmune responses. Since memory T cells appear to be less sensitive to anti-thymocyte globulin (ATG) induction therapy in clinical transplant patients, we generated a rabbit anti-mouse ATG and studied the impact of time of administration of the ATG on the depletion and reconstitution of memory CD4 and CD8 T cells. Administration of ATG 7 days prior to transplantation with a MHC-mismatched heart allograft confers much longer allograft survival than when the ATG is given at the time of transplantation. Mechanisms underlying these differences and the impact of recipient memory CD4 T cells on the reconstitution of recipient T cell repertoire will be discussed.
A proposed mechanism for microchimerism-based allograft tolerance

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Rare allogeneic cells of fetal or maternal origin are part of the normal composition of placental mammals. How these cells, usually <1:10,000 in frequency in adults, can profoundly influence the immune system of the host, as in the case of the beneficial effect of non-inherited maternal antigens[NIMA] in kidney transplant recipients, is still a mystery. Based on studies of the NIMA effect in F1 backcross mice, we propose that when microchimerism reaches a critical threshold, an amplification mechanism via intact alloantigen acquisition by host myeloid dendritic cells takes hold. This mechanism appears to allow the semi-direct/semi-indirect pathway to overcome the priming effects of indirect pathway on T cells, via intrinsic/anergy and extrinsic/regulatory effects.
Notes
Biological Diversity Within The Self: Microchimerism

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Microchimerism (Mc) refers to harboring a small amount of cells or DNA that originated in another genetically distinct individual. Bi-directional maternal-fetal exchange occurs during normal pregnancy creating a legacy of durable Mc in both individuals. Mc can be acquired from a twin and probably from an older sibling transferred along with maternal cells to a later fetus. Iatrogenically, Mc can result from blood transfusion, and organ and hematopoietic cell transplantation can create a spectrum extending from Mc to high levels of chimerism. Mc of fetal and maternal origin is found within T and B lymphocytes, monocyte/macrophages, NK cells and granulocytes. Moreover, Mc appears to differentiate into tissue-specific cells, for example cardiac myocytes, islet b cells, hepatocytes and other cell types. Mc has the potential for beneficial as well as adverse effects. Naturally acquired Mc is usually disparate for one HLA allele and shares the other HLA allele with the person who harbors the Mc and is of interest in autoimmune diseases. In systemic sclerosis, Mc of fetal origin has been implicated in an adverse role and maternal Mc has been implicated in neonatal lupus and juvenile myositis. In rheumatoid arthritis some types of Mc may confer benefit and others risk depending upon the specific HLA alleles of the Mc as well as the HLA alleles of the person who harbors the Mc. Mc is common in healthy individuals and benefits such as tissue repair/regeneration have been proposed. Recent studies support a role for Mc of fetal origin in immune surveillance against breast cancer, analogous to the graft-versus-tumor effect of donor cells in transplantation. Moreover, maternal Mc present in cord blood transplant units may impart benefit against recurrent leukemia in some patients (transplant recipients). Overall, advancing understanding of the natural diversity within the self is likely to inform and be informed by advances in understanding Mc and chimerism in transplantation.
The Aryl Hydrocarbon Receptor:
A Novel Target for Immunomodulation in Organ Transplantation

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The aryl hydrocarbon receptor is well described as the receptor for the toxin dioxin. In addition to its role in responding to exogenous toxins, it also is necessary for normal physiology. A number of high-impact papers have recently identified its ability to modulate the acquired immune system. This receptor has the unique ability to enhance T cell differentiation towards regulatory cells when bound to certain ligands, and instead favors Th17 effector differentiation when bound to different ligands. In addition to these in vitro findings, the receptor can similarly either aggravate or ameliorate autoimmunity, tumor survival, or transplant rejection when exposed to these ligands. The aryl hydrocarbon receptor also serves as a sensor to the outside environment. A number of autoimmune diseases are known to be aggravated or caused by environmental exposures, although the mechanisms are unclear. This connection has been less defined in transplant, although data is emerging in lung transplantation. This talk will explore the possible mechanisms through which environmental exposures might alter the balance that a recipient is in years after a transplant, leading to unexpected episodes of rejection. In addition to describing the data in normal physiology and T cell differentiation, I will present examples of the importance of this receptor in preclinical models of disease, and highlight specific ligands that target the aryl hydrocarbon receptor and will have efficacy in treating transplant rejection and tolerance protocols.
A major objective in clinical practice, including transplantation, is to develop more focused and “personalized” approaches for both diagnosis and treatment. There have been several recent successes in correlating specific polymorphisms with drug responses; however, the goal of personalized medicine is challenging for multiple reasons. 1) Humans are outbred with numerous genetic polymorphisms, 2) each individual confronts different sets of environmental exposures, and 3) as analysis focuses on smaller subsets or an individual patient the statistical power is increasingly diminished. In addition, the “gold standard” for diagnosis following kidney transplantation is pathological analysis of a graft biopsy; however, studies have shown approximately 20% variation among diagnoses between blinded pathologists. To address these challenges we focused on the largest available dataset of microarray data analyzing kidney biopsies [Halloran et al] and combined samples from multiple studies to increase statistical power. After filtering the data, we used multidimensional scaling (MDS) to eliminate 11 samples which were statistical outliers (> 2 SD). The 11 outliers did not correlate with any of the diagnoses and appear to include excessive technical noise. Next, we identified subsets of each diagnosis based on silhouette width, Dunn index and connectivity criteria using 3 types of clustering algorithms (hierarchical, kmeans and pam). These results indicated that the dataset represented 21 distinct molecular diagnoses (expanded from the 11 pathological diagnoses). The subsets of a pathological diagnosis were often extensively different. For example, we identified 849 genes that were significantly different (fdr <0.05) between 2 subsets of T cell mediated rejection (TCMR) indicating substantial differences in gene expression despite the same pathological diagnosis between the 2 sub-clusters of TCMR. To identify the biological functions of each sub-diagnosis, we identified the significantly enriched Gene Ontology terms and KEGG pathways for each of the 21 molecular diagnoses. In future studies, we plan to validate our results in prospective studies.
Notes
abstracts

FOR POSTER PRESENTATIONS
Effects of Inhibiting C3 Convertase of Complement cascade on Alloantibody-Mediated Rejection

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Antibody-mediated rejection (AMR) has been one of the biggest challenges in organ transplantation. Complement activation causes tissue injury by forming membrane attack complex and interacting with various complement receptors on leukocytes and parenchymal cells. In the present study, we examined effects on AMR of inhibiting C3 convertase in complement cascade.

Heart or kidney transplantations were performed using B10A (H-2a) and C57BL/6J (H-2b) SCID (rag-/-) mice as donors and recipients, respectively. Recipients received treatment of CR2-Crry, a fusion product between the functional portion of mouse complement regulator Crry and complement receptor 2. Crry has dissociation and cofactor functions to regulate C3 convertases, and CR2 localizes the molecule to sites of C3d and iC3b deposition. CR2-Crry was equilibrated in the circulation for 1 hour after which a mixture of monoclonal antibodies targeting H-2a was injected. Models of single and multiple injections were tested. Mice were sacrificed 1 hour after the last alloantibody transfer.

In control mice, alloantibodies caused complement (C4d) deposition, non-occlusive platelet aggregation and monocyte margination. CR2-Crry decreased levels of C3a and C5a in the grafts. Stains for vWf and P-selectin showed that CR2-Crry decreased platelet aggregates, which correlated with decreased levels of platelet specific cytokine, PF4. Interestingly, CR2-Crry significantly increased numbers of both inflammatory macrophages (Mac2+) and alternative activated macrophages (YM1+) in the grafts, which correlated with cytokine profiles measured by ELISA and qRT-PCR. Our data demonstrate that inhibiting C3 convertase activity decreases multiple aspects of antibody induced inflammation including platelets activation and chemotaxic complement split products. Moreover, CR2-Crry increases M2 macrophages with potentially beneficial repair functions. The mechanism by which CR2-Crry increases M1 macrophages infiltrates requires further study.
Tracing the fate of alloreactive B cells using MHC Class I Tetramers

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Alloantibodies mediate acute antibody-mediated rejection (AMR) as well as chronic allograft rejection in clinic transplantation, and there is an urgent need for effective immunosuppression that can preferentially control alloreactive B cells while preserving protective humoral immune responses to pathogens. Understanding the cellular basis for alloantibody production in transplant recipients would greatly facilitate in achieving this goal of controlling donor-specific antibody responses. Because antibody responses are orchestrated, at least initially, in secondary lymphoid organs, we have focused on tracing the evolution of alloreactive B cell activation and differentiation in both the draining lymph node and spleen. We have used a modified staining approach with a single MHC Class I tetramer (K\textsuperscript{d}) bound to two different fluorochromes to discriminate against fluorochrome-streptavidin binding B cells. This approach, demonstrates a high degree of specificity and binding efficiency and has enabled us to determine that by day 7-8 post-exposure to alloantigen, there was a 1.5-3.2-fold increase in the total numbers of K\textsuperscript{d}-binding B cells, within which approximately half of the alloreactive B cells have down-regulated IgD and have upregulated expression of MHC Class II and CD86. Also approximately 30-45 % of the K\textsuperscript{d}-binding B cells expressed a germinal center (Fas\textsuperscript{+}GL-7\textsuperscript{+}) phenotype and 3-12 % were IRF-4\textsuperscript{hi} plasma cells. The array of GC B cells and plasma cells along with the changes in the expression of MHC and costimulation markers demonstrate that the alloantibody response directed against Kd is T dependent. These data demonstrate the utility of MHC Class I tetramers to specifically track the fate of a subset of donor Class I-specific B cells upon sensitization within a context of complete MHC and minor alloantigen mismatch.
CD8+ T cells reduce alloantibody production by eliminating alloprimed B cells through cytotoxic mechanism

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While it is well known that CD4+ T cells and B cells collaborate for antibody production, our group previously reported that CD8+ T cells downregulate alloantibody responses following transplantation. However, the exact mechanism involved in the CD8-mediated downregulation of alloantibody remains unclear. We also reported that alloantibody production is enhanced when either perforin or FasL is deficient in transplant recipients. Therefore, we hypothesized that alloprimed CD8+ cytotoxic T cells play a role in eliminating alloantibody-producing IgG1+ B cells through cytotoxic mechanisms resulting in a downregulation of alloantibody production. By ELISPOT, we show that CD8+ T cell-deficient mice exhibit a higher number of B cells that produce IgG1 than wild-type mice post-transplant. Allospecific CD8+ T cells required FasL and perforin to downregulate the alloantibody production. Furthermore, CD8+ T cells required FasL and perforin, but not TNF-alpha, to induce clearance of target IgG1+ B cells. In fact, alloprimed CD8+ T cells directly induced apoptosis in alloprimed IgG1+ B cells in vitro. These data are consistent with the interpretation that CD8+ T cells downregulate posttransplant alloantibody production by a mechanism that involves FasL- and perforin-mediated cytotoxicity to eliminate alloprimed IgG1+ B cells.
Role of MCP1 and macrophages in antibody-mediated rejection

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Introduction: Macrophages are members of the innate immune response and quickly infiltrate tissues during inflammation. However, their role in antibody-mediated rejection (AMR) remains unclear. We have reported murine CCR5−/−/CD8−/− recipients produce high titers of antibody and reject class I MHC-mismatched heart allografts. The current studies were conducted to investigate the role of the monocyte/macrophage chemoattractant MCP1 (Monocyte Chemoattractant Protein-1) in AMR and how macrophages may participate in antibody-mediated allograft injury.

Methods: Complete MHC-mismatched A/J or MCP1−/− A/J hearts were transplanted into CCR5−/−/CD8−/− C57BL/6 recipients. Recipients were treated with 250 μg anti-CD40L mAb (MR1) on days 0 and 1. Allografts were harvested on day 14 post-transplant and mRNA expression levels were determined by qRT-PCR and graft infiltrating cells were assessed by immunohistochemistry. Serum antibody titers were assessed by flow cytometry. Donor-reactive CD4 T cells producing IFN-γ were assessed by ELISPOT.

Results: The mRNA expression of MCP1 and IL-1β was decreased in MCP1−/− AJ grafts compared to AJ wild type grafts at day 14. The infiltration of macrophages and T cells into the allograft was decreased in MCP1−/− AJ compared to AJ wild type under MR1 treatment. The number of donor-specific CD4 T cells producing IFN-γ in the recipient spleen was decreased in MCP1−/− AJ graft compared to AJ wild type at day 12. MCP1−/− AJ allografts induced lower antibody titers than AJ wild type allografts although there was no difference in C4d deposition between the groups at day 14. Consequently, the survival of MCP1−/− AJ allografts was better than that of AJ wild type allografts in recipients treated with anti-CD40L mAb (MST: AJ wild type, day 17.5 vs. MCP1−/− AJ, day 46).

Conclusions: Our results suggest that endothelial MCP1 plays an important role in AMR for infiltration of macrophage and donor-reactive CD4 T-cells into the allograft and producing antibodies. MCP1 could be a therapeutic target for AMR.
A Split in the Loss of Established Allograft Tolerance: Maintaining B cell Tolerance During Acute Rejection

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It is well established that the precursor frequency of alloreactive T cells negatively impacts the ability to induce transplantation tolerance. Here we used alloreactive TCR75 CD4⁺ T cells that recognize a peptide from Kd presented on recipient I-Ab. We report that the adoptive transfer of 1.9 x 10⁶/mouse of TCR75 T cells into stably tolerant recipients (at ≥60 days post-transplantation and post-treatment with anti-CD154+donor spleen cell transfusion) precipitated the acute rejection of established allografts. This loss of tolerance was associated with an increase in the percentage of IFN-γ/TNFα double-producing TCR75 cells in the spleen and heart, and also of endogenous alloreactive IFN-γ-producing T cells. In contrast, TCR75 cells did not differentiate into PD-1hiCXCR5⁺ TFH cells, nor were there any signs of a humoral response, measured serologically and by the absence of activated or Germinal Center alloreactive B cells; distinctive sequelae of unmodified acute rejection. These observations underscore the robust control of humoral alloimmune responses, compared to effector Th1 cells, during the loss of tolerance induced by the adoptive transfer of large numbers of alloreactive T cells. Finally this model allows for future investigations into the mechanisms controlling endogenous alloreactive B cell tolerance in steady-state and under when the allograft is undergoing acute rejection.
Mice engrafted with human hematopoietic stem cells and thymic tissue develop pathology resembling chronic GVHD

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Chronic Graft vs. Host Disease (cGVHD) is a significant roadblock to long-term transplantation success. The pathogenesis of cGVHD remains poorly understood, in part because of a lack of animal models which fully reproduce the characteristics of clinical cGVHD. Here we present an analysis of immunodeficient mice engrafted with human fetal hematopoietic stem cells (HSCs) and fragments of autologous fetal thymus and liver. No earlier than 100 days after engraftment, the mice developed symptoms that resembled cGVHD, in that sites such as skin, eyes, lungs and liver showed evidence of fibrosis and substantial infiltrates of human T cells, B cells and macrophages. Analysis of the impact of irradiating the mice prior to transplantation revealed that eliminating the irradiation step resulted in a selective reduction in B lymphocyte development without affecting the timing of GVHD symptom onset. Lowering the dose of human HSCs administered to the mice also reduced B lymphocyte development without affecting GVHD symptom onset timing. However, mice that were engrafted with human fetal tissue of a younger gestational age (14 vs. 20wks) showed a delay in the onset of GVHD symptoms. Mice with severe GVHD symptoms typically showed extensive fibrosis and degradation of the engrafted thymic organoid, as well as significantly reduced frequencies of Foxp3+ thymocytes. Thus, impaired thymic selection or Treg production may be involved in the pathogenesis of GVHD in these mice, as has been suggested for clinical cGVHD. This model provides a new basis for investigating mechanisms and testing treatment strategies for clinical cGVHD.
Abstract #7

STAT1-deficient bone marrow prevents GVHD through induction of STAT3 in plasmacytoid DCs

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Graft-versus-host-disease (GVHD) therapies have focused on directly inhibiting T cell function, also abrogating the beneficial graft-versus-tumor (GVT) effect. Because of prior studies demonstrating a critical role for both type I and type II interferons on GVHD/GVT, the effects of using T cell depleted (TCD) STAT1−/− bone marrow (BM) after minor histocompatibility antigen-mismatched allogeneic bone marrow transplant (alloBMT) was studied. By giving STAT1+/+ T cells as a delayed donor lymphocyte infusion (DLI), the timing and intensity of GVHD was controlled while leaving interferon signaling on T cells intact. TCD STAT1−/− BM prevented GVHD induced by DLI, leading to expansion of B220+ cells and regulatory T cells. Treating BM donors and recipients with Exenatide, an off-target inhibitor of STAT1, prior to DLI replicated the effects of using STAT1−/− BM. TCD STAT1−/− BM expanded a CD9 Siglec Hi subset of plasmacytoid dendritic cells (pDCs) in the BM and spleen of alloBMT recipients. Depletion of pDCs after TCD STAT1−/− alloBMT improved low grade GVHD. STAT1−/− pDCs were found to produce low levels of free radicals, IFNα and interleukin (IL)-12, while producing elevated levels of IL-10. Combining STAT1−/− pDCs pulsed with the antigen WT-1 with WT-1 TCR transgenic T cells decreased IFNγ production. STAT1−/− pDCs isolated after TCD alloBMT showed increased gene expression of S100A8 and S100A9, and usage of TCD S100A8/9−/− BM exacerbated GVHD-free survival after alloBMT. Elevated levels of STAT3 protein were found in pDCs isolated after TCD alloBMT. TCD STAT1−/− BM preserved GVT activity in terms of controlling tumor volume, but enhanced overall survival in the setting of GVHD. Understanding interferon signaling in non-T cell populations, like pDCs, provides a critical interface by which regulate the GVHD/GVT axis. Therapies that inhibit STAT1 in TCD BM may be useful toward inducing STAT3 in pDCs and affecting their biology during immune reconstitution after alloBMT.
Infusion of haploidentical NK cells expanded with 4-1BBL causes GVHD

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Expansion of natural killer (NK) cells for adoptive cell therapy of relapsed tumors is being explored after allogeneic bone marrow transplant (alloBMT). Because interleukin (IL)-15 is typically presented in trans by IL-15 receptor alpha (Ra), utilization of a recombinant IL-15/IL-15Ra complex was studied to expand murine NK cells ex vivo. The objective of this study was to determine if the presence or absence of an artificial antigen presenting cell (aAPC) expressing 4-1BB ligand (4-1BBL) would potentially improve NK cell expansion and activity induced by IL-15. In addition, the impact of using NK cells expanded by this combination after alloBMT has not been explored. NK cells were harvested from either Balb/c or CB6F1 spleens and cultured ex vivo for 1 week with IL-15 alone or with IL-15 plus 4-1BBL⁺ aAPCs. NK cells cultured with IL-15 showed a 20-fold expansion, but this expansion was attenuated with the addition of 4-1BBL⁺ aAPCs. There was no difference in the expression of the inhibitory receptor Ly49A or activating receptor Ly49D between Balb/c and CB6F1 NK cells expanded with IL-15 alone, or IL-15 and 4-1BBL⁺ aAPCs. Infusion of NK cells expanded ex vivo with IL-15 alone was well tolerated after T cell depleted major histocompatibility complex (MHC)-mismatched alloBMT (CB6F1 à B6), and led to improved survival after rhabdomyosarcoma (RMS) challenge. Surprisingly the addition of 4-1BBL⁺ aAPCs to cultures caused IL-15-expanded NK cells to induce GVHD lethality (CB6F1 à Balb/c), leading to poor survival after RMS inoculation. By contrast, after MHC-matched, minor histocompatibility antigen-mismatched alloBMT (DBA à Balb/c), infusion of NK cells expanded either with IL-15 or with IL-15 and 4-1BBL⁺ aAPCs were well tolerated, implying MHC disparities between donor and host cells is critical in driving 4-1BBL-stimulated NK cell alloreactivity. In summary, IL-15 expanded murine NK cells demonstrate robust expansion in vitro and do not cause toxicity when infused after alloBMT in vivo, leading to a graft-versus-tumor (GVT) effect. The presence of 4-1BBL⁺ aAPCs attenuated NK cell expansion with IL-15 in vitro. Remarkably, NK cells expanded by IL-15 in the presence of 4-1BBL⁺ aAPCs demonstrate increased propensity to cause GVHD after MHC-mismatched alloBMT, causing a loss of GVT. Further studies exploring the interaction of 4-1BB and IL-15Ra signaling on NK cells will be critical, particularly in the context of MHC mismatched alloBMT.
Acquisition of Allogeneic MHC-I Antigen is Related to Induction of Anergy of CD4+ T Cells in Mice

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Background: The two classical pathways leading to immune response in the context of transplantation are called indirect and direct. The former one implies the normal phagocytosis, processing and expression of peptides derived from allogeneic MHC molecules by recipient antigen-presenting cells (APC) as peptide-self MHC complexes. The direct one consists in the response of recipient CD8+ and CD4+ T cells against allogeneic MHC molecules expressed by donor cells. In the last decade the semi-direct pathway was described. In that one, dendritic cells (DC) of the recipient acquire MHC molecules from donor cells via exosome exchange and/or trogocytosis. Antigen acquisition pathways seem to be physiological important in the regulation of the normal immune response, greatly amplifying the response to viruses and other pathogens by lateral spread of the antigenic signal through host APCs. Maternal-fetal microchimerism may benefit from the same amplification mechanism to increase tolerance to an allogeneic solid organ graft in the offspring, even though the chimeric maternal cells represent a proportion of total cells in the offspring as small as 0.005%. We propose that the acquisition of non-inherited maternal antigens (NIMA) by the offspring cells from chimeric maternal cells is an important mechanism responsible for the tolerogenic effect of microchimerism.

Objective: to determine the nature of the subset of antigen-acquiring cells (AAC) in spleen and peripheral blood. Also, to study the effect of NIMA-acquisition on the T cell mediated alloimmune response.

Material and Methods: NIMA\textsuperscript{d}-exposed b/b offspring were obtained from the breeding B6 males (b/b) with BDF1 females (b/d). Peripheral blood mononuclear cells (PBMC) and splenocytes were screened for the expression of H2K\textsuperscript{d} using flow cytometry. It was also used antibodies anti CD11b, CD11c, B220 and CD3 in order to characterize the kind of AAC. The antibody YAe, specific for the complex peptide Eα(d)-MHCII(b), was also used for cytometric analysis. An in vivo MLR was performed by retroocular injection of 20x10\textsuperscript{6} CFSE-labeled CD4+ splenocytes from TEa mice (which only have CD4+ T cells with the same specificity than YAe antibody) in NIMA\textsuperscript{d} mice, whose statuses of H2K\textsuperscript{d} acquisition in peripheral blood were previously determined. After euthanizing the recipient, the proliferation of TEa cells in spleen and lymph nodes was assessed by the CFSE dilution method. In every case B6 mice (b/b) and BDF1 mice (b/d) were used as proper negative and positive controls.

Results: It was demonstrated that roughly 25% of the NIMA\textsuperscript{d} mice acquire H2K\textsuperscript{d} in the spleen and peripheral blood. There was a perfect match between the statuses in these two tissues. In the spleen the ACC were only myeloid DC (CD11c high CD11b low B220 neg) whereas all DC seem to have the ability to acquire the YAe epitope (myeloid B220neg and plasmacytoid B220pos). In PBMC, the H2K\textsuperscript{d} ACC subpopulation was CD11b high CD11c med, which is an immunophenotype of mDC too. In the in vivo MLR we observed proliferation in the TEa cells injected in NIMA\textsuperscript{d} mice previously screened as positive for YAe but negative for H2K\textsuperscript{d} acquisition. On the other hand, no proliferation at all was observed in NIMA\textsuperscript{d} mice positive for H2K\textsuperscript{d}, even though the TEa/YAe epitope was present.

Conclusions: The myeloid DC seems to be the subpopulation responsible for a)allogeneic antigen acquisition, and b)as a consequence, for the semi-direct pathway in spleen and peripheral blood. On the other hand, all DC have the potential to participate in the indirect pathway, according to the expression of YAe epitope. The acquisition of allogeneic MHC\textsubscript{I} molecules seems to have a tolerogenic effect, since AAC seem to induce anergy on CD4+ T cells specific against the acquired antigen. The mechanism of this phenomenon is still unclear.
While regulatory T cells in the context of transplantation play an important role in maintaining graft tolerance, they have an opposing, detrimental effect in the context of cancerous malignancies. In several types of solid tumors, regulatory T cells have been shown to play critical roles in cancer development and progression by limiting the generation of innate and adaptive anti-tumor immunity. While most of this research has focused on antigen non-specific natural CD4+CD25+ Tregs and myeloid-derived suppressor cells, we hypothesized that tumor antigen-specific regulatory T cells interfere with the detection of anti-tumor immunity following immunotherapy. Using samples from prostate cancer patients immunized with a DNA vaccine encoding prostatic acid phosphatase (PAP) and a trans-vivo delayed type hypersensitivity (tvDTH) assay, we identified PAP-specific effector responses following immunization that were actively suppressed by PAP-specific regulatory cells. These cells were CD8+CTLA-4+, and their suppression was relieved by blockade of CTLA-4, but not IL-10 or TGF-β. Moreover, antigen-specific CD8+ regulatory T cells were detected prior to immunization in the absence of PAP-specific effector responses. These PAP-specific CD8+CTLA-4+ suppressor T cells expressed IL-35, which was decreased following blockade of CTLA-4, and inhibition of either CTLA-4 or IL-35 reversed PAP-specific suppression of tvDTH response. PAP-specific CD8+CTLA-4+ T cells also suppressed T-cell proliferation in an IL-35-dependent, contact-independent fashion. Taken together, these findings suggest a novel population of CD8+CTLA-4+ IL-35-secreting tumor antigen-specific regulatory T cells arise spontaneously in some prostate cancer patients, persist during immunization, and prevent the detection of antigen-specific effector responses by an IL-35-dependent mechanism.
Inducible Costimulator (ICOS) Expression Promotes Proliferation but not Survival of Regulatory T Cells

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Introduction: The development of obliterative bronchiolitis severely limits the long-term survival of lung allograft recipients and is thought to be promoted by acute rejection. During episodes of acute rejection, CD8+ T lymphocytes damage the vascular endothelium and small airway epithelium of the lung as part of a cell-mediated immune response against alloantigens. Using an in vivo model of CD8-mediated lung injury, we have shown previously that regulatory T (Treg) cells expressing high levels of inducible costimulator (ICOS) are important for the resolution of inflammation; however, the mechanism underlying this observation is not known. We hypothesized that ICOS expression affects proliferation and survival of Treg.

Methods: To test this hypothesis, single cell suspensions were prepared from spleens of C57BL/6 wildtype and Icos−/− mice. Cells were resuspended at 50 x 10^6 c/ml in PBS +5% FCS and labeled with CFSE. Labeled cells were left unstimulated or stimulated with 0.01 μg/ml or 0.1 μg/ml anti-CD3ε antibody (2C-11) for 72 hrs. Cells were harvested, and dead cells were labeled using near-IR LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen). Cells were stained for flow cytometry with PerCP/Cy5.5 anti-mouse CD4 (GK1.5) and PE/Cy7 anti-human/mouse/rat CD278 (C398.4A). For intracellular staining, cells were fixed and permeabilized using anti-Mouse/Rat Foxp3 Staining Set buffers (eBioscience) and subsequently stained with APC anti-mouse/rat Foxp3 (FJK-16s). Samples were collected using an LSRII 561 nm laser flow cytometer (BD), and data were analyzed with FlowJo software (Tree Star).

Results: As expected, CD4+Foxp3+ cells stimulated with anti-CD3ε proliferated more than unstimulated cells. When stimulated with anti-CD3ε, CD4+Foxp3+ cells from wildtype mice proliferated better than CD4+Foxp3+ cells from Icos−/− mice. For each concentration of αCD3ε utilized, the mean fluorescence intensity of CFSE was increased in the Icos−/− sample compared to the wildtype sample, indicating that the Icos−/− samples had proliferated less. This result suggests that Icos−/− Treg have a defect in proliferation. When CD4+Foxp3+ cells were separated into live and dead populations, no difference was observed in the percentage of dead CD4+Foxp3+ cells from wildtype mice compared to Icos−/− mice for each concentration of anti-CD3ε tested. This result suggests Icos−/− Treg do not have a defect in survival compared to wildtype Treg.

Conclusions: The results of these experiments indicate a possible role for ICOS expression in Treg proliferation potential in vitro; however, ICOS expression does not seem to be important for the overall survival of Treg in vitro. ICOS-expressing Treg may be able to promote the resolution of CD8-mediated lung injury better than ICOS-deficient Treg due to an increased ability to proliferate.
Abstract #12

Tolerance-promoting regimens permit extensive T cell activation but alter differentiation in vivo

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Short-term perturbation of the costimulatory molecule CD154 and/or the adhesion molecule LFA-1 can induce allograft tolerance. However, the effects of these agents on antigen-specific T cells are unclear. Therefore, we determined how anti-CD154 or anti-LFA-1 monoclonal antibody (mAb) therapy impacted the reactivity of antigen-specific CD4 and CD8 T cells in vivo. We monitored responses of ovalbumin (OVA)-specific CD8 (OT-I) or CD4 (OT-II) TCR transgenic T cells and alloreactivity by polyclonal T cells (C57BL/6 anti-BALB/c). CD45.1 OT-I, OT-II or B6 T cells were transferred into CD45.2 B6 hosts locally challenged with mAct-OVA or BALB/c APCs, respectively, in the presence of mAb therapy. Anti-CD154 and anti-LFA-1 each inhibited the magnitude of OT-I and OT-II responses but permitted extensive proliferation and conversion to an antigen-experienced CD44hiCD62Llo phenotype. Both treatments decreased expression of granzyme B in OT-I T cells and interferon-gamma in OT-II T cells. Polyclonal alloreactivity also was markedly inhibited by either anti-LFA-1 or anti-CD154 therapy, though activation and proliferation still occurred in both cases. Interestingly, anti-LFA-1 but not anti-CD154 treatment resulted in a striking increase in the percentage but not absolute number of regulatory T cells in reactive lymph nodes. Overall, neither blocking CD154 nor LFA-1 prevented T cell reactivity in vivo. However, LFA-1 blockade exhibited a more pronounced ability to skew ratio of regulatory T cells versus effector T cells in vivo.
Abstract #13

IL-33 Facilitates ST2⁺ T Regulatory Cell Expansion Directly and Indirectly Through Actions on Myeloid Dendritic Cells

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Overview: IL-33 signals via the IL-1R/TLR family member, ST2, which is expressed on both T cells and myeloid cells. Historically, IL-33 has been ascribed Th2 promoting capacities, particularly through its action on ST2⁺ myeloid dendritic cells (mDC). However, IL-33 administration potently increases the number of CD4⁺ Foxp3⁺ regulatory T cells (Treg), which mediate cardiac allograft survival. As the precise mechanisms by which IL-33 supports expansion of Treg were undefined, we examined if IL-33 expands Treg by targeting them directly or indirectly through its actions on mDC.

Methods: The ability of IL-33 to facilitate anti-CD3/CD28-stimulated proliferation of BALB/c St2⁺/⁺ or St2⁻/⁻ CD4⁺ CD25⁺ T cells was directly compared to IL-2 by flow cytometric analysis. Conversely, the impact of IL-33 on the Treg-expanding capacity of mDC was defined in vitro on mDC generated from BALB/c St2⁺/⁺ or St2⁻/⁻ bone marrow and in vivo using CD11c-DTR eGFP B6 bone marrow chimeras administered diphtheria toxin (DT; 5 ng/g) to selectively deplete CD11c⁺ cells during IL-33 treatment. The capacity of sorted splenic or ex vivo expanded RFP⁺ Foxp3⁺ T cells to suppress CD3/CD28-stimulated proliferation and polarization of syngeneic CD4⁺ T cells was also assessed.

Results: The presence of IL-33 during CD3/CD28-stimulation of CD4⁺ CD25⁺ Treg promoted their expansion. Interestingly, IL-33 directed the proliferation of a population of suppressive ST2⁺ Foxp3⁺ that were absent from those expanded in IL-2-treated cultures. IL-33-exposed mDC, although supporting Th2 polarization, could also generate a ST2⁺ population of CD4⁺ Foxp3⁺ cells in naïve T cell populations. Importantly, although IL-33 can directly augment Treg proliferation, addition of IL-33 to St2⁻ mDC cultures revealed that the most significant contribution of IL-33 to ST2⁺ Treg expansion results from the impact of IL-33 on mDC. This was further supported by the failure of administered IL-33 to expand Treg, especially ST2⁺ Treg, in the absence of CD11c⁺ mDC in vivo.

Conclusions: IL-33 promotes the expansion of functional Foxp3⁺ Treg, including an ST2⁺ subset in vitro and in vivo. This results from IL-33 activity directly on ST2⁺ CD4⁺ CD25⁺ Treg and indirectly through actions on ST2⁺ CD11c⁺ cells. Thus, IL-33, in addition to supporting Th2 responses, can also mediate Treg expansion through its action on T cells and mDC.
Our lab has previously shown that pre and post transplant infusions of donor splenocytes treated with the chemical cross-linker ECDI (ECDI-SPs) is highly efficacious in inducing long-term protection of full MHC-mismatched islet allografts in the absence of immunosuppressive drugs. We have also recently shown that donor ECDI-SP infusions also significantly prolong cardiac allograft survival, and with addition of short course low dose rapamycin, indefinite graft protection was observed. Mechanistic studies revealed that graft protection was concomitant with the presence of intragraft CD11b⁺ cells expressing the tryptophan metabolizing enzyme indoleamine 2,3 dioxygenase (IDO), and Gr1, although co-localization of IDO⁺ and Gr1⁺ cells was not observed. Depletion of Gr1⁺ cells or inhibition of IDO activity using an IDO inhibitor 1-methyl-d-tryptophan (1-MT) abrogated graft protection by ECDI-SP infusions. Further phenotypic analysis of intragraft and spleen CD11b⁺ cells from ECDI-SP- treated mice revealed the increased presence of Ly6C high, intermediate and low cells expressing varying levels of Ly6G, CCR2 and F4/80, some of which co-localized with IDO⁺ cells as determined by immunohistochemistry. Multiplex analysis of splenocyte lysate showed an increase in myeloid differentiation factors and chemokines such as CCL2, M-CSF and GM-CSF. Further studies are currently ongoing to determine the function and exact nature of these intragraft and spleen CD11b⁺Ly6C^hi/+ cells, whether they themselves express IDO or induce it in a distinct population, and how they contribute to a favourable graft outcome.
Immunological memory has evolved to protect against repeated infections, but it also develops to other foreign antigens and thus is a liability for transplantation. Early transplantation experiments showed T cell-dependent accelerated rejection of secondary skin transplants following rejection of primary grafts. Methods to circumvent memory cell function or generation are highly sought after in transplantation because memory cells, once they are generated, are more difficult to control with current immunosuppressive therapies. Our data show that mice that have rejected previously long-term accepted cardiac allografts following infection with *Listeria monocytogenes* accept second donor heart grafts after the infection is cleared in the absence of any immunosuppression. This challenges the prevailing paradigm of immunological memory of rejection, and instead supports one of memory of tolerance. Maintenance of tolerance in these transplanted mice prior to infection was characterized by high percentages of intra-graft regulatory T cells and effector T cells that expressed the inhibitory receptor PD-1. However, only in the presence of increased alloreactive cell precursor frequencies did combined blockade of these two pathways precipitate rejection. *Listeria* infection transiently increased graft-infiltrating cells and lowered percentages of regulatory cells and PD-1+ cells in the primary graft. However, both of these latter populations reappeared in high percentages in the second tolerated grafts. The restored tolerance in the second grafts was dependent on regulatory cells and signals from PD-L1, as blockade of either of these pathways was sufficient to precipitate rejection of the second tolerated graft. These data support a model in which a dominant memory of tolerance exists to reestablish a tolerant state following infection-mediated rejection.
Abstract #16
Combined computational and experimental analyses of IL-6 and IL-1 reveal distinct mechanisms of action in the modulation of regulatory T cell suppressive activity

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Introduction: The development of autoimmune diseases, cancer, and transplant outcomes are all influenced by the ability of regulatory T cells (Treg) to suppress the activity of conventional T cells (Tconv). Therefore, rational modulation of Tconv susceptibility to suppression by Tregs is highly desirable. However, our understanding of the factors and mechanisms that underlie this modulation is limited. We believe that inflammatory mediators, by contributing to the activation of Tconv while reducing Treg activity, limit the effectiveness of tolerogenic regimens. We aimed to identify principal inflammatory mediators, released by innate immune cells, that lessen T cell regulation and infer their mechanism of action.

Methods: Using a CFSE-based, in vitro mouse T cell activation system, we quantified Treg suppressive activity and assessed the influence of the supernatant of maturing dendritic cells sampled at multiple times after stimulation with LPS. In parallel, 24 cytokines were quantified using Luminex™ in each sample tested. Principal Component Analysis (PCA) was utilized to delineate likely modulators of Treg suppression; the results were subsequently validated experimentally.

Results: PCA suggested IL-6, a known modulator of Treg suppression, as one of the cytokines with the most relevant contribution to the variance of the system. Interestingly, the same analysis revealed a similar pattern of variance between IL-1α and IL-1β with IL-6 suggesting a modulatory role. The CFSE assay indicated that IL-6 did not directly interfere with the susceptibility of Tconv to suppression (as originally suggested), but counteracted regulation by promoting a higher frequency of proliferating cells (that remained susceptible to suppression). Conversely, IL-1α and IL-1β each interfered with Treg suppression by directly affecting their suppressive activity. Using combinations of IL-1R-knock-out Tconv or Treg, we established that this effect was exerted by acting on both cells. As the in vitro activity of Treg is counteracted by IL-2 availability, we next tested if the differential effect of IL-6 and IL-1 correlated with differences in IL-2 secretion by Tconv following activation (in the absence of Treg). While IL-6 did not significantly increase the frequency of IL-2-secreting cells, IL-1 doubled this number to values comparable to those seen upon exogenous costimulation, further underscoring the separate mechanisms of action of these cytokines. In a mouse model of inflammatory bowel disease, preliminary results indicate that blockade of IL-6 signaling delays disease development through inhibition of colitogenic T cells expansion allowing tighter control by Treg.

Conclusions: Our results suggest that combining computation with experimentation may help to dissect the complex inflammatory milieu that determines T cell susceptibility to suppression. Specifically, this approach identified complementary as well as synergistic activities of IL-6 and IL-1 (α and β). Elucidation of the differential intracellular mechanisms used will aid in identifying targets for therapeutic approaches to selectively promote (autoimmunity and transplantation) or prevent (cancer and vaccination) immune regulation.
All-trans Retinoic Acid and Rapamycin Synergize with Transforming Growth Factor-β1 to Induce Regulatory T Cells but Confer Distinct In Vivo Migratory Capacities

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Introduction: Regulatory T cells (Treg) play important roles in maintaining immune homeostasis and, for this reason, therapies based on Treg are promising candidates for the treatment of a variety of immune-mediated disorders. However, therapies based on naturally-occurring Treg face the challenge of obtaining sufficient numbers of these cells from peripheral blood. Inducing Treg from non-Treg has been suggested as a viable alternative. Different methods to induce Treg have been proposed, mainly involving use of TGFβ (TGFβ-iTreg). Although TGFβ alone is not sufficient to induce stable Treg, all-trans retinoic acid (RA) or rapamycin (Rapa) has been shown to synergize with TGFβ to induce stable Treg. While RA-induced Treg (RA-iTreg) have been well described in the literature, the phenotype, function and migratory characteristics of rapa-induced Treg (Rapa-iTreg) have yet to be elucidated.

Methods: iTreg were induced from mouse CD4+CD25- T cells by in vitro stimulation in presence of TGFβ alone or together with: RA, Rapa, or a combination thereof. Expression of Foxp3, Treg-associated markers, and chemokine receptors was assessed by flow cytometry. In vivo migration was determined by adoptive transfer of congenic iTreg followed by flow-mediated enumeration or by adoptive transfer of luciferase-expressing iTreg (Luc) and monitoring of the bioluminescent signal.

Results: Addition of RA, Rapa, or both increased the frequency of Foxp3+ cells over the proportion obtained with TGFβ alone. Interestingly, no significant differences were found in the expression of Treg-associated markers like CTLA-4, CD25, and GITR, although a significant increase in folate receptor-4 (FR4) was evident on Rapa- and RA+Rapa-iTreg. All 4 iTreg types were comparable in their ability to inhibit T cell activation in vitro. Major differences were evident in the surface expression of chemokine receptors and integrins: TGFβ-iTreg were CCR7medCCR9loCD103hi; RA-iTreg were CCR7medCCR9hiCD103hi; Rapa-iTreg were CCR7hiCCR9loCD103lo; finally, RA+Rapa-iTreg were characterized by heterogeneous composition with the two most abundant populations being CCR7medCCR9hiCD103hi and CCR7medCCR9hiCD103lo. This suggested different migratory properties for each population. Monitoring the intensity of bioluminescent signal from adoptively-transferred iTreg-Luc (3, 6, 8, and 10 days after transfer) confirmed that Rapa-iTreg had a greater tendency to accumulate in lymph nodes compared with TGFβ- and RA-iTreg, with the latter population mainly accumulating in the gut. These results were reproduced by enumeration of congenic adoptively transferred iTreg.

Conclusions: These results indicate that although RA and Rapa are considered interchangeable in their promotion of stable iTreg, they confer a different migratory potential onto these cells. It is conceivable that these differences in migratory capacities result in different abilities to control local immune responses in vivo, a hypothesis that we are currently testing using models of inflammatory bowel disease and other immune-mediated disorders.
Anti-VLA-4 mAb Abrogates Early Infiltration of Endogenous Memory CD8 T cells into Cardiac Allograft and Donor-Reactive T Cell Priming

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Introduction: We have reported the rapid infiltration and activation of endogenous memory CD8 T cells with donor-reactivity into cardiac allografts expressing class I MHC disparities. Surface expression of VLA-4 is upregulated on memory vs. naïve T cells, suggesting VLA-4 as a target to inhibit endogenous memory CD8+ T cells in allograft recipients. The current studies were conducted to analyze the efficacy of anti-VLA-4 mAb to inhibit this early CD8 T cell allograft infiltration and activation in cardiac allograft rejection.

Methods: Complete MHC-mismatched A/J hearts were transplanted into C57BL/6 recipients. Recipients were treated with 200 μg control IgG or anti-VLA-4 mAb on days -1 and 0. Allografts were harvested on day 2 or 7 post-transplant and mRNA expression levels of inflammatory mediators determined by qRT-PCR and numbers of graft infiltrating cells assessed by flow cytometry. Donor-reactive CD4 and CD8 T cells producing IFN-γ assessed by ELISPOT.

Results: Anti-VLA-4 mAb treatment decreased CD8 T cell and macrophage infiltration into allografts on day 2 post-transplant about 70% that observed into allografts from control treated recipients. Infiltration of CD4 T cells and neutrophils into allografts in control and anti-VLA-4 mAb treated recipients was the same. Treatment with anti-VLA-4 mAb also decreased the number of donor-specific CD4 and CD8 T cell producing IFN-γ in the recipient spleen when assessed at day 7. These effects combined to a modest prolongation in allograft survival (MST: day 12 vs day 7.5 in control-Ig treated recipients, p < 0.05).

Conclusions: Our results suggest that peri-transplant administration of anti-VLA-4 mAb modulates early infiltration of donor-reactive memory CD8 T-cells into the allograft and the accompanying inflammation and delays donor-reactive T cell priming. Therapeutic targeting of this pathway may reduce the negative impact of early CD8 T cell-dependent inflammatory events during the allograft rejection.

Keywords: VLA-4 antigen; Graft-infiltrating cells; Adhesion molecules; Heart/lung transplantation
Abstract #19

**Prolonged cold ischemia increases the early infiltration and effector functions of activated effector-memory T lymphocytes in cardiac allografts**

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**Introduction:** During organ transplantation, ischemia-reperfusion injury (IRI) is an inescapable pathological challenge that may detrimentally impact graft outcome. While I/R induced molecular mechanisms mediating injury of ischemic tissues are increasingly being delineated, potential cellular elements that contribute to IRI remain poorly understood. The presence of pre-existing alloreactive memory T cells in the transplant recipient is now widely recognized as a major barrier to tolerance induction and maintenance. We have recently reported that endogenous memory CD8 T cells with donor-reactivity infiltrate cardiac allografts within 24 hours of reperfusion and are activated to produce IFN-γ. The mechanisms promoting infiltration of these T cells into the graft remain poorly defined.

**Methods:** A/J (H-2\(^a\)) hearts were subjected to 0.5hrs, 4hrs, or 8hrs of cold ischemia before being heterotopically transplanted to C57BL/6 (H-2\(^b\)) recipients. Graft infiltrating cells were quantified by flow cytometry, cytokine and chemokine mRNA expression levels measured by qRT-PCR analysis, and tissue damage and inflammation assessed by histology.

**Results:** CD8\(^+\)CD62L\(^{low}\) memory T cell infiltration into heterotopic cardiac allografts at 48 hours post-transplant was significantly influenced by the duration of ischemia imposed on the graft. Prolonged ischemia increased expression of the acute phase cytokines IL-1, IL-6, and TNFα within the graft 48 hours after reperfusion and anti-TNFα treatment at reperfusion inhibited CD8\(^+\) memory infiltration. Prolonged ischemia resulted in heightened production of IFN-γ and perforin by early infiltrating memory CD8\(^+\) T cells at day 5 which was associated with markedly increased graft inflammation, myocyte damage, and poorer graft survival compared to grafts subjected to minimal cold ischemia.

**Conclusion:** These data demonstrate that prolonged ischemic insult to cardiac allografts enhances the early infiltration and effector functions of CD8\(^+\)CD62L\(^{low}\) memory T cells post-transplant to mediate tissue inflammation and organ dysfunction. These studies provide novel insights into mechanisms underlying the poor outcomes associated with cadaver donor grafts.
Mechanisms underlying compartmentalization of acute versus chronic rejection in cardiac allografts

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Acute rejection of cardiac allografts is diagnosed by interstitial infiltrates in endomyocardial biopsies. In contrast, chronic rejection is defined by vasculopathy in large arteries that is diagnosed by angiography. Hearts transplanted from male to female C57BL/6, which differ only at H-Y minor antigens, undergo an acute rejection that transitions into chronic rejection. We defined the histopathological parameters of rejection 2 and 6 weeks after transplantation in this model. Diffuse interstitial infiltrates of CD3+ T-cells and macrophages that spared the large arteries were documented immunohistologically in the grafts at 2 weeks. However, by 6 weeks the interstitial infiltrates had resolved, about half of the large arteries developed prominent adventitial and intimal macrophage infiltrates together with neo-intimal proliferation of smooth muscle cells. These lesions resemble cardiac allograft vasculopathy (CAV) in human coronary arteries. In order to test the role of CD4 T-cells in orchestrating the acute interstitial infiltrates and the chronic macrophage rich cardiac allograft vasculopathy, male C57BL/6 hearts were transplanted into female B6 Rag1-/- mice, one week later recipients were injected intraperitoneally with purified CD4 T-cells isolated from female Marilyn CD4 T-cell receptor transgenic mice, in which all T-cells are specific for male minor transplantation antigen. This resulted in macrophage-rich interstitial infiltrates at 2 weeks and CAV at 6 weeks suggesting that CD4 T-cells are responsible for orchestrating acute as well as chronic rejection. To identify local mediators that cause this evolution of compartmentalization, we separated interstitial and arterial infiltrates by laser capture microdissection. RNA was isolated using an RNeasy Micro kit followed by cDNA preparation and preamplification of cDNA using RT2 PreAMP cDNA synthesis kit (QIAGEN). A customized PCR array kit (QIAGEN) was used to screen expressions of 86 genes by real time RT PCR. Among the most highly upregulated genes in the interstitium at 2 weeks were CD274 (PDL1) and a set of IFNg regulated genes: CXCL9 (MIG), CCL5 (RANTES) and CCL2 (MCP-1). By 6 weeks, the expression of these genes diminished together with the interstitial macrophage and T cell infiltrates. This pattern of gene expression correlated with intragraft protein levels as measured by ELISA. The upregulation of PDL1 in the interstitium at 2 weeks suggested that negative co-stimulatory signaling through PD1 on T cells might contribute to the resolution of acute interstitial infiltrates. Therefore, we blocked PD1:PDL1 interaction by 3 doses of 200µg of purified IgG2a rat monoclonal antibody to PDL1 (10F.9G2) or 200µg of isotype control (LTG-2) antibody (from BioXCell) in the second or sixth week after transplantation. Blocking PD1:PDL1 in the acute phase increased interstitial infiltrates leading to complete rejection of 2 of 4 cardiac allografts by 2 weeks. Delayed blocking of PD1:PDL1 increased interstitial infiltrates moderately, but did not alter arteriopathy at 6 weeks. A different set of genes was upregulated in the arterial compartment: IL1R2, IL1R1, TLR6 and TRAF6 at 2 weeks and TLR6, MyD88, CCR3, CCL5, CXCL9 and CCL1 at 6 weeks. The early sparing of arteries may be in part due to IL1R2 expression, which can compete with IL1R1 for binding IL-1. The expression of CCR3, CCL5, CXCL9 and CCL1 support the T cell dependency of the macrophage-rich arterial lesions. TLR6 and MyD88 indicate a possible role for endogenous or exogenous molecular pattern recognition in the development of arteriopathy.
Overview: Administration of IL-33 triples the survival of fully MHC-mismatched murine heterotopic cardiac allografts in the absence of any immunosuppression (mean survival time = 30 vs. 9 days). Although regulatory T cells are critical to the therapeutic benefit of IL-33 in this stringent model, these experimental heart transplant data are consistent with earlier identified direct cardiovascular protective properties of IL-33 and its receptor, ST2. Specifically, IL-33 promotes survival following myocardial infarction through ST2-dependent protection of cardiac myocytes. Likewise, IL-33 reduces the development of atherosclerosis. However, the impact that allograft expression of ST2 has on heart transplant outcome, particularly the development of allograft vasculopathy (AV), was undefined.

Methods: To assess the role of ST2 on the development of chronic AV, minor mismatched male wild type (WT) or St2-/- BALB/c (H-2d) vascularized heterotopic hearts were transplanted (HTx) into WT BALB/c females. WT BALB/c female hearts were transplanted into female WT BALB/c recipients as controls. Transplants were assessed daily by palpation and rejection defined as cessation of detectable HTx contraction. In separate experiments, WT or St2-/- BALB/c aortic allografts were transplanted (ATx) into WT C57BL/6 (B6; H-2b) recipients. Additional B6 mice received syngeneic control grafts. At post-operative day (POD) 28 (ATx) or 100 (HTx), transplants were explanted and assessed following H&E, Masson’s trichrome, or Verhoeff-van Gieson elastic staining. To quantitate infiltrating CD3+ cells, immunohistochemical (IHC) staining of transplant sections was also completed. Whole slide images of histology and IHC slides were generated by scanning with a Mirax MIDI and captured with Panoramic Viewer. The WSI analysis program Image Analysis Environment was used to quantify T cell infiltrate and calculate ATx percentage of luminal occlusion.

Results: Both BALB/c WT and St2-/- male HTx into female BALB/c exhibited long-term survival (survival >100 days). However, at 100 POD, St2-/- HTx grafts display increased CD3+ cell infiltrate and exhibit indications of augmented development of AV. Relatively, compared to WT BALB/c ATx, St2-/- ATx displayed profound intimal proliferation, deposition of connective tissue, and obvious luminal narrowing. As such, the St2-/- ATx group exhibited a significantly increased percentage of luminal occlusion.

Conclusions. The absence of ST2 in both HTx and ATx increased immune infiltration and accelerated development of AV. Thus, our data identify an unappreciated direct protective role for local IL-33 and graft ST2 following transplant of cardiovascular tissue.
Abstract #22

Increased Serum sST2 And Graft IL-33 Identify Pediatric Heart Recipients Suffering Rejection

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Purpose: IL-33, a cytokine that signals through ST2, reduces pathology and improves outcomes in rodent models of cardiac transplantation and hypertrophy. ST2 exists as both a transmembrane form (ST2L) and an antagonistic soluble form (sST2). In rejecting mouse heterotopic cardiac allografts, IL-33 and ST2 is upregulated. After acute myocardial infarction, high levels of serum sST2 indicate severe damage. We extended the above observations in testing the hypothesis that IL-33 or ST2 is upregulated in pediatric cardiac transplant (Tx) patients during rejection episodes and can be utilized as a rejection biomarkers.

Methods: Based on review of clinical records, pediatric heart Tx patients were divided into groups (n=8-12) consisting of: 1. non-rejecting (NR) patients, which lacked circulating donor-specific alloantibodies (DSA) and did not have a C4d⁺ EMB or >2 ISHLT score, 2. patients suffering episodes of acute cellular rejection (ACR; >2 score) or 3. exhibiting a history of AMR (DSA⁺ and/or C4d⁺ EMB). Study material was archived EMB and serum samples and the project was approved by the IRB of the University of Pittsburgh. Quantum (Q) dot-based, multicolor immunostaining of EMB for CD4, CD31, IL-33 and ST2L was completed. Labeled slides were scanned with a Mirax MIDI camera system and image analysis programs used to quantify fluorescent intensity in each color channel. Commercial ELISA kits were used to quantitate serum sST2. Normal levels for these measures were established on samples from normal, non-transplanted controls.

Results: Serum levels of sST2 were significantly (p<0.001) increased in ACR and AMR patients compared to the NR group or normal controls. Furthermore, we find distinctly modulated express of IL-33 and ST2L in EMB of patients with a history of ACR or AMR. ACR grouped patient displayed ST2L in cardiac myocytes and endothelium and exhibit weak nuclear IL-33 expression. Whereas, AMR biopsies display greatly increased nuclear IL-33 and little ST2L. Stable, NR patients EMB display little IL-33 or ST2L.

Conclusions: These data provide the first evidence that both IL-33 and ST2L are modulated in clinical Tx samples. Further examinations are warranted assessing if serum sST2 or EMB staining for IL-33 and ST2L can facilitate pediatric heart Tx patient rejection diagnosis and treatment.
**Abstract #23**

**CD4 T cells induce recovery of CD8 T cell in heart allograft recipients treated with antithymocyte globulin**

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Antithymocyte globulin (ATG) is a lymphoablative agent used in transplant patients. However, ATG treated recipients eventually reconstitute their T cell repertoire, develop anti-donor immune responses and either reject transplanted organ acutely or develop signs of chronic graft injury. We used a mouse model of heterotopic cardiac transplantation to investigate the mechanisms of homeostatic and antigen driven recovery of T cells after depletion with a murine analog of antithymocyte globulin, mATG.

Following mATG administration, the remaining T cells are mainly composed of effector/memory CD4 T cells. Additional administration of depleting anti-CD4 monoclonal antibodies prior to mATG treatment eliminates up to 99.9% of residual CD4 T cells. Heart allograft recipients depleted of CD4 T cells and treated with mATG demonstrated significantly lower recovery of CD8 T cells by 10 days posttransplant in comparison to mATG monotherapy. The inhibition of CD8 T cell recovery was observed in CD4 depleted and genetically CD4-deficient recipients of iso- and allografts as well as in non-transplanted mice treated with mATG. These results suggest that the impairment in the recovery of CD8 T cells observed in the absence of CD4 T cells is independent of antigen driven proliferation or surgery-induced inflammation. The injection of blocking anti-CD154 antibody significantly impaired CD8 T cell recovery and prolonged heart allograft survival in mATG treated recipients. Conversely, treatment with agonistic anti-CD40 antibody rescued expansion of CD8 T cells in mATG treated CD4 KO recipients. Thus, CD40/CD154 signaling was necessary and sufficient for CD8 T cell recovery driven by CD4 T cells. Our results demonstrate that residual CD4 T cells provide CD40-dependent helper signals for the expansion of both donor-specific and non-specific CD8 T cells following lymphoablation with mATG. Interfering with helper functions of CD4 T cells should improve the efficiency of ATG induction therapy in sensitized transplant recipients.
IFNγ production by donor-reactive memory CD4 T cells is required for the induction of pathogenic alloantibody in CD40-independent manner

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Donor-reactive memory CD4 T cells are potent inducers of pathogenic alloantibody (alloAb). We have previously reported that Th1 and Th17, but not Th2 memory CD4 T cells induce high titers of anti-donor alloAb in the absence of the CD40/CD154 pathway.

The goal of this study was to determine the cytokine requirements for CD40-independent help by memory CD4 T cells. TCR transgenic Mar T cells recognizing male antigen HY_Dby plus I-A^b were activated in vitro and polarized toward Th1, Th2 and Th17 phenotypes with cytokines and cytokine neutralizing Abs. The resulting cells were injected into CD40-/- B6 (H-2^b) female mice followed by BALB/c (H-2^d) male heart allograft transplantation three weeks later. Th1, Th17 and unpolarized memory Mar cells elicited comparable titers of anti-donor Ab while Th2 memory Mar cells failed to induce IgG alloAb in CD40-/- recipients. However, the resulting anti-donor Abs differed in their specificity and the ability to mediate heart allograft pathology. Th17 memory CD4 T cells induced inferior Ab responses against donor MHC class I molecules compared to Th1 and unpolarized memory CD4 T cell subsets. Furthermore, while passive transfer of anti-donor sera induced by either unpolarized or Th1 memory Mar cells into RAG2 -/- recipients of BALB/c male heart allografts caused diffuse C4d deposition in the graft capillaries, transfer of sera from recipients containing Th17 memory cells did not lead to capillary C4d deposition.

We next tested whether IFNγ is required for CD40-independent helper functions of memory CD4 T cells. Administration of blocking anti-IFNγ Ab to CD40-/- heart allograft recipients containing unpolarized memory Mar T cells drastically reduced serum IgG alloAb titers and prevented generation of donor MHC class I-specific antibody secreting cells. In contrast, donor-reactive CD8 T cells were efficiently activated under these conditions indicating that, in the absence of CD40/CD154 interactions, memory CD4 T cells require IFNγ in order to provide help to B cells but that the help for CD8 T cells is IFNγ-independent.

Our data suggest that IFNγ secreted by pre-existing donor-reactive memory CD4 T cells not only determines donor-reactive Ab isotypes, but also influences alloAb specificity and can thus affect allograft pathology. This information may be valuable for identifying transplant patients with a higher risk for developing de novo pathogenic alloAb.
Abstract #25

Paradoxically Rapid Islet Allograft Rejection in Autoimmune NOD Recipients

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Type 1 diabetes (T1D) results in hyperglycemia due to an autoimmune attack on insulin-producing β cells within the pancreatic islets. Islet allografts can restore euglycemia in T1D patients, but such grafts have not demonstrated long-term survival. It is unknown whether autoimmune memory T cells, alloreactive T cells, or some combination constitute the primary response to islet allografts in such autoimmune recipients. Similar to humans, non-obese diabetic (NOD) mice develop insulitis and β cell-directed autoimmune destruction requiring both T and B cells. In this study we tested the hypothesis that the primary acute response to islet transplants in diabetic NOD recipients requires donor MHC H-2g7 expression as targets of self-MHC-restricted, autoreactive (islet-specific) T cells. Autoimmunity alone is sufficient to destroy islet transplants since diabetic NOD recipients reject autologous NODscid islets within 10-15 days post transplant. Donor islets from congenic strains that are MHC-matched (H-2g7) but minor histocompatibility mismatched (BALB.H-2g7 or B6.H-2g7) are also acutely destroyed in NOD recipients, confirming that MHC matching does not benefit islet transplant survival in the presence of autoimmunity. Importantly, this autoimmune recognition requires islet graft MHC expression since most NOD MHC-deficient (β2M-/- + C2ta-/- double-deficient) islet grafts enjoy dramatically prolonged survival (6/7 grafts > 60 days) in diabetic NOD recipients. This latter result strongly suggests that the acute response to islet grafts in diabetic NOD recipients is due to a direct, H-2g7-restricted targeting of the transplant. If so, then MHC-disparate islet allografts should be less sensitive to this acute response. However, C57Bl/6 (B6, H-2b) islet allografts are very rapidly rejected within 5-8 days in diabetic NOD recipients, a tempo that is actually accelerated relative to the rejection of NOD islet autografts. Moreover, whereas published studies (including our own) indicate that both islet autograft and allograft rejection in NOD mice is CD4 T cell dependent, we find that MHC class II-deficient B6 allografts are acutely destroyed in diabetic NOD recipients. Ex vivo analysis of T cells infiltrating rejected islet grafts at the time of graft destruction indicates the immune infiltrate of both NOD autograft and B6 allograft rejection are comprised primarily of activated CD4+ and CD8+ T cells, most of which produce both IFN-γ and TNF-α.

Conclusion: Results suggest that the nature of islet autograft and allograft recognition differ in NOD recipients, especially regarding the role of donor MHC expression. While MHC H-2g7 expression is essential for acute autoimmune-mediated autograft rejection, allografts initiate a vigorous response that does not require H-2g7 expression by the transplant. Thus, although diabetic NOD mice have an existing memory response to islet autoantigens, they also demonstrate a marked acceleration in their response to MHC-mismatched islet allografts despite a lack of prior exposure to allogeneic MHC molecules.
Dual Nanoporous Encapsulation and Local Drug Delivery for Pancreatic Islet Cell Transplantation

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Type I diabetes mellitus (TIDM) is a common disease of both humans and animals that requires complex, life-long management. Transplantation of pancreatic islets is recognized as a potential curative therapy for TIDM; however, islets are extremely susceptible to the mechanical, hypoxic and immunologic stresses associated with transplantation, causing a high rate of failure. We are evaluating a novel technique to improve islet cell survival, using dual nanoporous encapsulation and local delivery of the incretin hormone mimetic, exenatide, a clinically approved drug known to promote islet survival. To achieve dual encapsulation, the islets are microencapsulated with alginate-poly-L-lysine using an electrospray method, then placed inside a nanoporous device. Initial in-vitro studies showed that microencapsulation improved islet survival and insulin production over a 10 day period. Further studies were performed to titrate drug diffusion through the nanoporous membrane, demonstrating that a 10nM pore size is ideal for achieving local therapeutic concentrations of exenatide. While polyethylene glycol coating of the membrane appears to improve biocompatibility, this technique clogs the pores in the membrane, preventing drug diffusion. Having refined the implant design, we are now testing the effect of dual encapsulation and local drug delivery on islet viability and function in-vitro using three study groups: (I) unencapsulated islets (control), (II) dual encapsulated islets, (III) dual encapsulated islets + exenatide. Serial assessment of insulin production will be performed over a 10 day period of islet culture. At the conclusion of the experiment, islet viability will be compared using dual fluorescent (live/dead) and epifluorescence microscopy. Based on our preliminary data, we expect that dual encapsulation and local drug delivery will improve islet viability and function when compared to control groups. Future plans include the implementation of dual encapsulated islets and exenatide for in-vivo studies in mice and dogs, with the ultimate goal of curing human TIDM.
The Impact of Hyperglycemia and Metabolic Stress on Allograft Immunity

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Background: Though most studies on the pathogenesis of Type 1 diabetes focus on the autoimmune contribution to disease, recent evidence indicates that the excessive metabolic demand of hyperglycemia itself can induce inflammation in islet beta-cells independently of an adaptive immune response. Clinically, recipient hyperglycemia also has been correlated with increased incidence of acute rejection of kidney, liver, and heart transplants but its causal role has not been established. However, how hyperglycemia itself directly impacts the adaptive immune response is largely unknown.

Hypothesis: Hyperglycemia and associated metabolic distress exacerbate the allograft response leading to accelerated graft rejection and inhibited transplant tolerance induction.

Approach: In this study, we determined the impact of extreme host hyperglycemia on: 1) alloantigen-specific CD8+ T cell reactivity in vivo, 2) the tempo of primary acute islet allograft rejection and 3) the impact on induction of tolerance to islet allografts. Severe hyperglycemia was modeled in C57Bl/6 (B6) Ins2<sup>akita</sup> mice (Ak). Ak mice express a mutant insulin-2 gene that dominantly suppresses insulin secretion causing stable, severe hyperglycemia. For assessing alloantigen-specific T cell responses, congenic bulk B6 CD45.1 spleen and lymph node cells were transferred into wild-type and Ak B6 recipients (both CD45.2). Recipient mice were then challenged with allogeneic (BALB/c) spleen cells in the hind foot pad and the response of transferred CD45.1+CD8+ T cells was measured in the draining popliteal lymph node day 5 after challenge. For assessing the tempo of primary acute islet allograft rejection and for tolerance experiments, BALB/c islet allografts were grafted into Ak and B6 mice rendered acutely (and less severely) diabetic by the beta-cell toxin streptozotocin (SZ-B6). Monoclonal antibody therapy using α-LFA-1 and α-CD154 was used to induce allograft tolerance.

Results: Pilot studies indicated modest increases in the activation of CD45.1+CD8+ B6 responder cells after allo footpad challenge in Ak versus non-diabetic B6 mice. However, Ak mice rejected islet allografts significantly more rapidly than SzB6 mice (mean of 8.7 days versus 12.9 days, respectively; p=.003). Moreover, Ak recipients were markedly resistant to tolerance induction relative to conventional diabetic SZ-B6 recipients (0/6 versus 9/10 with long-term allograft survival, respectively,
Innate immune stimulation contributes to T-cell resistance to tolerance and pancreatic islet allograft rejection

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Pancreatic islet transplantation can restore normal glucose regulation in type 1 diabetic recipients, resulting in reduced acute and chronic morbidity associated with the disease. However, immune-mediated rejection represents a major barrier to the success of islet allografts. As such, inducing specific immunological tolerance is a major goal in the transplant field. Previous studies show that short-term treatment with monoclonal antibodies targeting the costimulatory molecule CD154 and/or the adhesion molecule CD11a (LFA-1) can induce long-term, donor-specific tolerance. However, many studies also show that microbial infections can disrupt this tolerance process and trigger allograft rejection. This inhibition of tolerance has been associated both with general innate immune stimulation driven by pathogen-associated molecular patterns (PAMPs), and with the generation of memory T cells that may also interfere with tolerance induction. This study sets out to determine the relative contribution of innate (e.g. TLR stimulation) versus adaptive (memory T-cell generation) immunity to the disruption of induced allograft tolerance. We model pathogen exposure through host immunization with intact ovalbumin (OVA) and an adjuvant comprised of poly I:C (a mimic of viral double-stranded RNA) and agonistic anti-CD40 antibody treatment. We find that peri-transplant (day 2 after grafting) treatment with either poly I:C/anti-CD40 or poly I:C alone triggers rapid islet allograft rejection despite recipient treatment with either anti-CD154 or anti-LFA-1 therapies. These results indicate that innate immune stimulation alone, without T cell-dependent immunity to pathogen-derived antigens, can inhibit early allograft tolerance induction. Importantly, this disruption of tolerance is time-dependent. Early (day 2 post-transplant) but not late (day 60) adjuvant treatment is effective at preventing tolerance. Thus, it appears that immune stimulation impairs the early outcome of graft-reactive T/B cells, inducing immunity rather than tolerance following treatment with tolerance-promoting agents. We are currently determining the direct impact of peri-transplant, non-specific innate stimulation on the fate of defined, antigen-specific T cells during early tolerance induction in vivo.
Donor management: IL-1 Receptor Antagonist as a Strategy to Attenuate Inflammation in the Pancreas and Islets of Brain Dead Non-Human Primates

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Introduction: Most pancreas and islet grafts are recovered from donors after brain death (BD). BD is known to compromise organ quality through activation of systemic inflammation, partly due to multiple hemodynamic, neuro-hormonal and inflammatory events associated with severe irreversible neurological injury. Prevention of the deleterious effects associated with BD may mitigate islet loss and improve islet transplant engraftment and survival. In this study we characterized early innate immune activation and the inflammatory response induced by BD in pancreata/islets in a hemodynamically controlled non-human primate (NHP) model. We hypothesized that targeting the IL-1 pathway using a specific receptor antagonist may decrease immune activation and increase pancreas/islet quality and function.

Methods: Ten BD NHP were maintained hemodynamically stable for 6h after BD induction then divided into 2 groups [Group 1: standard donor management, no additional donor pre-treatment (n=5); Group 2: standard donor management+donor pre-treatment with IL-1ra 10 mg/kg body weight (n=5)]. Five animals that did not have BD induction served as controls (Group 3). Flow cytometry was used to characterize the migratory pattern and level of activation of peripheral blood leukocyte before and 6h after BD induction. Intra-islet neutrophils, macrophages, myeloid dendritic and endothelial cell number and level of activation (IL-1, TNF, MCP-1, MIP-1 and IL-8) were also evaluated. Gene expression at the pancreas level and ELISA were performed to measure pro-inflammatory cytokine and chemokine expression in all groups. Immunohistochemistry was used to assess tissue infiltration by innate immune cells. Characterization of islet viability and function in vitro and in vivo were determined by flow cytometry and transplant into the kidney capsule of immunodeficient animals.

Results: IL-1ra reduced BD-induced release and activation of neutrophils and monocytes from bone marrow and reduced emigration of myeloid dendritic cells to end organs. IL-1ra also prevented accumulation of CD45+ and CD68+ leukocytes occurring within the pancreas after BD induction. Pre-treatment with IL-1ra significantly decreased the level of activation of microvascular endothelial cells within the islets (reduction of MCP-1, MPI-1, IL-8 and IL-1β expression), also reducing the activation state of neutrophils and macrophages (decreased expression of CxCR1, CXCR2, CCR6 and CCR7 and reduction of IL-1β and MCP-1 expression). A complete blockade of myeloid dendritic cell migration was also observed after IL-1ra treatment. As a result, IL-1ra dramatically reduced the apoptosis rate induced by BD and significantly improved islet mitochondrial membrane polarity, with a reduction of time to cure when islets were transplanted under the kidney capsule in STZ-diabetic NOD-scid mice.

Conclusions: Treatment with IL-1ra prevented chemokine secretion by the endothelium and pro-inflammatory cytokine release by circulating and tissue monocytes and DC’s. Intra-islet endothelial cells, neutrophils, macrophages and dendritic cells displayed a heightened state of activation following BD that is significantly attenuated by donor pretreatment with IL-1ra. Together, these results suggest that donor treatment with IL-1ra may represent a new therapeutic strategy to improve islet quality and function prior to transplantation through the modulation of the innate immune response.
T cell epitope analysis of autoimmunity to the α1 chain of collagen type V

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After lung transplantation, the development of chronic rejection in the form of obliterative bronchiolitis (OB) is the major cause of graft loss and death. We have previously reported that Th17 cellular immune responses to collagen type V (ColV) are strongly associated with development of bronchiolitis obliterans syndrome (BOS), the clinical correlate of OB, following lung transplantation. Recently we have shown an association between a cellular immune response to ColV and coronary artery disease (CAD), another fibro-obliterative disease. The analysis of these Th17 autoimmune responses at the single-epitope level will be addressed. Examining immune responses to ColV before and after lung transplant and correlating responses to patient and donor MHC class II haplotypes, we found that 30% of the patients exhibited an immune response to ColV pre-transplant and more than half of these expressed HLA-DR-15 (p=0.02, compared to the frequency of DR15 in the lung transplant population) and patients with HLA-DR1 and DR17 developed responses post-transplant (p=0.04 and 0.01, respectively). Interestingly, patients with HLA-DR17 that developed immune responses to ColV post-transplant rarely developed BOS while those patients who received a lung from a donor that was HLA-DR15 were at significant risk of developing BOS (p=0.03). Using the services of ProImmune, Inc, we generated a peptide library of the triple helical region of the α1ColV protein and screened these peptides for binding to the HLA-DR1, 15, 17 and associated DQ molecules, HLA-DQ2 and 6 in addition to the mouse MHC class II I-Ab. The activity of the peptides identified by the ProImmune binding assay as well as those identified by website based algorithms, such as RANKpep, were then used in a trans-vivo delayed type hypersensitivity (tvDTH) assay and in vitro culture followed by ELISPOT to determine if patients or mice reactive to the whole ColV molecule respond to the identified peptides. From our results, it is clear that the algorithm predicted peptides did not elicit immune responses in ColV reactive patients or mice comparable to that obtained with the intact ColV. However, HLA-DR1/15 binding peptides identified by Proimmune, p799 and p1439, both elicit tvDTH swelling responses in HLA-DR1- or DR15-expressing ColV reactive patients, but not other MHC class II-typed patients. With the characterization of these epitopes we can now generate research tools to further examine and track the T cell response to ColV and its role in fibro-obliterative diseases.
Increased inflammation and fibrosis in MLK-3 deficient mice in response to acute lung injury induced by bleomycin

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Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease of unknown etiology that responds poorly to currently available pharmacologic therapies and usually progresses to respiratory failure and death. The pathogenesis of IPF is characterized by progressive, multifocal fibrosis that diffusely distorts lung architecture due to extensive remodeling of the lung parenchyma and obliteration of gas exchange surfaces. Because the c-Jun HN2-terminal kinase (JNK) gene is upregulated in pulmonary fibrosis and controlled by TGF-b and JNK inhibition of JNK has a wide range of biological effects, we investigated the role of the upstream kinase, mixed lineage kinase-3 (MLK3), on inflammatory and fibrotic responses to bleomycin.

Bleomycin (0.04 U per mouse) was instilled intratracheally into C57Bl/6 wild-type and age matched MLK3 knockout mice. Lungs were excised and analyzed via immunohisto-chemistry for the expression of collagen, presence of macrophages and myofibroblasts, presence of IκBα. Levels of the pro- and anti-apoptotic proteins, Bax and Bcl2 respectively, were determined via western blotting, and the cellular composition in the lung was analyzed by flow cytometry. Additionally, lung tissues from patients with IPF were analyzed for JNK phosphorylation.

JNK phosphorylation was increased in human IPF lungs. In mouse lungs, tissue inflammation as well as MLK3 and JNK phosphorylation were increased one week after bleomycin instillation as compared to saline controls. In contrast, MLK3 deficient mice showed reduced JNK phosphorylation but increased inflammation and decreased concentration of IκBα compared to wild-type mice at the same time point. The presence of cleaved caspase 3, Bax to Bcl2 expression ratio, and PARP expression indicated that cellular apoptosis was reduced in MLK3 knockout as compared to wild-type mice. In addition, the number of macrophages and myofibroblasts was higher in MLK3 knockout as compared to wild-type mice. In contrast, epithelial apoptosis at day 1 after bleomycin instillation was not different between the two groups. In conclusion, the MLK3 deficient mice showed significantly less apoptosis following bleomycin-induced lung injury, but lung inflammation and fibrosis was increased, suggesting that MLK3 activation is required to reduce inflammation by inhibiting NFkB activation, which may be linked to the effects of MLK3 on cellular apoptosis.
Th17 cells are not necessary for cellular rejection after orthotopic mouse lung transplant

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Obliterative bronchiolitis (OB) in the lungs is characterized by immune mediated fibrous obliteration of airways and has been a major impediment to long term survival in lung transplant recipients. To understand the mechanisms involved in development of OB, we use a mouse model of minor MHC mismatch orthotopic lung transplantation, which develops reproducible obliteration of the airways similar to the lesions in humans. Previous studies in our model have indicated that IL-17 contributes to OB and its blockade prevents cellular rejection, airway fibrosis and obliteration in allografts. In the present study, we hypothesized that IL-17 producing CD4+ T-cells (Th17) may be one of the main inducers of rejection and OB in the mouse model. The left lungs from minor histocompatibility Ag system (mHA) mismatched C57Bl/10 mice were transplanted orthotopically into C57Bl/6 mice and lung cells were evaluated by flow cytometry for infiltration of T-cells and IL-17 production at different time-points after lung transplant. The immune response after transplant is characterized by increased infiltration of CD4+ and CD8+ T-cells at 14 days after lung transplant in allografts. In contrast, IL-17A+ T cells are minimal at early time-points but are increased by day 21 in lung allografts. Interestingly, IL-17A was found to be produced by CD4+ T-cells (Th17) and γδ+ T-cells, and these IL-17A+ T-cells were higher in allografts than syngeneic grafts on day 21. Consistent with molecular findings, allografts developed cellular rejection by day 14 after transplant and showed evidence of OB at day 21 as evaluated by lung histology. To, further, test the hypothesis that IL17A+ CD4+ T-cells (Th17) are required for OB development, left lungs were transplanted from C57Bl/10 mice to C57Bl/6 CD4.Cre STAT3 fl/fl mice which cannot differentiate CD4+ T-cells in to IL-17A producing T-cells. Contrary to expectations, CD4.Cre STAT3 fl/fl allografts developed cellular rejection on day 21 after transplantation as well as fibrosis that was similar to control allografts. However, none of the Cd4.Cre STAT3 fl/fl allografts developed OB at this time-point. Although not significant, Real Time PCR showed a trend towards increased mRNA expression of IL-6 and TGF-β, which are required for Th17 differentiation, in CD4.Cre STAT3 fl/fl allografts as compared to control allografts, however, IL-17A remained undetectable on day 21 in both groups. Our data suggest that Th17 cells are not necessary for cellular rejection and fibrosis in our model, but may be required for the development of OB lesions.

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Environmental Pollutants Enhance Th17 Deviation in an AHR Dependent Manner

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The Aryl Hydrocarbon Receptor (AHR) is a cytosolic transcription factor with numerous xenobiotic and endogenous ligands, most notably TCDD. Recent evidence indicates that T-cell differentiation can be affected by Ahr activation. Air pollutants contain significant amounts of polycyclic aromatic hydrocarbons (PAHs), a class of compounds known to be Ahr ligands. The current study examined the effects of exposing T cells to known quantities of a standardized lot of air-derived particulate matter (Urban Dust Particles; UDP) containing known amounts of PAHs. We hypothesize that activation of the AHR by these toxicants leads to increased Th17 cell differentiation and activity.

Methods: To assess the effect of UDP on T-cell differentiation, naïve CD4+ murine T-cells were plated in Th17 polarizing conditions (TGF-β, IL-6) and stimulated with CD3/CD28 beads for 3 days with varying concentrations of UDP. Alternatively, varying concentrations of UDP were added to a standard MLR. In both experiments, changes in T cell differentiation were monitored by qRT-PCR and measurement of cytokine levels in the culture supernatant. In addition, B6 mice were given UDP intranasally over a 1 week period. The lung was harvested at the time of sacrifice and cytokine mRNA was measured by qRT-PCR.

Results: Differentiation of naive CD4 T cells from B6 mice in presence of UDP under TH17 conditions resulted in increased expression of IL-17 and IL-22 by both RT-PCR and ELISA. Comparable experiments done with AHR null mice demonstrated no significant change in cytokine expression with UDP addition. Both syngenic and allogenic MLRs cultured with UDP resulted in increased TNFα demonstrating an antigen non-specific effect of UDP. Only allogenic cultures presented with increased IL-2, IL-17, IL-22 and INFγ in cultures containing UDP. Intranasal administration of UDP resulted in increased IL-17 expression.

Conclusions: These preliminary experiments suggest that PAH-containing UDP can alter T cell differentiation and function toward a TH17 phenotype. This finding suggests a mechanism via which air pollutants may alter T cells responses and effect clinical outcome.
CARMA1 in T-cells is required for experimental autoimmune encephalomyelitis but not for skin allograft rejection

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Multiple sclerosis is a neurodegenerative disease resulting from immune insult by IL-17-expressing CD4 T cells in the central nervous system (CNS). The transcription factor NF-κB is required for T cell proliferation and survival. Stimulation through the T cell receptor (TCR) activates NF-κB through the adaptor molecule CARMA1. In vitro, we observed that CARMA1 is required for expression of the Th17 effector molecules IL-17A, IL-17F, IL-21, IL-22, IL-23R and CCR6, but not for the Th17-associated transcription factors RORγ, AhR and IRF4. In vivo, CARMA1-KO mice immunized with MOG$_{35-55}$ peptide (MOGp) were resistant to development of experimental autoimmune encephalomyelitis (EAE), while they rejected fully mismatched skin allografts. As analyzed by ELISpots, MOGp immunization failed to generate MOG-specific IL-17 producing splenocytes, while skin allograft promoted a robust IFNγ alloresponse. Analysis of the mononuclear CNS-infiltrating cells in MOG-immunized mice demonstrated that CARMA1 is required for T cell infiltration. Dendritic cells are professional antigen presenting cells involved in T cell activation, raising the possibility that CARMA1-KO DCs were not able to activate T cells. Interestingly, CARMA1-KO DCs had no defects in upregulation of costimulatory molecules upon LPS stimulation, or in antigen presentation to WT T cells. More importantly, CARMA1-KO mice adoptively transferred with 2D2 TCR-transgenic WT T cells and immunized with MOGp developed EAE, suggesting that the defect in EAE generation in CARMA1-KO mice is T cell-intrinsic. Together, our results indicate that CARMA1 is required for Th17 but not Th1 responses in vivo and point to a potential therapeutic target for Th17-dependent diseases.
Role of commensal microbiota in skin allograft rejection

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Skin transplants, along with transplants of intestine and lung, are notoriously difficult to tolerize. We hypothesize that the commensal bacteria associated with each of these tissues contribute to their greater immunogenicity than sterile organs. However, it is unclear if microbe-associated molecular patterns are absolutely required to precipitate rejection of skin grafts, and how these molecules, encountered physiologically, influence the quality or tempo of rejection. Previous studies by us and others showed that MyD88-dependent signaling through Toll-like receptors (TLRs) is required to cause rejection in both a minor mismatched skin transplantation model and in a major mismatched model with immunosuppression by costimulation blockade. Moreover, a multitude of recent studies have demonstrated that the intestinal microbiota influences systemic autoimmunity and response to infection. Bacterial molecules, in the intestine or locally at the transplant site, could therefore play an important role in the rejection of skin grafts. Our preliminary results show that altering the intestinal microbiota by oral antibiotic treatment tended to attenuate rejection of male skin by female recipients, though the effect was not statistically significant. Additionally, antibiotic treatment targeting the skin microbiota significantly slowed rejection of BALB/c skin by C57Bl/6 mice treated with costimulation blockade. These experiments suggest that both intestinal and skin bacteria can be influential in rejection of skin grafts. Future plans include carrying out the same 2 transplant models in germ free mice, to establish if the complete absence of live bacteria can prevent rejection of minor mismatched skin grafts and facilitate anti-CD154-mediated tolerance of fully mismatched skin transplants. We also plan to colonize germ free mice with different defined skin or gut microbes to narrow down the contribution of different bacteria to rejection. This study will be important in understanding the requirements for achieving transplantation tolerance, particularly in organs associated with microbial communities.
Abstract #36

Cytokine Profiles in Patients with *Staphylococcus Aureus* Skin Infections

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Our previous studies have shown that bacterial infections and microbial products sensed at the time of transplantation can prevent the induction of cardiac transplantation tolerance by costimulation-blockade therapies in mice. *Staphylococcus aureus* (*S.* aureus*)-mediated rejection was dependent on IL-6 signaling, which is a necessary cytokine for Th17 differentiation, and CpG-induced rejection was prevented by the combined blockade of IL-6 and IL-17. These results suggest that *S.* aureus infections may promote Th17 differentiation that can interfere with transplantation tolerance and have prompted us to study the immune responses occurring in humans in response to *S.* aureus infections.

Methicillin-resistant *S.* aureus (MRSA) causes skin infections in epidemic proportions among otherwise healthy individuals. Although the importance of antibodies in combatting the infection is appreciated, the role of T cells in anti-*S.* aureus responses is less well-defined. Several lines of evidence suggest that Th17 cells may be crucial to prevent localized *S.* aureus infections, as they occur in patients deficient in STAT3 or mice lacking IL-17A and F. In this study, we tested the hypothesis that patients presenting to the emergency room (ER) with skin infections but no known genetic deficiency may have a reduction in Th17 differentiation, either globally, or specifically to *S.* aureus antigens because of inhibitory factors produced by MRSA strains. Blood samples from 71 patients presenting to the ER with skin infections, and 142 controls were stimulated with MRSA lysates, or with the T cell mitogen OKT3, and supernatants were collected at 24h for detection of cytokines by ELISA. Upon stimulation with MRSA lysates, PBMCs of infected patients displayed increased production of IL-1β and IL-6, innate cytokines that can drive Th17 differentiation. While production of IFNγ and IL-17 in response to OKT3 was similar in patients and controls, secretion of IL17 but not of IFNγ upon stimulation with MRSA lysates was decreased in patients compared to controls. These data suggest that our otherwise healthy ER patients with skin infections do not have a global defect in Th17 differentiation but may have a specific reduction in acquisition of IL-17 in response to *S.* aureus antigens. Future experiments will determine whether factors produced by MRSA can antagonize Th17 differentiation.
Forcing NF-kB in T cells promotes tumor rejection

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T cells play an important role in the elimination of tumors. Tumor-specific T cells can be found in cancer patients despite tumor growth. However, in tumor-bearing hosts, tumor-specific T cells can have reduced viability, be intrinsically anergized, extrinsically suppressed, or lack sufficient effector function to successfully reject tumors. Therapeutic strategies aimed at promoting T cell survival and amplifying T cell differentiation/effector function would be extremely desirable as novel cancer therapies. NF-κB activity has been reported to be reduced in T cells from tumor-bearing hosts. Our previous results indicate that reduced NF-κB activation results in impaired survival of T cells, decreased Th1 and Th17 differentiation and increased iTreg differentiation. Mice with reduced T cell-NF-κB activity fail to reject cardiac and pancreatic islet allografts in the absence of any pharmacological treatment. We hypothesize that forced activation of NF-κB in T cells should have the opposite effect and promote T cell survival, facilitate Th1/Th17 differentiation and prevent iTreg differentiation, which would be beneficial to reject tumors.

We generated mice expressing a constitutively active form of IKKβ (CA-IKKβ) in T cells. Ectopic expression of CA-IKKβ resulted in phosphorylation of NF-κB. Transgene expression was limited to CD4+, CD8+ and NKT cells and T cells showed increased NF-κB activation and nuclear translocation. T cell numbers were comparable to littermate controls, but CA-IKKβ mice had fewer Tregs and increased frequency of activated T cells that produced IFNγ upon re-stimulation. When B16-SIY melanoma cells were injected subcutaneously, tumors grew progressively in control littermates, whereas they were rejected by mice expressing CA-IKKβ in T cells. CA-IKKβ expressing T cells were necessary for tumor control, as shown by antibody-mediated depletion of CD4+ and CD8+ T cells. Furthermore, adoptive transfer of CA-IKKβ-expressing, but not wild-type, T cells into immune-compromised (RAG-deficient) hosts prior to inoculation of tumor cells was sufficient for tumor control. Tumor control was associated with a massive increase in the number of tumor-specific IFNg-producing CD8+ T cells and CA-IKKβ+ CD8+ T cells were able to control tumor growth in the absence of CD4+ T cell help. Interestingly, on the other hand, IKKβ-CA+ CD4+ T cell help was sufficient to induce tumor control by WT CD8+ T cells. Finally, enhanced tumor control was observed in immune-competent mice when fewer than 5% of T cells expressed CA-IKKβ. Our results demonstrate NF-κB to be at the cross-roads of major T cell fate decisions that uniquely synergize for control of tumor growth and may be translatable to the clinic.
Abstract #38

FOXO3 Restricts the CD8 T cell Response to a Chronic Viral Infection

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Over 500 million people in the world are currently afflicted with chronic infections from viruses such as hepatitis B, hepatitis C, and HIV. A common denominator underlying viral persistence in the aforementioned chronic viral infections (CVIs) is the dysregulation of virus-specific T cell responses; virus-specific T cells either undergo clonal deletion or lose their ability to express the full spectrum of effector functions, termed functional exhaustion. During CVIs, there is a continuum of T cell proliferation and apoptosis, and the balance between these cellular processes controls the abundance of virus-specific CD8 T cells. However, the homeostatic mechanisms that control the number of virus-specific T cells under conditions of protracted antigenic stimulation are poorly defined. We have identified the transcription factor FOXO3 as a potential negative regulator of the magnitude and effector function of CD8 T cells during a chronic viral infection in mice. Ablation of FOXO3 in T cells reduced apoptosis, increased the abundance of polyfunctional virus-specific CD8 T cells, and improved viral control. Thus, FOXO3 is a promising candidate target for immunotherapies of chronic viral infection.
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