Induction of Tolerance: Mechanisms and Implications for Clinical Transplantation

by

Wendy Huei-Ping Shyu

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Immunology University of Toronto

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Wendy Huei-Ping Shyu

Master of Science
Graduate Department of Immunology
University of Toronto
2013

Abstract

Therapies that promote tolerance will improve outcomes in solid organ transplantation by eliminating the need for long-term immunosuppression. This thesis investigates two possible tolerance induction mechanisms: rapamycin induced expression of regulatory T cells and re-education of the immune system using syngeneic hematopoietic stem cell transplantation. Fibrinogen-like protein 2, a effector molecule of regulatory T cells, was also determined as a key mediator in the tolerance induction pathway as depletion of fibrinogen-like protein 2 lead to allograft rejection. The feasibility of using syngeneic hematopoietic stem cells for inducing allograft tolerance was studied by setting up a murine heart and bone marrow transplant model. Syngeneic T-depleted bone marrow transplantation resulted in a slight prolongation of the graft survival time compared to the animals reconstituted with total bone marrow cells. We provide compelling evidence to suggest that fibrinogen-like protein 2 and syngeneic hematopoietic stem cells can possibly be used to induce transplantation tolerance.
Acknowledgement

First and foremost, I would like to thank my supervisor Dr. Gary Levy for giving me this great opportunity to work as a master’s student in his lab. His support and guidance helped me to overcome many challenges and barriers I faced as a student and a scientist. I have learnt to work independently and evaluate hypotheses carefully by designing various experiments. Dr. Levy has inspired me to achieve my goal and become a better scientist. I would also like to thank my supervisory committee members, Dr. Michael Ratcliffe and Dr. Li Zhang. Their expertise and advice greatly helped me in completing this work and I really appreciated how they were always approachable and eager to assist. Likewise, I would like to acknowledge many collaborators who contributed to this work through the expert knowledge they brought: Dr. Harold L. Atkins, Dr. M James Phillips, Dr. Oyedele Adeyi, Dr. Reginald Gorczynski, and Dr. David Grant.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A2AR</td>
<td>Adenosine receptor 2A</td>
</tr>
<tr>
<td>ACR</td>
<td>Acute cellular rejection</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC*</td>
<td>Allophyocyanins</td>
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<td>AHR</td>
<td>Acute humoral rejection</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<td>BMCs</td>
<td>Bone marrow cells</td>
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<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
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<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Constant</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
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<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<td>Common lymphoid progenitors</td>
</tr>
<tr>
<td>CMPs</td>
<td>Common myeloid progenitors</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine</td>
</tr>
<tr>
<td>D</td>
<td>Diversity</td>
</tr>
<tr>
<td>DAB</td>
<td>3′,3′-diaminobenzidine</td>
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<td>Dendritic cell(s)</td>
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<td>Double negative</td>
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<td>Constant region fragment</td>
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<td>Fc receptor</td>
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<td>Fibrinogen-like protein 2</td>
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<td>FITC</td>
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<td>FKBP12</td>
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<td>Foxp3</td>
<td>Forkhead box P3</td>
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<td>FRB domain</td>
<td>FKBP12-rapamycin-binding domain</td>
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<td>FRED</td>
<td>Fibrinogen-related domain</td>
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<td>g</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GSK3</td>
<td>Glycogen synthase kinases 3</td>
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<tr>
<td>GZ-B</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>HAR</td>
<td>Hyperacute rejection</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen egg lysozyme</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>HSC(T)</td>
<td>Hematopoietic stem cell (transplantation)</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible costimulator</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2, 3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome</td>
</tr>
<tr>
<td>IVC</td>
<td>inferior vena cava</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>J</td>
<td>Joining</td>
</tr>
<tr>
<td>KlrG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
</tr>
<tr>
<td>L</td>
<td>Liter (includes micro [µ] and milli [m])</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte activating gene-3</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>Meter (includes milli [m], micro [µ], and centi [c]), membrane</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>M</td>
<td>Molar (includes nano [n], micro [µ], and milli [m])</td>
</tr>
<tr>
<td>ME</td>
<td>Mercaptoethional</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagles Media</td>
</tr>
<tr>
<td>mH</td>
<td>minor histocompatibility antigens</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>MHV-3</td>
<td>Murine hepatitis virus-3</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light-chain enhancer of activated B cells</td>
</tr>
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<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NKT cells</td>
<td>Natural killer T cells</td>
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<td>OVA</td>
<td>Ovalbumin</td>
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<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PerCP</td>
<td>Peridinin-chlorophyll proteins – cyanine dye</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>Secreted</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficiency</td>
</tr>
<tr>
<td>SMAD3</td>
<td>Mothers against decapentaplegic homolog 3</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SVC</td>
<td>Superior vena cava</td>
</tr>
<tr>
<td>TBI</td>
<td>Total body irradiation</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
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<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF</td>
<td>Tissue necrosis factor</td>
</tr>
<tr>
<td>Treg(s)</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
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List of Publications

Itay Shalev, Nazia Selzner, Wendy Shyu, David Grant, Gary Levy. Role of regulatory T cells in the promotion of transplant tolerance. *Liver Transpl* 18: 761-70

Peter Urbanellis*, Wendy Shyu*, Andrzej Chruscinski, Jihong Wang, Anna Zakharova, Wei He, Itay Shalev, M. James Phillips, Oyedele Adeyi, Heather Ross, David Grant, Gary A. Levy. Rapamycin induced tolerance of fully mismatched murine cardiac allografts is dependent on CD4−CD25−FoxP3+ regulatory T cells expressing the immunoregulatory cytokine fibrinogen-like protein 2 (Manuscript submitted, Chapter 4 in this thesis)

* These authors contributed equally to this work
Chapter 1

1. Introduction: The Immune System and Tolerance

1.1 The immune system

The immune system is composed of heterogeneous populations of cells and complex regulatory pathways that allow the discrimination between self and non-self (1). A functional immune system is able to recognize foreign molecules and activate specific pathways leading to clearance of pathogens while remaining non-reactive to self-antigens. The immune responses to the pathogens can be divided into innate and adaptive components. These two components are closely associated and highly interactive; both are important in the defense against pathogens. The general characteristics of innate and adaptive immune systems are summarized in table 1-1.

The innate immune system is activated immediately upon antigen exposure, acting as the first line of defense within the body. An important component of innate immunity is the presence of a variety of cell types which includes macrophages, dendritic cells, natural killer (NK) cells and granulocytes (2). A family of receptors known as pattern recognition receptors (PRRs), mediate innate immune recognition. Common PRRs include toll-like receptors (TLRs), RIG-I-like receptors and NOD-like receptors (3). Activation of innate immunity relies on these receptors recognizing conserved structures on or within the microbes known collectively as pathogen-associated molecular patterns (PAMPs). These PAMPs include bacterial lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and glucans (4). Because these PAMPs are usually essential for the survival of these microbes they are ideal targets for innate immune system recognition. After PAMP recognition, surface or intracellular PRRs are responsible for signalling the presence of infection to the interior of the cell through adaptor proteins, kinases and transcriptional factors, eventually leading to the production and release of pro-inflammatory cytokines (3).
The adaptive immune system is responsible for creating antigen specific cells to eliminate pathogens and generating immunological memory. The major cellular components involved in this arm of the immune system are the B-lymphocytes and T-lymphocytes. Each lymphocyte expresses a unique receptor on its surface with distinct specificity. The receptor is produced by somatic recombination of DNA at specific regions of the genome for T and B cells separately. Both the T and B recombination regions contain variable (V), diversity (D), joining (J) and constant (C) segments, which are rearranged to produce the cell-unique receptors. Gene rearrangement is orchestrated by a number of nucleases and ligases such as recombination-activating gene-1 (RAG-1) and RAG-2. After the recombination process, each lymphocyte has a receptor with a unique specificity (5). It has been estimated that a mature adaptive immune system in mice contains approximately $10^{14}$ and $10^{18}$ different somatically generated immunoglobin (Ig) and T cell receptors (TCRs) respectively (5). The large diversity of the receptor repertoire ensures that the lymphocytes can recognize a vast array of foreign antigens. Activated lymphocytes can proliferate and produce Ig, or produce cytokines to direct the other cells of the immune system to control infection. After clearance of the infection, lymphocyte populations decrease returning to near normal levels, leaving behind some “memory” lymphocytes. These memory lymphocytes can expand in numbers more quickly than the naïve cells, protecting the individual from subsequent infections by the same pathogen (3).

Together, the innate and adaptive arms of the immune system provide efficient protection against most pathogens. The immune system, however, can be a significant barrier to allogeneic transplantation, because donor tissue is identified as foreign by the immune system. This process will be further discussed in section 1.2.2.
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<thead>
<tr>
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<th>Innate immunity</th>
<th>Adaptive immunity</th>
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<tr>
<td><strong>Evolutionary origin</strong></td>
<td>Early</td>
<td>Late</td>
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<tr>
<td><strong>Receptors encoding</strong></td>
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</tr>
<tr>
<td><strong>Receptor recombination</strong></td>
<td>No</td>
<td>Yes</td>
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<tr>
<td><strong>Reaction kinetics</strong></td>
<td>Fast</td>
<td>Slow</td>
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<tr>
<td><strong>Receptor target</strong></td>
<td>Conserve (PAMPs)</td>
<td>Various peptides and allo-antigens</td>
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<td><strong>Memory response</strong></td>
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<td>Yes</td>
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<tr>
<td><strong>Receptor repertoire</strong></td>
<td>Hundreds</td>
<td>$10^{14}$~$10^{18}$</td>
</tr>
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<td><strong>Cellular components</strong></td>
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<td>T cells</td>
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<td>Basophils</td>
<td>B cells</td>
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<td>Mast Cells</td>
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<td></td>
<td>Eosinophils</td>
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<td></td>
<td>Macrophages</td>
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<td>Natural Killer Cells</td>
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<td></td>
<td>γδ T lymphocytes</td>
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<tr>
<td></td>
<td>Dendritic cells</td>
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<td><strong>Soluble factors</strong></td>
<td>Complements</td>
<td>Antibodies, cytokines</td>
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<tr>
<td></td>
<td>Interferon</td>
<td></td>
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</tbody>
</table>

Table 1-1: General characteristics of innate immunity and adaptive immunity.

A functional immune system is composed of innate and adaptive immune systems. Innate immune response is activated immediately after antigen exposure and is composed of a number of germ-line encoded receptors. The adaptive immune response plays an important role during later phase of infection. T and B cells express highly diverse receptors that are able to bind to foreign antigens. The two components of immune system work together in protecting the host from pathogen invasions.

### 1.2 Transplantation

#### 1.2.1 Current progress and challenges in transplantation

Currently, there are three major challenges for solid organ transplantation: the shortage of organs, the complications associated with long-term immunosuppressive treatment and poor long-term graft survival rates. Overcoming these challenges is an essential requirement to move the transplant field forward. In 2010, 2,153 patients received kidney, liver, pancreas, intestine, heart or lungs transplantation, while 4,529 patients were still on the waiting list (6). In order to increase the availability of donor grafts, hypothermic machine perfusion preservation methodology has been tested in several organs including lungs, kidneys and livers (7-9).
preservation method appears to result in better allograft function and may rehabilitate organs that may have been discarded previously. As a result, there may be more grafts available for transplantation.

Combinations of immunosuppressive agents were developed to allow allogeneic transplantation to be performed. The immunosuppressive drugs that are used in the clinic to treat organ transplant patients include: the calcinurin inhibitor, tacrolimus, the anti-proliferative, mycophenolate mofetil and the mammalian target of rapamycin (mTOR) inhibitor, sirolimus (10). With current immunosuppressive treatment, one-year graft survival rates in renal, cardiac, liver, lung and pancreatic transplantation are 80-95% (11). However, it is also known that the therapies induce a global immune suppression in the host and are associated with severe complications such as nephrotoxicity, new-onset post transplantation diabetes mellitus, hyperlipidemia, hypertension, susceptibility to opportunistic infections and malignancies (12, 13). Therefore, new therapies are being examined. Use of antibodies to deplete T cells and block co-stimulatory signals are examples of these new therapies. Cellular therapies including the expansion of regulatory T cells (Tregs) and the use of donor cells to establish mixed hematopoietic chimerism have also been used to allow the allografts to be accepted by recipients (14).

Another major challenge currently in solid organ transplantation is poor long-term graft survival. The frequency of late graft loss is approximately 7% per year in Canada and the United States (15, 16). There are multiple reasons for late phase of graft lost and the main cause is due to chronic allograft rejection (reviewed in section 1.2.2.4) (17). It was also found that chronic rejection result from long-term usage of immunosuppressive drugs. New treatments are, thus, needed to treat chronic rejection. The goal of transplantation is to reduce the requirement of
long-term immunosuppressive therapy while preventing all types of rejection and to allow the allografts to be accepted by the recipients.

1.2.2 Immunology in graft rejection

Organ rejection is a major barrier in the field of transplantation. A functional immune system recognizes the allograft as ‘non-self’ organ and mounts an allo-specific immune response towards the graft, leading to organ rejection (18). Approximately 0.1 ~ 10% of T cells are able to react directly to allogeneic major histocompatibility complex (MHC) molecules. Additionally, it has been shown that complete MHC matching is not sufficient to prevent rejection, because ‘minor’ histocompatibility antigens (mH) are recognized by the immune system. mHs are polymorphic proteins that are not encoded in the MHC. Mismatch in multiple mH antigens can also lead to significant organ rejection albeit at a slower rate (11). Allo-rejection can be grouped into four categories: hyperacute, acute cellular, acute humoral (antibody-mediated) and chronic rejection (19). The characteristics and mechanisms of rejection are further summarized in Table 1-2 and described in this section.
<table>
<thead>
<tr>
<th>Hyperacute Rejection</th>
<th>Acute Cellular Rejection</th>
<th>Acute Humoral Rejection</th>
<th>Chronic Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset</td>
<td>Minutes to hours</td>
<td>Within days to 3 months</td>
<td>Within days to 3 months</td>
</tr>
</tbody>
</table>

**Mechanisms**

<table>
<thead>
<tr>
<th>Hyperacute Rejection</th>
<th>Acute Cellular Rejection</th>
<th>Acute Humoral Rejection</th>
<th>Chronic Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preformed anti-donor antibody</td>
<td>Delayed-type hypersensitivity</td>
<td>Development of anti-donor antibody after transplantation</td>
<td>Unclear</td>
</tr>
<tr>
<td>Complement fixation/activation</td>
<td>Activated CD8⁺ T cells</td>
<td>Complement fixation/activation</td>
<td>Involvement of B cells, antibodies, and T cells</td>
</tr>
<tr>
<td>Direct cellular lysis</td>
<td></td>
<td></td>
<td>Chronic inflammation</td>
</tr>
</tbody>
</table>

**Treatments**

<table>
<thead>
<tr>
<th>Hyperacute Rejection</th>
<th>Acute Cellular Rejection</th>
<th>Acute Humoral Rejection</th>
<th>Chronic Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening of donor reactive antibodies prior to transplant</td>
<td>Inhibition of T cell activation by immunosuppressive agents</td>
<td>Inhibition/depletion of B cells</td>
<td>No effective treatments</td>
</tr>
<tr>
<td>Plasmapheresis</td>
<td></td>
<td>Plasmapheresis</td>
<td></td>
</tr>
<tr>
<td>Antibody depletion</td>
<td></td>
<td>Complement inhibition</td>
<td></td>
</tr>
<tr>
<td>Inhibition of complement</td>
<td></td>
<td>Rituximab</td>
<td></td>
</tr>
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<td></td>
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<td></td>
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</tr>
</tbody>
</table>

**Table 1-2: Rejection mechanisms and treatment options.**

Rejection can be further grouped into categories: hyperacute rejection, acute cellular rejection, acute humoral rejection and chronic rejection. Each group is induced by different mechanisms. Due to the differences in the mechanistic pathways and cell types involved, different treatments are developed to treat the patients. However, not all types of rejection (e.g., chronic rejection) can be effectively treated.

### 1.2.2.1 Hyperacute Rejection

Hyperacute rejection (HAR) is rapid in onset and occurs within minutes to hours post transplantation (20). It is known that HAR is caused by the presence of alloreactive antibodies. B and T cell memory allows for accelerated response in an individual during a second exposure to antigen-bearing pathogens such as bacteria and viruses. Alloreactive long-lived memory B-cells and antibody-secreting plasma cells play an important role in producing antibodies prior to transplantation (21, 22). The presence of anti-human leukocyte antigen (HLA) antibody or sensitization typically results from blood transfusions, a previous transplant, or pregnancies (22,
HAR is caused by antibody deposition in the graft and the resulting activation of complement. Pre-screening of recipients for the presence of pre-existing anti-donor antibody has markedly reduced the incidence of HAR. (23).

Some of the common antigens that can result in HAR in an individual are ABO blood group antigens and α-galactose (for xenotransplant) (24). Antibodies specific to these molecules are presented in all humans and the formation of these antibodies can be caused by previous exposure to microorganisms and environment antigens that contain the epitope (25). For instance, antibodies specific to A and B antigens can be generated through immunologic cross-reaction to similar carbohydrate antigens on non-pathogeneic gut bacteria (22). Most natural antibodies are encoded by germline genes and mostly present in the form of IgM and IgG. More importantly, 1-2% of all Ig are natural antibodies (25). Hence, these antibodies play a critical role in mediating AHR.

Furthermore, antibodies specific to allogeneic MHC molecules also play an important role in promoting AHR (22). The production of antibody specific to the allogeneic MHC requires previous exposure of the antigens. MHC class I molecules are expressed by all nucleated cells; blood-group antigens are expressed on the surface of most cells including erythrocytes and endothelial cells (20). Because these antigens are abundance within the individual, the presence of pre-existing antibodies against these antigens in the recipients can cause serious immune response and promote the development of AHR.

The pre-formed antibodies bind to the antigens on the donor endothelium, and activate complement to further lead to graft destruction. C1q binds to the antibody-antigen complex resulting in the production C4a and C4b. The C4b, C2b and C3b bind covalently to the surface of damage cells, and are recognized by phagocytes that express complement receptors through a process called opsonization (26). Additionally, C3a, C4a and C5a have the ability to promote
vasodilation and migration of leukocytes to the damaged site. Lastly, a membrane attack complex (C5b-9) is formed which directly destroys the endothelial cells by promoting cellular lysis (26). The cellular damage process leads to platelet activation and fibrin deposition resulting in thrombosis and tissue necrosis (22). These processes result in the loss of graft function within minutes to a few hours after the transplant procedure (26).

Identifying patients with preformed anti-graft antibodies can prevent HAR. Therefore, several antibody-testing systems have been developed. The complement-dependent lymphocytotoxic crossmatch is the first test developed to detect the presence of anti-donor HLA antibodies. The donor lymphocytes are mixed with recipient’s serum in the presence of complement (23). A positive crossmatch is defined by cellular lysis in the mixture. More recently, the flow cytometric technique and the bead immunoassay have been developed to detect the preformed antibodies (22). Flow cytometry uses immunofluorescence rather than cellular lysis as the read out for the test and has a higher sensitivity compared with the complement dependent test. Bead immunoassay uses fluorescent-impregnated beads bound with HLA molecules to detect the presence of anti-HLA antibody using a fluorocytometer. All of these detection methods are useful in preventing HAR and are commonly done prior to organ transplantation (27).

Sensitization is a serious problem for patients who are on the wait list for an organ transplant. For kidney transplant candidates, it has been reported that sensitized patients wait twice as long for a kidney transplant than unsensitized patients (23). Hence, several desensitization treatments have been developed to reduce anti-HLA antibody. For instance, plasmapheresis with intravenous immunoglobulin (IVIG), antibody depletion and high-dose IVIG with rituximab can be used as desensitization treatments (28, 29). Furthermore, a kidney-paired donation program has been developed, which allows the exchanged of live donors to be
done between compatible recipients (30). With testing for anti-HLA antibody and the development for desensitization protocols, HAR is now a rare event in solid organ transplantation.

1.2.2.2 Acute Cellular Rejection

Acute rejection occurs within days after the transplantation procedure (19). Based on the mechanism, acute rejection can be separated into acute cellular rejection (ACR) and acute humoral rejection (AHR). This section will cover ACR. T cells play an essential role in organ rejection as illustrated by experiments with athymic rats, which accept fully MHC mismatched kidney transplant allografts (31). Both CD4\(^+\) and CD8\(^+\) T cells have been shown to be involved in ACR (32). CD4\(^+\) helper T cells are required for B cell activation and antibody production. CD8\(^+\) T cells can be activated through recognition of donor-derived antigens and further differentiate into effector cells, which migrate to the graft and promote tissue destruction.

The percentage of T cells that react to allogeneic MHC molecules is much higher than the percentage of T cells that react to foreign antigen peptides loaded on self MHC molecules (33). It has been reported that the fraction of T cells that react to a foreign peptide antigen is \(1/10^5\). The fraction of T cells that react to allogeneic MHC molecules can be as high as \(1/10^3\) (34). The high percentage of alloreactive T cells may be explained by various donor MHC/peptide combinations, creating an enormous repertoire of targets (35). Furthermore, TCRs are not selected against hundreds of possible MHC alleles during thymic development. Therefore, many of the post-selected T cells can interact with peptides presented by allo-MHC molecules (36). It has been suggested that alloreactivity can be a consequences of T cells reacting with the MHC surface, which is conserved among the MHC alleles (37). Due to the abundance of alloreactive T cells, cellular rejection is almost always observed if patients stop their immunosuppressive medications.
The allore cognition process can be further separated into the direct and indirect pathway. In the direct pathway, recipients T cells recognize the donor MHC/peptide complex on the allograft directly. The direct evidence of this pathway is the identification of the crystal structure of a TCR binding to an allogeneic MHC molecule (38). In the indirect pathway, donor-derived antigens from the grafts are processed by recipient APC and presented to the recipient’s T cells (39). The direct pathway can be observed immediately after transplantation, however, donor APCs diminished over time with only donor endothelial cells left to stimulate the direct pathway at late time point. On the other hand, indirect pathway tends to be dominant during late stage of rejection (18).

CD4+ T cells alone are able to mediate ACR. After antigen recognition, CD4+ T cells are activated and can further differentiate into different populations of T helper cells including T helper 1 (Th1), Th2, Th17, Th9 and T follicular helper (fh) depending on the surround cytokine environment (18). It has been shown that allogeneic skin graft rejection can occur by isolating CD4+ T cells from CD8−/− mice and adoptively transferring them into severe combined immune deficiency (SCID) mice which are deficient in both T and B cells (40). Th1 cells are instrumental in orchestrating the innate immune system, which also plays a key role in tissue rejection. After activation, Th1 cells secret cytokines including interferon-γ (IFN-γ) and tumor necrosis factor (TNF). These cytokines promote inflammatory responses, such as activation and recruitment of monocytes and macrophages (41). More cytokines and chemokines can be produced by the recruited macrophages and lead to the migration of lymphocytes to the graft which can cause more tissue damage and directly influence the vascular tone, permeability and integrity of the graft (42). In contrast to Th1 cells, activated Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are critical for initiating antibody-mediated rejection. Furthermore, IL-4, IL5 and IL-13 are able to activate eosinophils, which migrate to the graft and release granules containing
cytotoxic substances, such as superoxides and peroxidase to damage the graft directly (43). In short, activated CD4\(^+\) T cells are able to produce a variety of cytokines and chemokines, which trigger or initiate the organ rejection process.

Evidences show that CD8\(^+\) T cells also play an important role in promoting ACR. CD8 deficient mice fail to reject the cardiac graft in a mH antigen mismatch mouse model (44). In general, CD8\(^+\) cells are activated and differentiated into cytotoxic T lymphocytes (CTLs) after encountering donor peptide/MHC class I complex and co-stimulatory molecules (45). These MHC I molecules are found on virtually all cells of the body, including cells of the donor organ. CD8 deficient mice fail to reject the cardiac graft in a mH antigen mismatch mouse model and provide direct evidence that CD8\(^+\) T cells play a role in mediating ACR (44). CTLs can directly damage the graft through the secretion of serine proteases, granzymes, pore-forming protein, perforin, and the expression of Fas ligand (46). CTLs migrate to the graft and recognize their targets through MHC class I/TCR recognition. Perforin creates holes in the membrane of the grafts cells and facilitates granzyme uptake into the cells, which further induce cellular apoptosis through activation of caspases (42). Binding of Fas to Fas ligand can also induce cellular apoptosis and lead to tissue destruction. Granzyme expression has been associated with acute rejection, and studies have shown that granzyme and perforin expression can be used as markers for acute rejection (47, 48).

ACR can be prevented through the use of immunosuppressive therapies. Calcineurin inhibitors, such as cyclosporine and tacrolimus prevent production of various cytokines by blocking gene transcription, such as nuclear factor of activated T cells (NFAT) in lymphoid cells and has been shown to be very powerful in preventing ACR as NFAT regulates the secretion of IL-2, IL-4 and IFN-\(\gamma\) (49). Rapamycin, another immunosuppressive drug, is a mTOR pathway inhibitor and can down-regulate the expression of co-stimulatory molecules and inhibit cytokine
production (49). Additionally, ACR can be prevented by administering antibodies specific to thymocytes (49). In conclusion, with the success of immunosuppressive treatment, ACR is controllable in the field of transplantation.

### 1.2.2.3 Acute Humoral Rejection/Acute Vascular Rejection

Humoral response refers to the soluble factors that mediate the immune system such as complement and antibodies (22). The observation of the presence of circulating antibodies and the deposition of C4d within the first few days post transplantation illustrates that the humoral response plays an important role in acute rejection, and is known as acute humoral rejection (AHR). Clinical diagnoses of AHR rejection are based on histological evidence of acute tissue injury, the presence of circulating donor specific antibodies and positive C4d staining in the grafts. (50).

Antibodies can mediate tissue damage through several methods. One is through activation of the complement system. The complement fixation pathway was described previously in section 1.2.2.1. In short, complement activation can cause direct damage to the cells by forming pores via the membrane attack complex (C5b-9) and indirect damage via recruitment of effector cells to the graft, which further damage the graft by inducing inflammatory responses. Antibodies can also cause tissue damage in a complement-independent pathway. Antibodies bind to targets and recruit NK cells, neutrophils and macrophages through binding to the low-affinity Fc receptor FcγRIII (CD16) and FcγRII (CD32) which can further cause cell death through secretion of granules containing perforin and granzymes or promote cell death by triggering apoptotic pathways. This is known as the antibody dependent cell mediated cytotoxicity pathway (20). Additionally, antibodies can stimulate endothelial cell proliferation, which is important in the development of chronic rejection and will be reviewed in section 1.2.2.4 (20).
Treatments have been developed to target B cells specifically to treat AHR. Both plasmapheresis and immunoadsorption have been used to deplete circulating anti-HLA antibody and isoheamagglutinins (22). Rituximab, an anti-CD20 antibody, is also used to treat AHR. Although Rituximab is effective at depleting B cells, it is not effective in depleting plasma cells. However, antibody rebound is common in patients with these kind of treatments because the plasma cells are not removed. Therefore, patients usually need multiple treatments in order to get the effect. Splenectomy is a procedure that has been shown to be effective in reducing the plasma cell population in patients. Newly formed plasma cells need to receive niche-derived trophic and anti-apoptotic signals from the spleen before they migrate to the bone marrow to become long-lived plasma cells (51). Therefore, it is common to observe a dramatic drop in the plasma cells level after spleen removal. Lastly, proteasome inhibitors and complement inhibitors are used to treat AHR (22). Proteasome inhibitors disrupt protein degradation in the endoplasmic reticulum and have been shown to inhibit/deplete antibody producing plasma cells. The complement inhibitor (eculizumab) is a humanized anti-C5 antibody, which can prevent the formation of the membrane attack complex (C5b-C9) and directly protect the graft. In general, these treatments are well tolerated with minor side effects (22). With the development of these treatments, AHR can be treated but with time some of the patients still develop chronic rejection and can eventually lose their grafts.

1.2.2.4 Chronic Rejection

Chronic rejection is a slow process, which occurs months to years after the transplant (20). Immunosuppressive therapies have been shown to be effective in preventing acute rejection, but not chronic rejection. Every year, 3-5% of heart, liver and kidney transplant patients develop late graft failure (52). The pathophysiology of chronic rejection is still poorly understood, however, it is known that both T cells and B cells play important roles in promoting
the development of chronic rejections.

CD4+ T lymphocytes, cytotoxic T lymphocytes (CTLs) and B cells are the main cell types that are responsible for causing chronic rejection (52). Using a rat kidney transplant model, Hancock et al. showed that cytokines, such as IL-1, TNF-α and IL-6 produced by CD4+ T cells are critical for the promotion of chronic rejection (53). Another group using a mouse cardiac graft transplant model showed that cytokines produced by the Th2 cells (e.g., IL-4, IL5 and IL-10) can also lead to chronic graft rejection by attracting eosinophils, large mononuclear cells and fibroblast-like cells to the graft (54). Moreover, it has been shown that skin allograft chronic rejection can be prevented by administering an anti-IL-4 antibody into the recipients further confirming the importance of cytokines in promoting chronic rejection (55).

CD8+ T cells also play an important role in mediating chronic rejection. The main mechanism that CTLs use to initiate tissue damage is through direct cytolysis of graft parenchymal or vascular cells via the production of granzymes and perforins (52). Furthermore, it has been shown that CTL-specific mRNA expression is associated with graft failure in both humans and in an animal kidney transplant model (56). Immunosuppressive agents, such as the calcineurin inhibitors cyclosporine and tacrolimus, block CD4+ T cell function more effectively than blocking CTL activity (52). As a result, CTLs may be relatively more available to promote chronic allograft rejection than CD4+ T cells.

Antibodies also contribute to the progression of chronic rejection. A mouse strain with a defect in antibody production was used in an MHC-mismatched murine model of vascular transplantation to demonstrate that chronic allograft vasculopathy (narrowing of the lumen of the arteries which is a hallmark of chronic rejection) was prevented in the absence of humoral response (57). Another group has demonstrated that anti-HLA antibody binds to the MHC class I molecule expressed on both endothelial cells and smooth muscle cells (58). Anti-HLA antibodies
can activate mouse endothelial cells to produce TNF and chemokines that can recruit leukocytes to the vasculature (59). Furthermore, antibodies can also activate the complement system, leading to tissue destruction.

Immunosuppressive drugs themselves can contribute to chronic rejection. In kidney transplant it has been shown high dose of cyclosporine and tarcolumus promote progression to chronic rejection by triggering vasoconstriction in the afferent arteriole of the glomeruli (60). Also, long term usage of cyclosporine leads to interstitial fibrosis, tubular atrophy and inflammatory cell infiltration (60). These toxic effects of cyclosporine occur in 65% of the patients after 5 years and 100% of patients after 10 years of the treatment. Therefore, one of the treatments for chronic rejection is to minimize the use of the calcinerin inhibitors, by replacing them with other drugs, such as belatocept, sirolimus and mycophenolate that do not have toxic effects on the vasculature; however, there are complications associated with these drugs as well (60). To summarise, immunosuppressive therapies are critical in preventing acute rejection. However, chronic rejection still remains a challenge as it can be a product of cellular immunity, humoral immunity, and also a product of the immunosuppressive drugs.

**1.3 Tolerance**

The immune system protects the individual from pathogen invasions while being tolerant to self-antigens. In organ transplantation, the goal is to induce donor specific tolerance in the recipients. This way, complications associated with long-term immunosuppressive treatment can be avoided. In order to induce tolerance, it is important to first understand tolerance induction mechanisms. By using genetically engineered transgenic, knockout and mutagenized mice, tolerance mechanisms are well understood now and are divided into the central and the peripheral tolerance compartments (61). They are further reviewed in the following section.
1.3.1 Central Tolerance

The functional immune response is tightly regulated to ensure that self-reactive leukocytes do not cause autoimmune diseases. In a process known as central tolerance, self-reactive immature leukocytes are deleted in primary lymphoid organs, such as the thymus and bone marrow prior to their exit to the periphery (62).

In the thymus, immature CD8⁺CD4⁺ double positive thymocytes must go through both a positive and a negative selection process before they can reach full maturation. Positive selection occurs to ensure that the T cell receptor (TCR) is capable of interacting with self-MHC molecules, while negative selection occurs to prevent the maturation of self-reactive T cells. Functionally, negative selection selects against T cells whose reactivity is strong with self-antigen through sampling their TCR against self-peptides on the thymic epithelium (36). Cells that react to self-peptide in the context of MHC with a low to intermediate affinity will continue to obtain survival factors, while cells that do not bind or that bind too strongly are eliminated. The selected double positive T cell precursors then continue to develop into single positive CD4⁺ or CD8⁺ T cells based on the preferential binding of TCR to MHCII or MHCI molecules, respectively (36). To demonstrate the phenomenon of central tolerance, a murine study was conducted using an antibody specific to Vβ17a of the TCRs. T cell reactivity to I-E class II MHC molecules is associated with TCRs containing the Vβ gene segments, the Vβ17a and Vβ5 (63). It was found that C57BL/6 mice which express I-E class II molecules in thymic epithelium, have no Vβ17a⁺ receptor T cells, but if the I-E class II molecules were expressed in pancreatic β cells (not in the primary lymphoid organ), no tolerance to the self antigen was observed (63). Furthermore, differentiation antigens, L3T4 and Lyt2 (immature thymocytes express both antigens and mature thymocytes only express one or the other) were used to demonstrate the
presence of the Vβ17a⁺ immature thymocytes (L3T4⁺Lyt2⁺). These studies directly illustrate that central tolerance of T cell occur in the thymus.

Expression of tissue specific antigens on thymic epithelial medullary cells is also important in the clonal deletion process (64). Autoimmune regulator (AIRE) is the central mediator for regulating the expression of tissue antigens in the thymus (65). In human and mice, a defect in the AIRE gene leads to the development of autoimmune diseases (66). However, not all the peripheral antigens are controlled by AIRE and subsequently expressed in the thymus. A subset of autoreactive T cells escape into the periphery where they are regulated by peripheral tolerance mechanisms.

In the bone marrow, self-reactive B cell clones are also eliminated by induction of program cell death (apoptosis) through a process called clonal selection (67). It was found that 20-25% of splenic B cells from an H-2d mice express membrane IgM directed against H-2k MHC molecules (68). However, if H-2k and H-2d mice are mated, the F1 generation lack B cells that are reactive toward H-2k, suggesting the self-reactive B cell clones were deleted during the development process (68). Additionally, it was found that immature self-reactive B cells can trigger a regulatory process called receptor editing (67). Receptor editing utilizes a DNA recombination process to replace self-reactive BCR with a non-reactive BCR (69). Chen et al. used a transgenic mouse model, which have the JH locus replaced with a rearranged VDJ coding for the heavy chain of an anti-DNA B cell receptor (70). It was found that the JH locus in these animals was replaced with upstream endogenous VH or DH in order to generate a new B cell repertoire. Now, it is known that these self-reactive B cells can also become anergic after migrating out of the bone marrow. Not all of the self-reactive cells are deleted in the primary lymphoid organs, therefore, peripheral tolerizing mechanisms further play an important role in preventing the development of autoimmune diseases in the individual.
1.3.2 Peripheral tolerance

Clonal deletion is important in eliminating autoreactive lymphocytes in the primary lymphoid organs. However, some autoreactive T and B lymphocytes escape central tolerizing mechanisms and migrate into the periphery. It is estimated that 25%~40% of T cells escape thymic clonal deletion and must be tolerized in the periphery (71). Such autoreactive cells, if left unchecked, have potential to initiate autoimmune diseases. For instance, mutation of the foxp3 gene in mouse and human result in lethal autoimmunity because the mutation impair in the peripheral tolerance system by disrupting the development of regulatory T cells (72, 73). Furthermore, a number of peripheral tolerizing mechanisms work together to maintain tolerance to self, and these mechanisms will be reviewed in the following sections.

1.3.2.1 Anergy/Deletion

The induction of anergy is one of the primary mechanisms of tolerizing autoreactive T cells in the periphery (74). Autoreactive T cells that capable of recognizing antigens, but do not receive co-stimulation will not be activated (61). Instead these T cells progressively lose their function or become eliminated. The factors that influence the induction of anergy or deletion are dependent on the abundance of the self antigens and the degree of crosslinking that occur on the autoreactive T cells. Transgenic mice expressing either low or high level of the ovalbumin (OVA) protein in the pancreas were utilized to demonstrate this process. Deletion of these self-reactive clones occurred when the OVA-specific CD8+ T (OT-I) cells were transferred into the high-OVA expressing mice. In contrast, OT-I cells become anergized when adoptively transferred into the low-OVA expressing host (75). Therefore, the concentration of the self-antigen in the host is critical in directing the anergy or deletion of self-reactive T cell clones.

1.3.2.2 Tolerogenic Antigen-Presenting Cells
Dendritic cells (DCs) play an important role in presenting antigens to T cells to promote T cell activation, but DCs also play a critical role in inducing peripheral tolerance. Immature DCs express low levels of MHC and co-stimulatory molecules (76). Upon infection, DCs present the foreign peptides on MHC molecules and undergo maturation by up regulating the expression of MHC and co-stimulatory molecules to activate T cells (76). DCs maturation process is depended on the antigens and the microenvironment. For instance, regulatory cytokines such as IL-10 can block DC maturation (77).

Incomplete maturation of DCs can generate tolerogenic APCs. The first study that shows DCs can be tolerogenic in vivo was done using an antibody specific to hen egg lysozyme (HEL) to deliver the HEL antigen to DC-restricted endocytic receptor (78). When HEL antigen is delivered to DCs without the up-regulation of MHC class II and CD80 expression, a short period of T cell proliferation is observed, but T cells progressively lost function and become deleted. In contrast, co-injection of HEL and a co-stimulatory antibody to CD40 found on APCs induced DC antigen presentation and T cell activation (78). The study suggests that DCs can be tolerogenic and play a role promoting peripheral tolerance.

1.3.2.3 Regulatory T Cells

The presence of suppressive T cells was first described by Gershon et al. in 1970 (79). However, due to the lack of specific markers and techniques to isolate these suppressive T cells, this area of research became less popular until Sakaguchi et al. discovered CD25 as a marker for these regulatory T cells (Tregs) (80). Nude mice develop autoimmune disease after adoptive transfer of effector CD4^+CD25^- T cells which can be prevented by co-transferring of regulatory CD4^+CD25^- cells into the animals (80). Furthermore, it was found that forkhead box P3 (Foxp3) defective mice, scurfy mice, lack Treg function, suggesting Foxp3 can be used as a marker for Tregs (81). Moreover, Immunodysregulation, polyendocrinopathy and enteropathy, X-linked
syndrome (IPEX) is a disorder found in humans who lack functional Foxp3 expression. These individuals do not have sufficient numbers of Treg and suffer autoimmune disease (81).

A number of different Tregs subsets have been reported, including regulatory T cell type 1, T helper 3, CD8+ regulatory T cells, double negative (DN) T cells, γδ T cells, NK T cells and CD4+CD25+ Foxp3+ regulatory T cells (82). All of these cells possess the capacity to suppress the immune response, but were separated into different groups based on their phenotypes and expression of surface markers. This study will be focusing on the CD4+CD25+ Foxp3+ regulatory T cells (Tregs). Tregs are important in transplant tolerance due to their suppressive activities. Understanding the mechanisms by which Tregs exert their suppressive activities can provide an insight into the process of peripheral tolerance and allow for the development of a potential therapy to induce tolerance of the transplanted organ. The mechanisms of Treg function can be further separated into suppression by inhibitory cytokines, induction of cytolysis, suppression by metabolic disruption, and inhibition of DC maturation (83).

Inhibitory cytokines, such as IL-10, TGF-β and IL-35, have been shown by several investigators to be mediators of Treg induced suppression and can facilitate the development of induced (adaptive) Tregs (Figure 1-1A) (83). IL-10 and TGF-β producing Tregs are critical in controlling the immune response in airway inflammation, hyper-reactivity and Th2 dependent allergic diseases (84). It was found that allergic diseases can be controlled by adoptively transferring allergen-specific Tregs, and this effect can be disrupted by the administration of anti-IL-10 antibody (85). In transplantation, Hara et al. used a murine heart transplant model to show that injection of Tregs can prevent rejection. IL-10 neutralization using an antibody however accelerates rejection (86). Similarly, TGF-β can suppress effector T cell proliferation and B cell antibody production in vitro (87). TGF-β secreted by the Tregs can directly suppress T effector cells and prevent the development of colitis in a murine model of inflammatory bowel disease.
Recently, IL-35 was discovered as another inhibitory cytokine produced by Tregs (89). Mouse Foxp3\(^+\) cells expressed high level of IL-35 compared with activated or resting CD4\(^+\) T cells (89). It was also found that ectopic expression of IL-35 in naïve T cells promote regulatory function in naïve T cells and recombinant IL-35 can suppress T cell proliferation. These studies suggest that IL-10, TGF-β and IL-35 are all important in facilitating the suppressive function of Tregs.

Induction of cytolysis through secretion of granzymes and perforin in activated human Tregs is another Treg mechanism of action (Figure 1-1B) (90). In mice, it was found that granzyme B (GZ-B) was highly expressed by Tregs using gene expression array analysis and GZ-B\(^-\) Tregs have reduced suppressive ability compared to the WT Tregs (91). It has also been reported that Treg can kill B cells in a GZ-B and perforin dependent manner and result in suppression of B cell function (92). Additionally, galectin-1 was found to be highly expressed by mouse and human Tregs and this molecule can induce T cell apoptosis to further suppress the immune response (83).

Metabolic disruption is another proposed Treg suppression mechanism (Figure 1-1C). Tregs induce CD4\(^+\) effector cell apoptosis through deprivation of IL-2 due to expression of CD25. With very high level of IL-2 receptor (CD25) expression, Tregs may be able to use up all the local IL-2, starving actively dividing effector T cells, leading to suppression of T effector proliferation. A study demonstrated that, IL-2 levels decreased in a Tregs and responder T cells co-culture system where responder cells continue to produce IL-2 to demonstrate that the depletion of IL-2 after addition of Tregs is due to consumption of IL-2 and not lack of production of IL-2. It was found that IL-2 \(^{-/-}\) and IL-2α (CD25) \(^{-/-}\) Tregs still maintain normal suppressive function, suggesting that deprivation of IL-2 is not the only mechanism that Tregs use to suppressive T effector cell proliferation (93).
Tregs can suppress effector T cell proliferation via generation of adenosine and cyclic adenosine monophosphate (cAMP), molecules that can suppress T cell proliferation and cytokine production (Figure 1-1C). CD39 and CD73 expressed on the surface of Tregs generate adenosine, which bind to adenosine receptor 2A (A<sub>2A</sub>R) on effector T cells to suppress proliferation and cytokine secretion of the effector cells (94, 95). Tregs also inhibit T cells proliferation and IL-2 production by transferring cAMP into T effector cells through gap junctions (96). IL-2 provides survival signals for effector T cells, and without sufficient IL-2 being produced the immune system is supressed.

Treg can act on DCs to modulate DC maturation process and DC function (Figure 1-1D). Microscopy techniques were used to demonstrate that T cells interact less with DCs in the presence of Tregs suggesting Tregs affect the immune response via altering the length of T cell and DCs interaction time (97). It was found that Tregs express high level of cytotoxic T lymphocyte – associated antigen 4 (CTLA-4) and this molecule plays an essential role in suppressing DCs function in vivo (98). Injection of CD45RB<sub>low</sub>CD4<sup>+</sup>CD25<sup>+</sup> T cell into SCID mice protects the animal from getting colitis and anti-CTLA-4 antibody disrupted this protection. Tregs also down-regulate CD80 and CD86 expression of DCs in a CTLA-4 dependent pathway (99).

Tryptophan catabolism by DCs through the enzyme indoleamine 2,3-dioxygenase (IDO) is another Tregs induced suppressive mechanism. IDO catalyzes tryptophan degradation and induces the production of proapoptotic metabolites and can directly inhibit T cells acticity (100). It was found that resting Tregs or anti-CD3 activated Tregs induced tryptophan catabolism in vitro through a CTLA-4 dependent mechanism (101). The evidence that CTLA-4 is able to induce IDO expression was also demonstrated in vivo (102). These studies suggest that CTLA-4 play an essential role in initiating the suppressive function of Tregs.
There are many mechanisms that Tregs use to suppress immune responses by modulating DC maturation and T cell activation through secretion of immunoregulatory cytokines or effector molecules. Tregs play a critical role in controlling peripheral tolerance and more studies are required to be done to understand the mechanisms of Tregs suppressive function. These mechanistic studies can be beneficial to transplant patients and assist in the development of alternative therapeutic options.
Tregs play a critical role in suppressing the immune system. (A) Tregs suppress effector T cell proliferation by the secretion of immunosuppressive cytokines, IL-10, IL-35 and TGF-β. (B) Tregs secrete granzyme A or granzyme B to induce cellular lysis. (C) Metabolic disruption is another suppressive mechanism. Tregs consume IL-2 in the microenvironment and further induce effector T cell apoptosis. Tregs can transfer cAMP via gap junction into effector T cells to disrupt the effector T cell function. Expression of CD73 and CD39 on Treg surfaces can suppress cytokine secretion of Th1 and Th2 effector cells via production of adenosine. (D) Tregs interact with DCs and inhibit DC maturation through binding of CTLA-4 to CD80/CD86 complex. CTLA-4 interaction between DCs and Tregs induce IDO secretion of DCs. IDO catalyzes Tryptophan degradation and induces the production of proapoptotic metabolites, which inhibit T cell function directly. Teff, effector T cells. Figure adapted from Vignali, D.A., et al. (83).

### 1.4 Fibrinogen-like Protein 2 (FGL2)

Current studies have been focused on investigating new immunoregulatory molecules in inducing tolerance in patients. Fibrinogen-like protein 2 (FGL2), also known as fibroleukin, was first cloned as a T-lymphocyte specific gene and it is a member of fibrinogen protein superfamily due to its carboxyl-terminal homology (36%) to the β and γ chains of fibrinogen (103).
The fgl2 gene has two exons that are separated by an intron, and the gene localizes on chromosome 7 and 5, and encodes for 432 and 439 amino acids in human and mouse respectively. The protein contains a globular fibrinogen-related domain (FRED) at its C-terminus and a linear α-helical region in the N terminal. The molecule also contains five N-linked glycosylation sites and conserved cysteine residues, which promote disulphide linkages between different FGL2 subunits and result in a tetrametric FGL2 complex under normal physiological condition (Figure 1-2) (104, 105). Human FGL2 shared 78% identity with the mouse FGL2, but the two proteins share 90% homology in the FRED region (104, 105). FGL2 has been shown to exist both as a membrane associated protein (mFGL2) and a secreted immunosuppressive molecule (sFGL2). The functions of the two forms are further discussed (Table 1-3).

Figure 1-2: The structure of FGL2.

FGL2 contains a globular FRED domain at the carboxyl terminal region and the amino terminus of the protein contains a linear α-helical region. Several conserved cysteine residues and five N-linked glycosylation sites are found throughout the molecule. Figure adapted from Lui, H., et al. (106).
Table 1-3: The comparison table between membrane-associated FGL2 and secreted FGL2.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Membrane-associated</th>
<th>Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages and endothelial cells</td>
<td>Prothrombinase activity, involved in coagulateon</td>
<td>Immunoregulatory activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibit T cell proliferation and induce B cell apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treg effectors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibition DC maturation</td>
</tr>
</tbody>
</table>

FGL2 has been shown to exist in two forms: the membrane-associated FGL2 and the secreted FGL2.

1.4.1 Membrane-associated FGL2

It is now known that the membrane associated form of FGL2 (mFGL2) plays a role in regulating the coagulation process, and this activity is only found in the amino-terminus of the protein. mFGL2 is expressed in macrophages and endothelial cells possessing prothrombinase activity and can produce thrombrin directly from prothrombin (107). Site-directed mutagenesis was performed to demonstrate that serine 89 residue is essential for the coagulation activity of FGL2. Furthermore, the study demonstrates that phospholipids, calcium and factor Va are required for efficient thrombin catalytic activity of FGL2 (107).

Murine hepatitis virus-3 (MHV-3) infection studies demonstrated that mFGL2 has coagulation activities (108). Post MHV-3 infection, fgl2 mRNA transcripts and FGL2 proteins were elevated in susceptible BALB/C and C57BL/6 mice, which was associated with fibrin deposition leading to ischemic hepatocellular necrosis. A/J mice (produce less FGL2 in response to MHV-3) and Fgl2−/− mice both have prolonged survival rate post MHV-3 infection compared to susceptible WT C57BL/6 mice. Furthermore, anti-FGL2 neutralizing antibody treatment prevents fibrin deposition by inhibiting coagulation activities. FGL2 prothrombinase activity has
also been implicated in the pathogenesis of experimental xeno- and allotransplant rejection and Th1 cytokine-induced fetal loss syndrome (109, 110).

1.4.2 Secreted FGL2

Secreted FGL2 (sFGL2) are detected in peripheral CD3+ , CD4+ and CD8+ populations and is an immunosuppressive molecule (105). sFGL2 binds specifically to FcγRIIB and FcγRIII receptors expressed on B lymphocytes and DCs and further inhibits DC maturation and induces B cell apoptosis (Figure 1-3) (104). sFGL2 had the ability to inhibit T cell proliferation stimulated by alloantigens, anti-CD3/anti-CD28 mAbs and Concanavalin A in a dose-dependent manner (107). Neutralizing FGL2 activity using an antibodies restores this proliferation effect in the T cell proliferation response. Additionally, sFGL2 promote a Th2 cytokine profile in alloantigen-stimulated cultures and inhibited DCs maturation by preventing NF-κB nuclear translocation (107). These studies provide direct evidence of the immunosuppressive function of the sFGL2 and demonstrate the FGL2 inhibitory mechanism.

To further study the immunoregulatory role of FGL2, mice with targeted deletion of fgl2 were generated (111). These mice spontaneously develop autoimmune glomerulonephritis with increasing age. Similarly, wild-type animals reconstituted with fgl2−/− bone marrow also develop autoimmune glomerulonephritis. It was found that DCs from the fgl2−/− mice have increased activity when stimulated with LPS compared to the DCs harvested form the WT mice. Most importantly, this study demonstrates that FGL2 is mainly expressed by Tregs as the fgl2 mRNA level is 6-fold higher in the Tregs than the CD4+CD25− cells (111).

Several genome-wide association studies were done on Tregs and fgl2 was found as a putative Treg effector gene. Green fluorescent protein (GFP)-Foxp3 knockin mice were created to study the mRNA profile of CD25+Foxp3gfP+ T cells and the study demonstrated that Foxp3gfP+ T cells express high level of Treg characteristic genes such as IL-10, KlrG1, Icos, CTLA-4 and
fgl2 compared with CD25 Foxp3<sup>naïve</sup> naïve T cells (112). Moreover, the same group used a Cre-lox foxp3 allele conditional deletion system to study the effect of deleting foxp3 in mature peripheral Treg on the expression of suppressive genes (113). Tregs lost suppressive function after foxp3 deletion, and downregulated several suppressive effector genes expression, including fgl2, simultaneously (114). The genome-wide association studies done on Tregs demonstrated that FGL2 is a Treg effector molecule.

1.4.3 FGL2 and transplantation

In organ transplantation, several animal experimental models have demonstrated that FGL2 plays a role in suppressing the immune system. A BALB/C to C57BL/6J skin transplant model was adopted to show that giving recombinant FGL2 treatment prolongs the survival of the skin graft and this phenomena was not observed when FcγRIIB<sup>−/−</sup> recipients were used (104). The data suggests that FGL2 is able to prolong graft survival rate and that FcγRIIB receptors are required for its function. Moreover, it has been reported that FGL2 is highly expressed in CD8<sup>+</sup> Tregs using a Rat cardiac transplant model (115). The study used microarray to compare the gene expression profile between tolerogenic CD8<sup>+</sup> Tregs and naïve CD8<sup>+</sup> Tregs, and found that tolerogenic CD8<sup>+</sup> Tregs expressed high levels of fgl2. It was further found that FGL2 neutralization resulted in restoration of CD4<sup>+</sup> T cell proliferation while co-cultured with DCs and tolerogenic CD8<sup>+</sup> Tregs. These organ transplant animal models studies suggest that FGL2, an effector of Tregs may potentially play a role in inducing tolerance in organ transplantation.
Figure 1-3: FGL2 mechanisms of action.

FGL2 is an effector molecule of Tregs. The sFGL2 is able to bind to FcγRIIB receptor on B cells and DCs. Binding of the molecule to the receptor can inhibit DC maturation and induce B cell apoptosis. Treg, regulatory T cells, Teff, effector T cells.
1. Hypothesis and Aims

Through a previously established rapamycin induced tolerance murine heterotopic heart transplant model, it is possible to examine tolerance induction mechanisms. Previous studies done by our group have reported elevated Treg number and FGL2 plasma levels in the tolerant recipients compared with the rejecting recipients. Additionally, through a Treg associated gene screening study, FGL2 was found to be highly expressed in the tolerant allografts compared to the rejecting allografts. With these observations, we hypothesized that Tregs and FGL2 play critical roles in controlling the immune response in solid organ transplantation and allow donor specific tolerance to be induced in the recipient mice.

To further study the role of Tregs and FGL2 in this model, the aims of the present study were:

a) To determine the contribution of Treg in the tolerance induction process in our mouse model.

b) To investigate the immunoregulatory effect of FGL2 and the contribution of FGL2 in the development of tolerance.

c) To study the mechanism of FGL2 suppressive function on T cells.
Chapter 2

2. Introduction: Hematopoietic stem cell Transplantation

2.1 Hematopoietic stem cells

Hematopoiesis is a process that homeostatically maintains the numbers of myeloid and lymphoid cells from a single hematopoietic stem cell (HSCs) population residing within the bone marrow (116). The parameters that define such cells are namely their capacity to self-renew and ability to differentiate into all the cellular components of blood (117). Till et al. discovered that transplantation of HSCs results in hematopoietic reconstitution and protection of mice after lethal irradiation, whereas untreated mice quickly succumb to the effects of the irradiation (118). Moreover, HSCs were capable of forming colonies of cells within the spleen representing all the blood cell types and serial passage of those cells from one mouse to another could further protect the recipient animal from lethal irradiation (118). HSCs are characterized as Lin−cKit+Sca-1+ for mice and Lin−CD34−Thy1+ for humans (119, 120). The cell surface phenotypes of the HSCs are further summarized in Figure 2-1. These markers allow HSCs to be further isolated and HSC transplantation to be performed.

An important characteristic of HSCs is their ability to differentiate into all of the blood cell types (121). During the differentiation process, HSCs gradually lose the self-renewal ability and only sustain hematopoiesis in vivo for six weeks (122). The short-lived HSCs then differentiate into the multipotent progenitors (MPPs), losing their capacity for self-renewal. The MPP can differentiate into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). CLPs are responsible for the production of T cells, B cells and NK cells, while CMPs give rise to megakaryocytes, erythrocytes, macrophages and granulocytes (122). Hematopoietic lineage map is shown in Figure 2-2.
Figure 2-1: The surface phenotype of murine and human HSCs.

Human and murine HSCs express different surface markers. Both HSCs are characterized as negative for all the differentiated lineage markers (Lin'). Murine HSCs express Sca-1 and c-kit; human HSCs express CD34 and Thy-1.

Figure 2-2: The lineage map of hematopoiesis.

During the hematopoiesis process, HSCs gradually lose the self-renewal ability and differentiate into MPP. The MPP can differentiate into either CLPs or CMPs. CLPs then differentiate into T cells, B cells and NK cells, while CMPs are responsible for producing megakaryocytes, erythrocytes, macrophages and granulocytes (122).
2.2 Bone marrow transplantation

The first step in bone marrow transplantation (BMT) is an immune ablation treatment, such as chemotherapy and irradiation, which deplete the recipients’ hematopoietic cells. The next step is injecting bone marrow cells, which contain the HSCs, into the patient to allow reconstitution of the hematopoietic compartment (123). Studies on mice have shown that donor-derived monocytes, dendritic cells and neutrophils could be found by day 7 post BMT and the peripheral hematopoietic compartment were fully reconstituted by 3-4 weeks post BMT (124, 125). B cells appear soon after the irradiation treatment, and T cells have a slower reconstitution rate compared to B cells (125). The reconstitution rate of the different cellular components following irradiation is further summarized in figure 2-3.

In 1955, Thomas and colleagues were the first group to begin a human whole BMT program (126). The first trial involved six patients and only two patients showed a transiently detectable marrow graft, but none of the patients survived beyond 100 days. Although the study was not successful in treating the patients, it was the first study to demonstrate that bone marrow can be processed to remove fat and large bone particles and then inject into human (126). It was not until the development of HLA-typing in 1964 that BMT became clinically available (127).

BMT can also be termed hematopoietic stem cells transplantation (HSCT), because the HSCs in the bone marrow are responsible for reconstituting the host immune system (128). Currently, most HSCT are performed by isolating peripheral-blood-mobilized stem cells to reconstitute the hematopoietic system of the patients following radiation treatment or chemotherapy (126). The success of allogeneic HSCT is limited by the development of graft-versus-host-disease (GVHD) in the recipient, which results from donor allogeneic T cells attacking the recipients’ organs or tissue, by the same mechanism as in rejection in solid organ transplantation (reviewed in section 1.2) (129). It has been reported that GVHD is fatal in
approximately 15% of allogeneic HSCT recipients (129). GVHD does not occur in autologous BMT because there is no HLA mismatch between the donor and the recipient. HSCT is now used widely as a common therapeutic treatment option for patients with leukemia, lymphoma and autoimmune diseases (130).

![Figure 2-3: Schematic representation of hematopoietic reconstitution over time.](image)

After total body irradiation (TBI), neutrophils, monocytes and DCs are the first population to be detected in the recipients. B and T cells reconstituted after neutrophils, monocytes and DCs. Plates and erythrocytes only reconstituted the peripheral compartment at later time point (124).

**2.2.1 Immune re-education with autologous HSCT**

Long-term immunosuppressive therapy has been used as a therapy to prevent organ rejection. However, immunosuppressive therapies are deleterious in the long-term because they increase the risk of cancer and infection. Therefore, a major goal of organ transplantation is to induce tolerance to the graft and avoid long-term immunosuppressive treatment. Studies on using HSCT to treat autoimmune diseases demonstrate the potential of using this treatment to re-educate the immune system and induce self-tolerance. By using the same rationale, HSCT may potential induce donor-specific tolerance.
Due to the capacity of HSCs to renew and differentiate into all the components of the blood, HSCT can be used in conjunction with immune ablation therapy to eliminate reactive or cancerous cells, while reconstituting the blood system in patients suffering from autoimmune diseases and lymphomas (131-133). Studies using HSCT to treat cancer and autoimmune diseases show that B, NK and CD8\(^+\) cells have rapid reconstitution rates, while the recovery rate of CD4\(^+\) cells is delayed (134, 135). These studies confirm that full immune reconstitution is possible in adult patients. Measurement of TCR excision circle DNA generated in the TCR rearrangement process reveals that thymopoeisis occurs following HSCT (136). These studies also suggest that adult thymus can remains active following HSCT and plays an important role in generating a new repertoire of T cells (137). Long term follow up studies in patients who have received an autologous HSCT following the immune ablation suggest that that the naïve CD4\(^+\) T cell population, which is derived from the transferred HSCs, can recover or even exceed pre-transplant levels 2 years post HSCT (138, 139). Analysis of the antigen-specific diversity of these naïve T cells demonstrated up to 90% of renewal in the T cell repertoire (138). Such a concept of re-educating the immune system has wide therapeutic benefit and may be applicable to solid organ transplantation.

Rejection in solid organ transplantation involves T and B cell recognition of donor antigens, which initiate a cell-mediated or/and humoral reaction to promote inflammation and vascular damage (as reviewed in section 1.2). Deletion of donor-specific cells concomitant to the establishment of a new immune system may lead to donor-specific tolerance (134). The antigenic environment that the immune cells develop in is critical for the re-programming of the immune repertoire. During this period of immune reconstitution, the presence of a variety of antigens, such as donor antigen, will promote the deletion of these donor-reactive cells and allow donor-
specific tolerance to be established (as reviewed in section 1.3). Therefore, it may be possible to create a new donor tolerant immune system in the recipients.

In addition to central tolerizing mechanisms, it was also found that autologous HSCT promotes peripheral tolerizing mechanisms to self-antigens during the early reconstitution phase. Studies on 5 autologous HSCT patients have shown that the patients have a higher number of circulating Treg cells and express higher levels of Treg associated genes, such as Foxp3 and CTLA-4, compared with Tregs in a normal individual (140). The study also demonstrated that the autoreactive T cells switched from a pro-inflammatory phenotype with high IFN-γ and T-bet mRNA expression to an anti-inflammatory phenotype with high IL-10 and gata-3 expression (140). This study illustrates that autologous HSCT not only can result in a new immune system but also promote the development of a tolerogenic immune system.

Autologous HSCT has been shown to promote re-education and establishment of a new immune system and were used to treat several severe autoimmune diseases. However, using autologous HSCT to promote tolerance in solid organ transplantation has not been explored. Therefore, more studies are necessary to determine if autologous HSCT can promote donor-specific tolerance.
2.3 Hypothesis and Aims

Based on the results of autologous HSCTs in autoimmune diseases, we propose that HCST could be utilized to re-educate the immune system promoting tolerance induction to alloantigens.

To further investigate this hypothesis we will:

d) Establish a BALB/cJ to C57BL/6J heterotopic heart and syngeneic bone marrow double transplant model.

e) Investigate the effect of syngeneic T cell-depleted bone marrow transplantation on cardiac allograft survival.
Chapter 3

3. Materials and Methods

3.1. Mice

Female C3H/HeJ (MHC haplotype H-2^k), BALB/cJ (MHC haplotype H-2^d) and C57BL/6J (MHC haplotype H-2^b) mice at 4-10 weeks of age were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The transplant procedure was done on 8-12 week old recipient mice. Mice were housed in a sterile animal facility at the Toronto Medical Discovery Tower according to policies provided by the University of Health Network. Animal protocols were approved by the University Health Network in accordance with guidelines of the Canadian Council on Animal Care.

3.2. Heterotopic cardiac transplantation

Heterotopic cardiac transplantations were performed by Dr. Wei He according to the protocol reported by Corry et al. (141). Mice were anesthetised with a single dose of pentobarbital administrated intraperitoneally (i.p.) and were shaved and cleaned with 70% ethanol. The donor’s heart was first removed from the BALB/cJ animals by placing the mouse under an operating microscope at 25x magnification followed by systematic heparinization by injecting 1mL of 300 unit heparin into the inferior vena cava (IVC) of the donor mouse. The heart was harvested by ligating the IVC and the right superior vena cava (SVC) using a 5-0 silk suture. Then the pulmonary veins and the left SVC were then ligated. The donor heart was harvested and placed in a preservation solution (Baxter Healthcare, Illinois, USA) at 4°C.

Recipient mice were anesthetised and placed under the operating microscope at 6-25x magnification. The gastrointestinal organs were moved to the left side of the animal and covered with saline soaked gauze to expose the abdominal aorta and the IVC between the renal vessels.
Iliac bifurcation was conducted using cotton swabs and micro-bipolar forceps. The lumbar vessel originating from the exposed great vessel were ligated using 7-0 silk sutures. A bulldog clamper was placed on to the exposed great vessels to interrupt the blood flow. Aortotomy, surgical incision into the aorta, was conducted on the abdominal aorta using a 30-gauge needle, and then the hole was extended longitudinally using micro-spring scissors to match this aortotomy to the width of the donor aorta. The donor heart was placed into the right side of the abdomen and the heart was covered with cold saline solution buffered gauze during anastomos (surgical connection of 2 vessels). The vessels were connected by amatosing the donor aorta to the recipient abdominal aorta in an end-to-side fashion using 10-0 nylon on 4 mm needles.

The donor’s pulmonary artery and recipient’s IVC were ligated in a similar manner. Lastly, the clamps on the recipient’s abdominal aorta and IVC were removed and the transplanted heart began to beat spontaneously. The total warm ischemia time of the procedure was approximately one hour. The gastrointestinal organs were then returned to their original location and the skin incision was closed using 6-0 silk sutures.

Daily assessment of graft survival was conducted through transabdominal manual palpation and a score from 0 to 3 was given based on the strength of the heart beats. A score of 3 indicates a strong constriction and a soft with little turgor graft. A score of 2 indicates mild contraction with mildly hard turgor graft, and 1 indicates a weak contraction and hard turgor heart. Rejection was defined as a score of 0, which is the complete cessation of the graft. Rejection was further confirmed by visual examination of the graft.

### 3.3. Tolerizing protocol

The tolerance protocol was adapted from that described previously (142). Recipient C3H/HeJ mice with the BALB/cJ allografts were treated with 0.4mg/kg rapamycin (Wyeth-Ayerst, Princeton, New Jersey, USA) by i.p. injection on day 0 and 1, 2, 4, 6, 8, 10, 12, 14, and
16 days post transplant. On the other hand, recipient C57BL/6J mice with BALB/cJ allografts were treated with 2 mg/kg rapamycin (Wyeth-Ayerst) by i.p. injection from day -1 to day 13 post cardiac transplantation (14 dosages in total). The rapamycin was stored at 4°C and was diluted with phosphate-buffered saline (PBS).

3.4. Histology and Immunohistochemistry

Transplanted grafts or non-transplanted hearts were harvested and dissected into sections less than 1/3 cm in size by cutting perpendicular to the vertical axis of the heart. The peri-suture area and the apex of the heart were discarded. The remainder of the graft was immersed in 10% formalin for 48 hours. The formalin fixed tissue was submitted to the Pathology Core facility at Toronto General Hospital. Tissue was embedded in paraffin, cut into 5µm thick sections, and stained with hematoxylin & eosin (H&E).

For immunohistochemistry, the 5µm thick tissue sections were stained with anti-mouse/rat Foxp3 (Clone FJK-16s; eBioscience, San Diego, California, USA) or anti-mouse CD3 (Clone 17A2, eBioscience). The tissues were then incubated with a Horse Raddish Peroxidase (HRP) conjugated secondary anti-rat IgG antibody that allowed for colour development after addition of substrate 3,3’-diaminobenzidine (DAB) (Zymed, San Francisco, California, USA). Histology pictures were taken by Dr. Oyedele Adeyi and selected slides were scanned digitally by the pathology department at Toronto General Hospital. Positively stained cells were identified and were quantified using the computerized morphometry program, spectrum version 10.2.2.2317 (Aperio Technologies Inc., Vista, California, USA).

3.5. Isolation of lymphocytes from spleens

Spleens were harvested from mice and washed with PBS. A plunger from a 1 mL syringe was used to mash the tissue against a 40 µm filter, which separated splenocytes from the
connective tissue. The isolated splenocytes were centrifuged at 1280 rpm for 10 minutes and treated with 3mL of Red Blood Cell lysis buffer (eBioscience) to remove the red blood cells, then washed twice with PBS before further processing.

### 3.6 Isolation of lymphocytes from bone marrow

Tibia and femurs were removed from the mice using surgical scissors. The surrounding muscles were removed using sharp scissors and blades. The cleaned tibia and femurs were placed in PBS. The knee and heel of the bones were removed using scissors. The bone marrow was flushed out from the tibia and femurs with a 18 gauge needle and a 5 mL syringe by drilling the needle into one end of the bone and flushing the bone with PBS. The bone marrow was collected in a 15 mL tube and further passed through a 40 µm filter to separate the connective tissue. The sample was centrifuged at 1500 rpm for 5 minutes, further treated with 3mL of Red Blood Cell lysis buffer (eBioscience), and then washed twice before further processing.

### 3.7. 5-carboxyfluorescein diacetate succinimidyl ester dye (CFSE) and cell proliferation dye eFluor 670 labelling

The CFSE and cell proliferation dye eFluor 670 were purchased from eBioscience and prepared as 5 mM stock (CFSE) and 10 mM stock (eFluor 670). Splenocytes from recipients were isolated and resuspended at 5x 10^6 cells/mL in PBS. CFSE stock solution was used in a 1:2000 dilution in PBS, and the cells were incubated in the dark for 10 minutes at 37°C. Splenocytes of the donor mice were isolated and resuspended at 10x10^6 cells/mL in PBS. The cells were incubated with 20 µM of cells of cell proliferation dye eFluor 670 in dark for 10 minutes at 37°C. The labelling process was stopped by diluting 5 times with cold Rosewell Park Memorial Institute (RMPI)-1640 media and incubated on ice for 5 minutes. The cells were then washed twice before further processing.
3.8. FGL2 blockage CFSE proliferating assay

Splenocytes were isolated from donor and recipient mice. The donor splenocytes were exposed to 2000 rad γ-irradiation to introduce double stranded breaks that prevented cell division. The responders and the stimulators were labeled with CFSE dye or cell proliferation dye eFluor 670 respectively. The responders (4x10^5 cells) were co-cultured with the stimulators (8x10^5 cells) in a 96 well plate in the presence of 10 µg/mL anti-FGL2 antibody (clone 6H9, Abnova, Taiwan) or 10 µg/mL IgG isotype control antibody (clone RTK2071, Biolegend, San Diego, California, USA). The cells were incubated at 37°C in RPMI-1640 solution (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, Utah), 1 mM L-glutamine (Invitrogen, Carlsbad, California, USA) and 50 µM 2- mercaptoethonal (2-ME) (Sigma-Aldrich, St. Louis, Missouri). Background proliferation levels were assessed by incubation of CFSE-labeled responder cells without the stimulators. At day 5, the cells were collected and stained with anti-CD3 and the fixable viability dye eFluor 450 (eBioscience) to study the proliferative response of T cells.

3.9. Regulatory T cell in vivo depletion protocol

PC61 5.3 hybridoma (ATCC, catalog No. TIB-222, ATCC, Manassas, Virginia, USA) that produced rat anti-mouse CD25 monoclonal antibody (mAb) and was grown in Dulbecco’s modified Eagle medium (DMEM) (Wisent Bioproducts, Quebec, Canada) supplemented with 10% low IgG FBS (Thermo Scientific HyClone, Logan, Utah, USA) and 1 mM L-glutamine. The antibody was purified using standard procedure by passing the culture supernatant through a protein A column (Millipore, Billerica, Massachusetts, USA). The depletion protocol was modified from that described by W. Li et al. (143). PC61 mAb (250 µg) was injected into the recipient mice two days before the heart transplant surgery, followed by 6 more additional
injections on day 0, 3, 6, 9, 12, and 15. A rat IgG1 isotype control (clone RTK2071, Biolegend) antibody was used following the same protocol as the control. On the day of graft rejection, allografts were collected for histology analysis. The depletion was confirmed on the day of graft rejection. Splenocytes of the depleted animals were isolated and the amount of regulatory T cells was further characterized by flow cytometry. The control animals were sacrificed at day 100.

3.10. FGL2 in vivo depletion protocol

The 9D8 hybridoma (Veritas, Toronto, Canada) that produced rat anti-mouse FGL2 mAb was grown in serum-free media. The antibody was purified using standard procedure by passing the culture supernatant through a protein A column. The depletion protocol was modified from that described by W. Li et al. (3). Anti-FGL2 mAb (250 µg) were injected into the recipient mice two days before the heart transplant surgery, followed by 6 more additional injections on day 0, 3, 6, 9, 12, and 15. An IgG isotype control (clone RTK2071, Biolegend) antibody was used following the same protocol as the control. On the day of graft rejection, allografts were collected for histology analysis. The recipients were sacrificed on the day of graft rejection and the control animals were sacrificed at day 100.

3.11. T cell purification

Splenocytes were isolated from male C57BL/6J mice (Jackson Laboratory) and resuspended in PBS with 0.5% FBS and 2mM Ethylenediaminetetraacetic acid (EDTA). T cells were first purified using a negative selection mouse pan T cell isolation kit II (Miltenyi Biotec., Auburn, California, USA) following the manufacture recommended protocols. In brief, the cells were incubated with biotin-antibody cocktail for 10 minutes and anti-biotin microbeads for 15 minutes. The cells were then washed once and passed through a LS column (Miltenyi Biotec.). The flow through was collected as the purified T cell fraction. The cells were then labelled with
APC-CD90.2 antibody (clone 30-H12, Biolegend), and further purified by fluorescence-activated cell sorting (FACS). The purity was determined by flow cytometry analysis.

3.12 T cell activation

Anti-CD3ε antibody (Clone 145-2C11, eBioscience) were diluted to 0.5 µg/ml in PBS and coated on a 96-well plate for two hours at 37°C. The CFSE labelled T cells (2x10⁴ cells) were then seeded into the anti-CD3ε pre-coated 96-well plates in triplicate. To further activate the T cells, anti-CD28 antibody (Clone 37.51, Biolegend) at a concentration of 2µg/mL was added into the appropriate wells. To test the effect of FGL2 on T cell activation, different concentrations (1-2 µg/ml) of the Histidine (His)-tagged FGL2 recombinant protein, His-FGL2 (Veritas), were added into the wells. At day 4, the cells were collected, and the proliferation profile was determined by flow cytometric analysis.

3.13 Bone marrow T cell depletion protocol

Bone marrow cells were collected by flushing out the cells from femur and tibias from C57BL/6J mice. The bone marrow cells were resuspended in cytotoxicity medium (Cedarlane, Hornby, Ontario, Canada) at a concentration of 1x10⁷ cells/mL. Anti-Thy1.2 mAb (0.2 µg /1x10⁷ cells, clone 30H12, BD Pharmingen) was added and mixed with the bone marrow cells. After 60 minutes of incubation on ice, the cells were washed once with the cytotoxicity medium and then resuspended in cytotoxicity medium containing 10-fold diluted Low-Tox rabbit complement (Cedarlane, Hornby, Ontario, Canada). Following by incubation at 4°C for 60 minutes, cells were washed twice. The efficiency of the depletion protocol was examined by flow cytometric analysis.

3.14 Bone marrow transplantation
Female C57BL/6J recipient mice were exposed to 2 doses of 650 rad γ-irradiation (6 hours apart) using a cesium irradiator (Nordion International Inc., Kanata, Ontario, Canada). Within one hour after the last irradiation, the mice were injected intravenously (i.v.) with either 2x10^6 T cell depleted bone marrow cells or total bone marrow cells. Mortality was recorded daily. Body weight was obtained weekly. The mice were monitored every other day for cardiac survival.

3.15 Flow cytometry and reagents

**Antibodies and reagents utilized for flow cytometry.** Detection antibodies recognized mouse antigens and included: fluorescein isothiocyanate (FITC)-CD4, allophycocyanin (APC)-CD8α, phycoerythrin (PE)-Foxp3, peridinin-chlorophyll proteins–cyanine dye 5.5 (PerCP-Cy5.5)-CD3ε, APC-CD3ε, APC-CD90.2, FITC-CD45.1, APC-CD45.2, PE-CD32 and PE-CD19. Isotype control antibodies included: FITC- rat IgG2a, APC–rat IgG2a, PE-rat IgG2b, FITC-rat IgG2b and PE-rat IgG2b. Propidium iodide (PI) and fixable viability dye eFluor 450 were used as the viability dyes. All antibodies and reagents were purchased from eBioscience and Biolegend.

**Cell suspension.** Single cell suspension of 10^6 splenocytes were prepared in the flow cytometry staining buffer that contained PBS supplemented with 1% FBS and 5 mM EDTA. Total of 10^6 cells were resuspended in 100 µl of flow cytometry staining buffer in a polypropylene test tube and stained.

**Treg labeling.** The protocol provided by the manufacturer (eBioscience) was followed. Splenocytes (10^6 cells) were resuspended and anti-mouse CD16/32 (eBioscience) was added to block Fc receptors for 15 minutes on ice followed by incubation of FITC-CD4 and APC-CD25
antibodies for 30 minutes to detect surface CD4 and CD25 expression. For intracellular staining, cells were incubated overnight with fixation and permeabilization solution (eBioscience) and stained for intracellular FoxP3 with a PE-conjugated anti mouse/rat FoxP3 antibody (eBioscience). After 3 washes, the cells were analyzed by flow cytometry.

*Analysis.* The BD LSRII flow cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA) was used for analysis. Data were analyzed using FlowJo software version 8.8.6 (Tree Star Inc, Ashland, Oregon, USA). First, the singlet gate was set up and forward and side scatter were used to gate on lymphocytes. Viable cells were gated based on the PI or eFluor 450 negative populations when appropriate.

### 3.16. Statistics

Log-rank tests were performed to assess the statistical significance of survival data plotted on Kaplan-Meier curves. Unless otherwise specified, statistical significance of other studies was assessed using the Analysis of Variance (ANOVA) test followed by a Tukey’s Honestly Significant Difference (HSD) test as a post-hoc analysis for group comparisons. Statistical analysis was performed using Prism 5 software (Graphpad Software Inc., La Jolla, California). Differences with \( P \leq 0.05 \) were considered statistically significant.
Chapter 4

4. Results: CD4⁺CD25⁺Foxp3⁺ cells or their effector molecule FGL2 alone, promote organ acceptance.

4.1 Anti-CD25 antibody depletes CD4⁺CD25⁺Foxp3⁺ cells in the spleen and cardiac allografts.

Heterotopic cardiac transplantsations were performed by implanting the BALB/cJ female donor hearts into the abdomens of female C3H/HeJ mice (BALB/cJ → C3H/HeJ). Transplants were accompanied by 4 different treatments: rapamycin alone; rapamycin with anti-CD25 antibody; rapamycin with isotype control antibody; or no immunosuppressive treatment.

Rapamycin was given in 10 doses over 16 days post transplantation, and our lab has previously reported long-term graft survival (survival time > 100 days) with this treatment regimen (manuscript submitted). Anti-CD25 antibody or the isotype control was administered beginning 2 days before transplantation and on -2, 0, 3, 6, 9, 12 and 15 post-operative day (POD).

Cardiac grafts were monitored for beating through transabdominal palpation and a cessation of beating indicated graft rejection. On the day of rejection or POD 100 for the control groups, cardiac allografts and spleens were harvested; Tregs were then enumerated by either flow cytometry or immunohistochemistry (IHC). Similar to results found earlier by our lab, the spleens of recipients treated with rapamycin had an increased percentage of CD4⁺CD25⁺Foxp3⁺ cells when compared with the spleens of the no-treatment rejecting group (11.90 ± 1.23% versus 8.86 ± 1.28%, respectively). The rejecting recipients had a similar percentage of CD4⁺CD25⁺Foxp3⁺ cells in the spleen compared with the non-transplanted group (8.81 ± 1.49%, Figure 4-1.1A). Anti-CD25 treatment (Rapa + anti-CD25) resulted in a significant decrease in the percentage of splenic CD4⁺CD25⁺Foxp3⁺ cells to approximately 1/5th of that found in either the rapamycin treated recipients or the isotype control treated recipients (Rapa + Isotype).
(Figure 4-1.1B). Mean of the percentage of CD25⁺Foxp3⁺ cells for all the data collected is summarised as a bar graph (figure 4.1-1.1B).

Furthermore, Tregs were enumerated by staining tissue in allografts with anti-Foxp3 antibody, after rejection or 100 days post transplantation. Figure 4-1.2A compares the anti-CD25 antibody treated (Rapa + anti-CD25) group with the isotype controls (Rapa + Isotype). Histologically, there were fewer Foxp3⁺ cells in the anti-CD25 antibody treated group, as expected. Morphometric analysis was conducted to quantify Foxp3⁺ cells, expressed as a percentage of total CD3⁺ allograft-infiltrating cells (Figure 4-1.2B). The Foxp3⁺ T cell percentage in the anti-CD25 treated group and the control group were 0.94 ± 0.39 % and 12.36 ± 1.81 % respectively. In summary, anti-CD25 antibody treatment resulted in a significant decrease in the percentage of CD4⁺CD25⁺Foxp3⁺ cells in the spleen and Foxp3⁺ cells in the allografts.
Figure 4-1.1: CD4⁺CD25⁺FoxP3⁺ Tregs are depleted in the spleen with anti-CD25 antibody treatment.

BALB/cJ hearts were transplanted into C3H/HeJ recipient mice and treated with rapamycin (Rapa) in combination with a monoclonal anti-CD25 antibody (Rapa + anti-CD25) or a IgG isotype control (Rapa + Isotype). Flow cytometry used to characterise the percentage of CD4⁺CD25⁺Foxp3⁺ cells in the spleen. A, Representative flow plots displaying splenic Treg population of the CD4⁺ gated splenocytes from (i) non-transplanted mice (ii) transplant recipients receiving no treatment (iii) Rapamycin treatment alone (iv) Rapamycin in combination with anti-CD25 or (v) Rapamycin with IgG isotype control. Data were collected on the day of graft rejection or POD 100 for all groups except the no treatment group where data were collected 30 POD. The graphs are representative of 3-4 mice per group. B, Representative result of the flow cytometric analysis of the splenic CD4⁺CD25⁺Foxp3⁺ cells of the non-transplanted, no treatment, rapamycin treatment/tolerant (Rapa), anti-CD25 (Rapa + anti-CD25) and isotype control (Rapa + isotype) groups. The data are representative of 3-4 mice per group. *p<0.05 compared to Rapa + anti-CD25 group. Data represents means ± SEM of minimum of 3 mice in each group. *p<0.05; Rapa, rapamycin.
Figure 4-1.2: CD4⁺CD25⁺FoxP3⁺ Tregs are depleted in the allografts with anti-CD25 antibody treatment.

BALB/cJ hearts were transplanted into C3H/HeJ recipient mice and treated with rapamycin (Rapa) in combination with a monoclonal anti-CD25 antibody (Rapa + anti-CD25) or a IgG isotype control (Rapa + Isotype). On the day of graft rejection, graft tissue was harvested and fixed. Immunohistochemistry were used to characterise the percentage of Foxp3⁺ cells in the allograft respectively. A, Representative Foxp3⁺ immune-peroxidase staining of (i) anti-CD25 treated allograft or and (ii) IgG isotype control treated cardiac grafts (magnification 200x). B, Morphometric analysis of allografts. Cells stained positive for Foxp3 are shown as percentage of CD3⁺ allograft infiltrating cells from anti-CD25 antibody (Rapa + anti-CD25) and isotype control (Rapa + Isotype). Data represents means ± SEM of minimum of 3 mice in each group. *p< 0.05; Rapa, rapamycin.
4.2 Anti-CD25 antibody treatment results in rejection of cardiac grafts.

Anti-CD25 antibody was used to deplete Tregs in vivo in order to determine the role of Treg in the cardiac transplant model described here. Five out of six allografts were rejected by mice that received anti-CD25 antibody (mean survival time=52.5 days), whereas all allografts from mice treated with the isotope control antibody survived indefinitely (survival time > 100 days) (recall, both groups received rapamycin) (Figure 4-1.3A). Allografts from recipient C3H/HeJ mice without immunosuppressive therapy were all rejected (mean survival time=9.0 days, data not shown). Allografts from the rejecting anti-CD25 antibody treated group showed pronounced mononuclear cell infiltrates with vasculitis and haemorrhage consistent with acute cellular rejection (Figure 4-1.3B, panel i and ii). Conversely, cardiac structure remained largely preserved in the rapamycin with isotype control treated tolerant grafts (Figure 4-1.3-B, panel iii and iv).
Figure 4-1.3: Depletion of Treg results in graft rejection.

BALB/cJ hearts were transplanted into C3H/HeJ recipient mice and treated with rapamycin. Concurrent with rapamycin treatment, the mice were treated with either monoclonal antibodies to CD25 or an isotype control antibody on days -2, 0, 2, 4, 6, 8, 10, 12, and 15 POD. A, Graft survival in anti-CD25 antibody (○, mean survival time = 52.2 days, n=6) and isotype control treated (●: survival time > 100 days, n=3) recipients. Treatment with anti-CD25 antibody resulted in graft rejection on POD 29, 31, 44, 54 and 57. *p< 0.05. B, Representative H&E staining of cardiac allograft are shown. Anti-CD25 treated graft showed severe vasculitis (panel i and ii). Allografts of IgG control treated grafts had normal histology with healthy myocytes (panel iii and iv) (All panels 200x magnification).
4.3 CD3⁺ cells from tolerant mice have reduced proliferation to donor antigen.

It had previously been shown in an *in vitro* assay, that splenocytes from tolerant mice have reduced proliferative capacity in response to donor antigen. To further clarify the mechanisms and cells responsible for tolerance in the accepting donors we isolated splenocytes from mice that had accepted heart allografts (POD 30) and compared them to splenocytes isolated from both rejecting recipients (POD30) and non-transplanted C3H/HeJ mice for their proliferative capacity *in vitro*. Cellular proliferation was evaluated by flow cytometry, staining for CFSE to detect cell division and for CD3⁺ to stain for T cells. Together these data confirmed that T cells obtained from the tolerant mice had reduced proliferation when compared to the no treatment group (9.46% versus 52.9% respectively, **Figure 4-1.4**). In fact, the tolerant group had similar T cell proliferation response to the naive T cells from the non-transplanted animal (9.46% versus 7.4%, respectively).

**Figure 4-1.4**: Reduced CD3⁺ cell proliferation in response to donor antigens in tolerant mice compared to rejecting mice.

CFSE labeled C3H/HeJ responder lymphocytes were isolated from spleens of a (i) non-transplanted, (ii) rapamycin treated and (iii) no treatment and, tolerant mice at 30 POD and were co-cultured in triplicate for 5 days with irradiated splenocytes from donor (BALB/cJ) mice. This graph is representative of three independent experiments. Similar results obtained with splenocytes taken at 100 POD. The grey shaded area represents the signal of unstimulated CFSE labeled responder cells.
4.4 Inhibition of FGL2 was sufficient to restore proliferation of T cells from the tolerant mice.

In order to characterise some of the molecular mechanisms involved in tolerance, an earlier study from this lab compared the expression of 22 Treg-associated genes in syngeneic, rejecting and tolerant allografts. Interestingly, it was found that FGL2 was up-regulated in the tolerated allografts. In order to further examine the role of endogenous FGL2 in inhibiting the T cell proliferation response in vitro, CFSE labeled tolerant C3H/HeJ (POD30) splenocytes were cultured with irradiated stimulators isolated from BALB/cJ splenocytes in the presence of 10µg/ml anti-FGL2 antibody or IgG isotype control. It had been previously shown that this antibody had a neutralizing effect on FGL2 (111, 115). The addition of anti-FGL2 antibody led to enhanced proliferation of tolerant splenocytes in a one-way MLR demonstrating that endogenous FGL2 was inhibiting proliferation (Figure 4-1.5).

![Figure 4-1.5: Treatment with anti-FGL2 antibody enhances proliferation of tolerant splenocytes.](image)

Proliferation of CFSE labelled tolerant splenocytes from anti-FGL2 antibody treated or IgG isotype control treated recipients is shown as CFSE proliferation plot. The right panels show the merged results from the two groups. This graph is representative of three independent experiments. Percentage of proliferative cells labeled with CFSE is shown. The grey shaded area represents the signal of unstimulated CFSE labeled responder cells.
4.5 FGL2 blockade reverses the tolerizing effects of rapamycin *in vivo* without reducing the number of Treg cells in the allograft.

The effect of blocking FGL2 *in vivo* on tolerance induction was next examined. Heterotopic cardiac transplantations were performed by implanting BALB/cJ donor hearts into the abdomens of female C3H/HeJ mice (BALB/cJ → C3H/HeJ). While receiving rapamycin, the mice were treated with monoclonal antibodies to FGL2 (9D8) or the IgG isotype control antibody (250 µg/ip injection) beginning 2 days before transplantation and on -2, 0, 3, 6, 9, 12 and 15 POD.

All seven anti-FGL2 antibody treated recipients rejected their grafts around day 43 ± 12.8 days; whereas all of the isotype control treated grafts continue to beat until time of sacrificed at 100 POD (Figure 4-1.6A). Histological examination of grafts from the anti-FGL2 antibody treated group showed cellular rejection with severe vasculitis (Figure 4-1.6B panel i). The IgG isotype treated showed near normal histology (Figure 1-1.6B panel ii). Foxp3 staining of the allografts were also perform. Allografts in the anti-FGL2 treated group were found to have a similar number of Foxp3⁺ cells compared with the allografts in the isotype control treated group (Figure 4-1.6C).
Figure 4-1.6: FGL-2 blockade reverses the tolerizing effects of rapamycin *in vivo*.

**A**, Allograft survival in mice following rapamycin treatment and administration of either anti-FGL2 (○: mean survival time = 47.7 days, n=7) or isotype control (▲: survival time > 100 days, n=3). *p<0.05. POD indicates postoperative day. Treatment with anti-FGL2 antibody resulted in graft rejection on POD 34, 36, 43, 44, 56, 58 and 63. **B**, Graft histology in recipients treated with (i) anti-FGL2 antibody or (ii) isotype control. Black arrow indicates severe vasculitis. (hematoxylin and eosin stains; magnification 200x). **C**, Representative Foxp3+ immune-peroxidase staining of (i) anti-FGL2 treated or (ii) IgG isotype control treated cardiac grafts (magnification 100x).
4.6 FGL2 has no direct effect on the inhibition of T cell proliferation.

To investigate the mechanism of FGL2-mediated inhibition, T cells were isolated using FACS to obtained a greater than 98% pure CD3\(^+\) T cell population. The isolated T cells were stimulated with anti-CD3 (0.5µg/ml) and anti-CD28 (2µg/ml) antibodies in the presence of 1 or 2 µg/ml of FGL2; equal volumes of PBS were added in control wells for the experiment. It was found that FGL2 treatment did not directly inhibit T cell proliferation (Figure 4-1.7A). In the absence of FGL2, 93.2% of T cells divided into daughter cells. With the addition of 1 or 2µg/ml of FGL2, 92.1% or 93.0% divided daughter cells, respectively (Figure 4-1.7A). The effect of FGL2 on T cell apoptosis was also examined by annexin V staining following incubation with FGL2 for 18 hours or 24 hours. There was no difference in the percentage of annexin V positive cells in the FGL2 incubated samples compared to the vehicle controls (Figure 4-1.7B).

Expression of FcγRII from activated T cells was also determined by flow cytometry. Total splenocytes stimulated with ConA was used as a positive control as it is known that both dendritic and B cells express FcγRII receptor. 22.2% of total splenocytes expressed FcγRII receptors when activated with Concanavalin A (Figure 4-1.7C). In contrast, only 0.15% of the isolated T cells stimulated by anti-CD3 and anti-CD28 were positive for the FcγRII receptor, similar to the unstained control (Figure 4-1.7C).
Figure 4-1.7: FGL2 does not inhibit T cell proliferation directly *in vitro.*

Purified C57BL/6J splenic T cells were isolated and stimulated with anti-CD3 (0.5µg/ml) and anti-CD28 (2µg/ml) antibodies for 4 days in the presence of 1 or 2 µg/ml FGL2 or PBS. **A,** CFSE proliferation profile of (i) 1 µg/mL FGL2 and (ii) 2 µg/mL FGL2 stimulated splenic T cells. **B,** Dying cells shown as a percentage of annexin V positive CD3\(^+\) T cells, recorded by flow cytometry. Graph shows mean of 3 independent experiments +/- SEM. **C,** Flow cytometric plot showing the expression of FcyRIIB positive cells of (i) total splenocytes, (ii) pure T cells expressed as a percentage of viable cells. Data for panels A-C are representative of 3 independent experiments performed in triplicate.
Chapter 5

5. Results: T-depletion of BMT leads to a trend of prolonged graft survival compared with total BMT.

5.1 Rapamycin treatment results in better-preserved cardiac allografts than cyclosporine A and FK-506.

It is known that rapamycin treatment alone is not sufficient in inducing tolerance in organ transplant patients. Therefore, our lab attempted to investigate another potential tolerance induction therapy. Autologous HSCT following intensive immune ablation treatment has been shown to be able to re-educate the immune system and create a new immune system (134). We hypothesized that following lethal dosage of irradiation treatment, the transferred cells will develop in an environment in the presence of donor antigen and further allow donor specific tolerance to be induced in the cardiac graft recipients.

We conducted further study with a different strain combination, BALB/cJ donors transplanted into C57BL/6J recipients. Once set up, we will use this model to investigate whether autologous HSCT could be used as a tolerance induction therapy for organ transplantation. We needed to determine the immunotherapy drug regimen required to maintain the transplant in this model. It is know that in this model, it is more difficult to induce tolerance. Prior to the bone marrow transplantation (BMT) procedure we tested three different drug immunotherapies. Cyclosporine (CsA, 20 mg/kg), rapamycin (2 mg/kg) or FK-506 (2mg/kg) were used as immunosuppressants and were administrated to the recipients daily from one day before the transplant to 13 POD, giving 14 doses in total. CsA treatment failed to protect the allografts from rejection as the grafts stopped beating while the animals were still on the CsA treatment. Allografts in the Rapamycin and FK-506 treated groups continue to beat until the day of sacrifice (POD13). Analysis of the histology of the POD13 allografts showed that rapamycin treatment
resulted in less infiltrating cells, compared with the FK-506 treated grafts (Figure 5-1.1).

Rapamycin treated grafts had nearly normal histology and only a few infiltrating lymphocytes, whereas, the FK-506 treated grafts had extensive myocyte damage with larger numbers of infiltrating cells. Furthermore, we also confirmed that rapamycin treatment will not result in long-term graft survival in this model (data not shown).

![Image](image.png)

**Figure 5-1.1: Rapamycin treatment preserves cardiac allograft.**

Representative hematoxylin and eosin (H&E) staining of cardiac allograft are shown. BALB/cJ donor hearts were transplanted into C57BL/6J recipients treated with (i) 2 mg/kg rapamycin or 2 mg/kg FK-506 daily from -1 POD to 13 POD. The allografts were collected on 13 POD. (hematoxylin and eosin stains; magnification 100x).
5.2 The T cell-depletion protocol is sufficient to deplete CD3$^+$ cells in the bone marrow.

Our intention was to promote tolerance by resetting the immune system through ablation of the hematopoietic cells of the recipient, replacing these cells with syngeneic progenitor cells. Syngeneic T cell-depleted bone marrow would be injected into lethally irradiated C57BL/6J cardiac recipients to examine if this treatment is able to prolong cardiac graft survival time. Our first step was to examine the efficiency of the T cell-depletion protocol. Bone marrow was harvested from the femur and tibia from the C57BL/6J mouse, incubated with anti-Thy1.2 plus rabbit complement to deplete the T cells in the bone marrow. Flow cytometry analysis of total bone marrow leukocytes before depletion showed 5.87% of the cells were positive for CD3; after depletion the CD3$^+$ population was reduced ~10 fold to 0.53% of CD3$^+$ cells found after the depletion procedure (Figure 5-1.2).

Figure 5-1.2: The T cell depletion protocol resulted in effective T cell depletion.

Bone marrow cells harvested from the femur and tibia bones of a C57BL/6J mouse were incubated with anti-Thy1.2 plus rabbit complement. The T cell-depleted bone marrow cells were stained with anti-CD3$\varepsilon$ antibody and the percentage of CD3$^+$ cells were determined by flow cytometry. Representative data of three independent repeats is shown.
5.3 T cell-depleted BMT results in a slower cellular recovery rate in the CD4\(^+\) compartment compared with total BMT.

A pilot study of bone marrow reconstitution was conducted using T cell-depleted and total bone marrow cells to determine the different cellular reconstitution rates between the two groups. T cell-depleted or total bone marrow cells were i.v. injected into lethally irradiated C57BL/6J recipients. Animals were weighed for 30 days to monitor their overall health. After total body irradiation, the bone marrow recipients experienced weight losts but their weights returned to the original levels by day 10 post BMT procedure (Figure 5-1.3A). The peripheral blood of these animals was collected every 10 days until day 30 post BMT to determine the percentage of CD4\(^+\), CD8\(^+\) and CD19\(^+\) cells by flow cytometry. Levels of CD4\(^+\) cells in the group that received T cell-depleted bone marrow were considerably lower compared with the group that received total BMT at day 20 (Fig 5-1.3B). Interestingly, there was no significant difference in CD8\(^+\) T cells between the groups. At 30 days post BMT, CD4\(^+\) cells returned to the similar level as the wild type (5.75%, data not shown) for both T cell-depleted (5.76%) and total bone marrow (5.11%) groups (Figure 5-1.3B). The reconstitution rate was very similar between total bone marrow and T cell-depleted BMT group in the CD8\(^+\) compartment. Very few CD19\(^+\) cells were detected at day 10 and this population increased rapidly, then stayed at the same level until day 60 for both T cell-depleted and total BMT groups (Figure 5-1.3B). After three months, the spleens of these animals were harvested and analyzed by flow cytometry to determine the percentage of CD4\(^+\), CD8\(^+\) and CD19\(^+\) splenocytes. There were no differences in the level of CD4\(^+\), CD8\(^+\) and CD19\(^+\) cells between the total bone marrow and the T cell-depleted group (Figure 5-1.3C).
Figure 5-1.3: Bone marrow transplantation with total body irradiation results in fully peripheral and splenic lymphocyte reconstitution.

The recipient mice were irradiated and reconstituted with 2x10^6 T cell-depleted of total bone marrow. A, The weight of the bone marrow transplanted mice was monitor for 30 days. B, Peripheral blood of the recipient mice were collected on day 10, 20, 30 and 60. The data are expressed as a percentage of leukocytes. C, Splenocytes were harvested at three months time point. The samples were stained with anti-CD4, CD8 and CD19 antibodies. The percentage of CD4^+, CD8^+ and CD19^+ cells were determined by flow cytometry. N=3 mice per group at each time point. Graph show mean level +/- SEM.
5.4 The reconstituted lymphocytes were derived from donor cells.

In order to determine the origin of the reconstituted cells, a lineage tracing BMT experiment was performed. Bone marrow cells were harvested from CD45.1\(^+\) donor C57BL/6J mice and injected into lethally irradiated CD45.2\(^+\) recipient mice. The peripheral blood of the recipient animals was collected on day 10, 20, 30 and 40, and the percentage of CD45.1\(^+\) and CD45.2\(^+\) lymphocytes were determined by flow cytometry. It was found that 10 days after the BMT over 95\% of the reconstituted cells expresses CD45.1 markers (donor origin) with less than 5\% cells expressing CD45.2 marker (recipient origin). This ratio was maintained stably up to 40 days after the BMT (Figure 5-1.4).

![Graph showing percentage of CD45.1\(^+\) and CD45.2\(^+\) cells in peripheral blood over time.]

**Figure 5-1.4: The reconstituted lymphocytes express donor origin phenotype.**

CD45.2\(^+\) recipient mice were irradiated and reconstituted with CD45.1\(^+\) bone marrow cells. Peripheral blood of the recipient mice was collected on day 10, 20, 30 and 40. The samples were stained with anti-CD45.1 and CD45.2 antibodies. The percentage of donor (CD45.1) and recipient origin (CD45.2) cells were determined by flow cytometry. N=3 mice per group at each time point. Graph show mean level +/- SEM.

5.5 Heterotopic heart transplantation followed by T cell-depleted syngeneic BMT results in prolongation of the cardiac graft compared with the group reconstituted with syngeneic total bone marrow.
We hypothesized that HSCs from the bone marrow of syngeneic donors could restore the immune compartment of lethally irradiated organ transplanted recipients leading to graft acceptance, due to the absence of mature cells which identify the graft tissue as foreign. As a preliminary investigation, we used T cell-depleted bone marrow cells to reconstitute the hematopoietic compartment of solid organ recipients that had undergone lethal irradiation to see whether this had any effect on graft survival.

C57BL/6J cardiac allograft recipients were treated with rapamycin daily for 14 days to prevent graft rejection during the early time point and to allow the recipients to recover from the heterotopic heart transplant. On POD13, the recipients were lethally irradiated and reconstituted with syngeneic T cell-depleted bone marrow cells. Cardiac grafts were monitored as described previously.

Cardiac allografts in the recipients that received rapamycin but no bone marrow transplant rejected on POD 36 ± 6 days (Figure 5-1.5-A). On the other hand, allograft recipients that received T cell-depleted or total bone marrow injections resulted in graft rejection on POD 48.3 ± 3.9 and POD 46.0 ± 1.7 days, respectively (Figure 5-1.5A); both group also received 14 days rapamycin treatment prior to bone marrow transplantation. Histological examination of grafts from total bone marrow and T cell-depleted bone marrow transplanted groups both showed signs of rejections, including cellular infiltrates, vasculitis and haemorrhage. However, grafts from the T cell-depleted group had reduced myocyte damage compared with total bone marrow group (Figure 5-1.5B).
Figure 5-1.5: Heterotopic heart transplant followed by syngeneic T cell-depleted bone marrow transplantation results in prolongation in the survival time of the allograft compared to the group reconstituted with syngeneic total bone marrow.

BALB/cJ hearts were transplanted into C57BL/6J recipient mice and treated with rapamycin for 14 days. The recipient mice were treated with total body irradiation and reconstituted with syngeneic T cell-depleted or total bone marrow cells. A, Graft survival in total bone marrow (●: median survival time = 46.0 days, n=3) or T cell-depleted bone marrow (☐: mean survival time = 48.3 days, n=6) reconstituted recipients. Rapamycin treated, no bone marrow reconstitution recipients (○=median survival time=35.6 days, n=3) were shown as the control. *<0.05, **<0.01 compared to rapamycin only treatment group. B, Graft histology in recipients (30 days post bone marrow transplantation) reconstituted with (i) total bone marrow or (ii) T cell-depleted bone marrow. (hematoxylin and eosin stains; magnification 200x).
5.6 Allografts from the T cell-depleted syngeneic bone marrow reconstituted group had similar level of T cells, but decreased level of macrophages and increased level of B cells compared with the allografts from total bone marrow reconstituted group.

To identify which cells contribute to the rejection process, allografts were harvested on the day of graft rejection and stained with immunohistochemical markers for CD3⁺, B220⁺ and F4-80⁺ to determine the level of T cells, B cells and macrophages respectively. The positively stained cells were then quantified by morphometric analysis. Allografts from the two groups (T cell-depleted bone marrow and total bone marrow) had similar levels of CD3⁺ T cells (15.55% and 16.17% respectively, Figure 5-1.6). Surprisingly, the group reconstituted with total bone marrow had lower levels of B220⁺ B cells and higher levels of intra-graft F4-80⁺ macrophages, compared to the T-cell depleted group (2.23% versus 9.43%; 24.58% versus 14.04%, Figure 5-1.6). Although the grafts from both groups were harvested at the day of rejection, the profiles seen in figure 5-1.6 suggests that the mechanisms of rejection might be different between the two groups.
Figure 5-1.6: Morphometric analysis of allografts from total bone marrow and T-depleted bone marrow reconstituted cardiac grafts.

Allografts were harvested from transplant recipients that had previously received either T cell-depleted (☐) or total bone marrow (□) reconstitution. Tissue was recovered on the day of graft rejection. Cells stained positive for i) CD3, ii) B220 and iii) F4-80 are shown as percentages of total cells in a given area. Data represents mean ± SEM. N= 3-6 mice in each group. *p<0.05.
Chapter 6

6. Discussion

Transplantation is currently the most effective treatment for patients with end-stage organ failure. With the development of immunosuppressive treatment, the short-term outcome of transplantation is extremely successful; however, the long-term outcomes of organ grafts suffer from limitations in presently used immunosuppressive therapies. These immunosuppressive treatments are associated with a range of complications that include: cancer, susceptibility to infectious diseases, organ failure, and inability to control chronic rejection, all resulting in increased morbidity and mortality of transplant recipients, as reviewed in section 1.2.

Therefore, an important goal of transplantation research is the development of donor-specific tolerance, that is, the ability to perform transplants without the need for long-term immunosuppression. This thesis investigates two approaches to induce donor specific tolerance. The first approach uses rapamycin to promote Treg development and explore the role of the Treg protein FGL2, an effector molecule that acts on various immune cells to reduce initiation of antigen-specific immune response. The second approach is to induce tolerance by ablating the existing immune system followed by reconstitution using autologous hematopoietic stem cell transplant (HSCT).

In order to investigate the role of Tregs and FGL2, we utilized a rapamycin induced tolerant murine cardiac transplant model previously described by Li et al. (142). In brief, donor hearts were harvested from BALB/cJ mice (MHC haplotype H-2^d) and transplanted into the peritoneal cavity of the C3H/HeJ recipient mice (MHC haplotype H-2^k). The full MHC mismatch resembles full mismatch observed in most transplant patients. Treatment of the recipient mice with rapamycin for 16 days following transplantation was sufficient to induce long-term graft function. This heterotopic heart transplant model allows for simple evaluation of
graft function via transabdominal palpation of the donor heart. The following covers some details about this model.

C3H/HeJ mice, it should be noted, have a mutation in the toll/interleukin-1 receptor domain of tlr4 resulting in a defect in lipopolysaccharide (LPS) response (144). We investigated the effect of this tlr4 deficiency in the graft survival in this model, and we found no differences in the graft survival rate between the tlr4−/− C3H/HeJ mice and the tlr4+/+ C3H/HeOuJ mice (manuscript submitted). This suggests that the tlr4 deficiency in C3H/HeJ does not contribute to the acquisition of long-term graft survival in the rapamycin-induced tolerant model. Consistent with our results it has been shown that TLRs play a relatively unimportant role in heart, kidney and pancreas transplants compared with lung, skin and intestine transplant models, presumably due to the lack of exposure to environmental antigens and commensal bacteria found in the former organs (145). In conclusion our data and previously published work suggests that tolerance induction in this mouse strain combination is not dependent on a mutation in tlr4.

Rapamycin is necessary for inducing long-term graft survival in this model, unlike in humans, after a short course with this immunosuppression there is no need to continue with the drug to maintain long-term tolerance in the C3H/HeJ recipients. Rapamycin inhibits rejection by blocking T cell cycle progression from G₁ to S phase after activation and by inducing T cell anergy. Thirdly, it has also been reported that rapamycin can expand and promote the generation of CD4+CD25+Foxp3+ Tregs (146). At the molecular level rapamycin exerts its effect by disabling the mammalian target of rapamycin (mTOR) pathway. mTOR is a kinase responsible for T cell mitogenesis. The kinase exists in two complexes, the rapamycin-sensitive complex 1 (mTORC1) and the rapamycin-insensitive mTORC2. In immune cells, mTORC1 regulates cell growth via phosphatidylinositol-3-kinases (PI3K), WNT/glycogen synthase kinases 3 (GSK3) and AMP-activated protein kinase (AMPK) signaling pathways (147). Rapamycin prevents
mTOR signaling by forming a complex with FKBP12; this complex then binds to the FKBP12-rapamycin-binding (FRB) domain of mTORC1. This binding disrupts the interaction between mTOR1 and regulatory associated proteins involving in the pathways described above, inhibiting cell growth and differentiation in several cell types of the immune system.

Rapamycin can also promote tolerance by promoting the development of Treg cells. A key transcription factor of Treg cells is Foxp3. Expression of Foxp3 is increased by the transcription factor SMAD3, which binds to the enhancer region of the Foxp3 promoter. SMAD3, together with another transcription factor NFAT, promote chromatin remodeling at the Foxp3 locus allowing the expression of Foxp3 (148). SMAD3 is inhibited when the mTOR-AKT pathway is active. As have been described previously, rapamycin binding to mTOR inhibits the mTOR-AKT pathway. Thus rapamycin indirectly leads to SMAD3 activation and subsequent induction of Foxp3 expression, increasing Treg numbers (147). A Treg increase is observed in our murine heterotopic heart transplant model, suggesting that Tregs play a role in promoting tolerance in this model.

In order to further examine the importance of Treg in this rapamycin induced tolerant cardiac transplant model, Treg cells were depleted by administering anti-CD25 antibody to the recipient mice for a period of 17 days. Anti-CD25 antibody treatment resulted in over 80% depletion of the CD25+ Tregs in the spleen. Binding of the antibody to CD25 possibly blocks activation by the endogenous ligand, IL-2. Another possible mechanism of Treg depletion with this antibody is suggested by the use of FcγRIII knockout animals: lack of this Fc receptor prevents the depletion of CD25+ Treg cells (149). Of all CD4+Foxp3+ cells in the spleen, it was observed that treatment with this antibody depleted only 44% of this population. This finding is consistent with the data reported by other investigators; Stiady et al. reported that anti-CD25 antibody treatment leads to ~47% reduction of the splenic CD4+Foxp3+ cells (149).
depletion has been reported to occur at day 8 after the injection, and the depletion continued for 2-3 weeks with the Treg number only beginning to increase again on day 22 after the last antibody treatment (149).

Treatment with anti-CD25 antibody lead to allograft rejection in rapamycin-treated animals. Rejection was not quite as rapid as in animals not given rapamycin (i.e. anti-CD25 did not completely reverse the inhibition of rejection due to rapamycin). The rejection of the anti-CD25 antibody treated cardiac grafts was observed between 14-42 days after the last antibody treatment. The delay in time of the rejection compared to the rapid rejection seen with no rapamycin might be explained by the presence of other suppressive cells. The anti-CD25 antibody treatment is only sufficient to deplete the CD25\(^+\) cells while maintaining a population of suppressor cells that are CD25\(^-\). These include CD25\(^{low}\) or CD25\(^{-}\)Foxp3\(^+\) Tregs, CD8\(^+\) regulatory T cells, double negative (DN) Tregs, NK T cells and regulatory B cells. These cells suppress the immune response through essentially similar mechanisms as the CD4\(^+\)CD25\(^{-}\)Foxp3\(^+\) Tregs (reviewed in section 1.3.2.3) of cytokine secretion and induction of immune cell death.

Treg are known to mediate graft tolerance through a number of mechanisms including generation of inhibitory cytokines, killing of effector cells, disruption of effector cell metabolism, and inhibition of DC function (83). FGL2, a member of the fibrinogen superfamily, is a newly described Treg-effector molecule with immunosuppressive properties (as reviewed in section 1.4). A possible role for FGL2 in promoting tolerance was raised by two findings in our rapamycin induced heart transplant model. First, plasma levels of FGL2 were increased in tolerant mice and secondly, the gene for FGL2 was increased in tolerant allografts (manuscript submitted). In vitro studies with depleting anti-FGL2 antibodies confirmed that FGL2 accounted for the inhibition of the effector cell responses in the CFSE proliferation assay. These findings are similar to a recent report showing that CD8\(^+\) Treg function was inhibited by anti-FGL2
antibodies in vitro (115). We also demonstrated that recipient mice treated with anti-FGL2 antibodies failed to develop tolerance and rejected their allografts.

It has been shown by others that FGL2 is secreted by CD4⁺CD25⁺Foxp3⁺ Treg and fgl2 mRNA level is increased in CD4⁺CD25⁺Foxp3⁺ Treg (103, 105, 111). Our lab previously identified a role for FGL2 in preventing DC maturation, inhibiting T cell proliferation, and skewing immune responses to a Th2 phenotype by binding FcγRIIB/III on APC including B lymphocytes, DC cells, and macrophages (104). In this study, we further showed that activated T cells do not express FcγRIIB receptor suggesting FGL2 affects APCs leading to suppression of T cell function. The finding that rapamycin-induced tolerance was blocked by either anti-CD25 or anti-FGL2 antibodies and that FGL2 is highly expressed by Treg supports the concept that FGL2 and Treg are important for the development and maintenance of tolerance in our rapamycin induced heart transplant model.

Our data further suggest that Treg induction therapy may be effective in preventing organ rejection. Human Tregs can be expanded through ex vivo or in vivo approaches (150). Ex vivo Treg expansion therapies have been tested on bone marrow transplant patients to prevent graft-versus-host disease (GVHD) (151-153). Early clinical trials showed that the Treg therapy reduces the incidence of GVHD; currently a large scale clinical trial of this protocol is on-going in Europe (the ONE study) to evaluate the role of different immune cells in the recipients (154).

Rapamycin administered to patients can be considered an in vivo Treg induction therapy. Similar to our mouse transplant data, rapamycin treatment in organ transplant patients had lead to an expansion of the number of Tregs (155). A recent study demonstrated that rapamycin treatment resulted in a significant increase in the intra-graft Foxp3⁺ cells in liver transplant recipients and allowed immunosuppression to be withdrawn in some patients (156).

In our mouse model, we showed that rapamycin treatment is sufficient to induce long-
term graft acceptance. However, we also found that the rapamycin treatment will not induce
tolerance in other mice strain combinations (e.g., BALB/cJ to C57BL/6cJ). We and others have
demonstrated that rapamycin will not induce tolerance in human organ transplant patients
(though it can promote graft acceptance through universal immunosuppression). Therefore, other
tolerance induction therapy is required to be developed.

Recently, allogeneic HSCT has been used in the clinic as a donor-specific tolerance
induction therapy for kidney transplant patients, wherein stem cells from the donor are
transplanted along with the solid organ (157). Through murine studies, it has been reported that
long-term tolerance to allogeneic donor tissues can be generated via induction of mixed
hematopoietic chimerism. Ildstad et al. demonstrated that donor-specific tolerance can be
achieved by reconstituting the irradiated host with T cell-depleted donor plus host bone marrow
cells (158). Donor-specific tolerance is confirmed by conducting a skin graft experiment. It was
found that 85% of the donor grafts were accepted. The third party allogeneic grafts rejected at a
similar time as the un-irradiated control (158). This is the first study to show that tolerance can
be induced through HSCTs using a mixture of donor and host cells. Subsequent studies on swine
and non-human primate provide further evidence that this protocol is sufficient to induce
tolerance. Allogeneic HSCT has been in clinical trails and two different groups have reported
successful bone marrow and kidney double transplant tolerant cases. These tolerant patients have
been free of immunosuppression for 2 – 13 years (157).

However, using allogeneic HSCT as a tolerance induction therapy can expose the patients
to the risk of GVHD a condition that can be fatal to as many as 15% of allogeneic HSCT
recipients (129). Although allogeneic HSCT has been shown to have some success with kidney
transplant patients, the mechanism of this protocol is still unclear; it is still important to develop
other therapies that minimize the risk to the organ transplant patients.
We have now initiated studies to determine the potential of using autologous HSCT to re-educate the recipient’s immune system to re-introduce tolerance in the recipients. Currently, autologous HSCT is used to treat patients with autoimmune diseases (159). Autologous HSCT has now been examined for its ability to treat autoimmune disease by re-educating the immune system and delete auto reactive B and T cells (reviewed in section 2.2). By applying the same rationale, we hypothesized that autologous HSCT can be used in organ transplantation to re-educate the immune system to promote donor specific tolerance. We set up a murine heterotopic heart and autologous bone marrow transplant model to investigate the potential of using syngeneic HSCT to induce allograft tolerance.

BALB/cJ donor cardiac grafts (MHC haplotype H-2^d) were transplanted into the C57BL/6J recipient mice (MHC haplotype H-2^b). In order to prevent early graft rejection, recipient mice were treated with rapamycin from day -1 to day 13 following cardiac transplantation. We showed rapamycin does not induce long-term graft survival in this model as opposed to the BALB/cJ to C3H/HeJ model. Two weeks post heart transplantation, the recipient mice received a lethal dosage of total body irradiation (TBI) and were reconstituted with T cell-depleted syngeneic bone marrow cells (BMCs). This radiation treatment depletes the donor reactive cells and creates space for the transferred cells to develop in the environment in the presence of donor antigens. The immune system is a tightly controlled system where the self-reactive clones are deleted or become anergic during the development process, as reviewed in section 1.3. In the normal state, foreign antigens are not present in the individual; therefore, the developing lymphocytes will not have a chance to react to the foreign antigens and will not be depleted. In contrast, if the lymphocytes have a chance to react to the donor antigens during the developing stage, the donor reactive cells may be deleted and allow donor specific tolerance to be induced in the host.
We did not find a significant difference in survival between the hearts grafted into mice reconstituted with T-depleted BMCs compared with hearts implanted into mice receiving total BMCs. However, there was a trend towards increased survival when T effector cells were removed. These results suggest that syngeneic T depleted bone marrow transplantation (BMT) is not sufficient to induce graft acceptance, and that other cells or factors in the BMT contribute to rejection.

Possible candidates contributing to rejection include cells from both the innate and the adaptive immune systems. Mature cells of innate immunity from the BMT could potentially respond to chemotaxic signals from the graft if it has already been damaged. Histology of the POD13 rapamycin-treated grafts revealed infiltrates and inflammation in the allografts before the bone marrow transplant procedure. These mature BMCs would include neutrophils and macrophages. Mouse bone marrow cells contain a large number of functionally competent neutrophils and these cells will be transferred into the recipients during the BMT procedure in our model (160). As well, neutrophils and monocytes that have recently developed from the BMT precursor cells introduced into the recipient can be observed in the reconstituted host by day 7 post bone marrow transplant (125). These innate immune cells will be the dominating cell type during the early reconstitution phase. Additionally, it has been reported that hearts are sensitive to radiation, thus the process of preparing the host to receive BMT might cause additional inflammatory damage to the allograft, promoting further innate immune cell recruitment (161).

It would appear that mature (i.e. CD3$^+$ cells) T cells from the bone marrow played some role in promoting macrophage activity. This is illustrated by the fact that the recipients of T depleted BMT had considerably lower numbers of macrophages in the allografts, as seen by morphometric analysis of histological slides of the grafts. The presence of more macrophages in
the tissue of total BMT recipients can lead to greater tissue damage, accelerating allograft rejection rate. It should be acknowledged that this is a cyclic process where damage tissue can recruit macrophages through engagement of PRRs, and macrophage activity through the production of reactive nitrogen and oxygen species can cause tissue damage (162).

Adaptive immune cells from both the host and the donor could be promoting allograft rejection in the T depleted recipients. After lethal dosage of irradiation, the number of lymphocytes in the host will drop dramatically, however T cells are the most radiation-resistant hematopoietic population (125). Even after a lethal dosage of irradiation, it is expected that there will be a fraction of T cells originating from the host (125). From the bone marrow donor, analysis of leukocytes by flow cytometry of the T cell-depleted bone marrow samples showed that there were still ~0.5% of T cells remained in the depleted sample, which originally would have had ~5.87% T cells. Recall that as much as 10% of total T cell population can be activated by exposure to donor tissue antigens (as reviewed in section 1.2.2), thus an enormous number of T cells are potentially reactive to the host that will have been transferred into the recipient suggesting that HSCs should be used for subsequent studies to avoid T cell mediated graft rejection.

B cells from the BM are another source of adaptive immune cells that could participate in the rejection of the allograft. Upon activation by alloreactive T cells (reviewed in section 1.2.2), donor-specific B cells produce anti-donor antibodies (163). Antibodies trigger tissue damage through recruitment of complement and independently by activating phagocytic cells through binding to Fc receptors (22). Morphometric analysis revealed that more B cells were in the allografts of T depleted BMT recipients compared with allografts from total BMT recipients. This data suggest that B cells may play a more important role in mediating allograft rejection in the T depleted BMT recipients due to the higher B cell to T cell ratio in the grafts compared with
the allografts from total bone marrow group. Lack of T cells during the early reconstitution phase may be responsible for the increase of B cells in the allograft, how the kinetics of T cell expansion would change B cell appearance in the allografts is unknown, but could be due to alterations in Th1 versus Th2 expression, which would impact B cell expansion and activity; this has not been explored, however. More studies are required to be performed to investigate the feasibility of using autologous HSCT as a tolerance induction therapy.
7. Future Directions

This study suggests that autologous BMT could be a promising tolerance induction treatment. Moreover, our group has reported some successful clinical cases using autologous HSCT treatment to induce tolerance in solid organ transplantation (unpublished data). We believe that allograft rejection that we see from the murine model would likely be avoided if primitive cells were transferred. Our data provide insights into how to refine future experiments. The subsequent studies should be conducted using HSCs instead of T cell-depleted bone marrow to reduce the possibility of transferring mature effector populations into the animals. To track expansion of the HSCs, CD45.2 HSCs could be transferred into CD45.1 recipients, allowing for characterization of the donor and host cellular components in the cardiac grafts. The immune ablation therapy should also be optimized to reduce possible cardiac damage of the allograft during the irradiation procedure. Furthermore, immunosuppressive treatment should be continued after radiation therapy to suppress the re-populated innate immune cells during the early reconstitution phase. Transferring HSCs, rather than mature cells into an environment with donor-antigens may allow re-education of the newly developed immune cells and further establish an immune system that is tolerance to donor antigens.
8. Conclusion

In conclusion, this thesis demonstrated two novel methods that could potentially be used for inducing tolerance in solid organ transplantation clinically. Chapter 4 showed that FGL2 could be a powerful tolerance induction mediator in a Treg dependent allograft transplant model. This study also provided the foundation in chapter 5 for using syngeneic BMT as a tolerance induction protocol. Future studies are needed to determine if FGL2-producing Treg can be used therapeutically to achieve tolerance in humans. Additionally, HSCs instead of T depleted BMCs should be used, with a goal of establishing a long-term graft survival model. Finding a tolerance induction therapy that does not depend on general immunosuppression will be beneficial to patients by avoiding the complications associated with long-term immunosuppressive treatment and further increases the long-term outcome of organ transplantation.
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