The Role of Notch Receptors in the Alloimmune Response

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Research conducted in the Transplantation Research Center, Brigham & Women’s Hospital and Harvard Medical School, 221 Longwood Ave, Boston, Massachusetts 02115, USA

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Thesis submitted February 2015
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- **Notch-2 Signaling is Critical to B Cell Development and Alloantibody Production**
  
  Magee CN, Shimizu T, Safa K, Najafian N, Riella LV
  
  Free Communications, World Transplant Congress, San Francisco 2014
  
  Abstract published: Am J Transplant 2014; 14 (Suppl. 3) and Transplantation 2014, 98 (Suppl. 1) 20–21

- **Notch-2 Plays a Crucial Role in B Cell Development and Alloantibody Production**
  
  Magee CN, Shimizu T, Safa K, Najafian N, Riella LV
  
  Free Communications, American Society of Nephrology, Atlanta 2013
  
  Selected as one of the Top Oral Abstracts by Trainees
  
  Abstract published: J Am Soc Nephrol 24, 2013: 23A

- **Critical Role of Notch-1 in the Alloimmune Response**
  
  Ohori S, Magee CN, Shimizu T, Siebel CW, Riella LV, Najafian N
  
  Plenary Session, American Transplant Congress, Seattle May 2013
  
  Abstract published: Am J Transplant 2013; 13 (Suppl. 5) 204

- **Notch-2 Plays a Critical Role in Alloantibody Production**
  
  Magee CN, Shimizu T, Ohori S, Siebel CW, Najafian N, Riella LV
  
  Free Communications, American Transplant Congress, Seattle 2013
  
  Recipient Young Investigator Award
  
  Abstract published: Am J Transplant 2013; 13 (Suppl. 5) 176

- **Critical Role of Notch Receptors in Alloimmunity**
  
  Magee CN, Shimizu T, Najafian N, Riella LV
  
  Free Communications, American Society of Nephrology, San Diego 2012
  
  Selected as one of the Top Oral Abstracts by Trainees
  
  Abstract published: J Am Soc Nephrol 2012:
- The Emerging Role of Notch Receptors in T cells in the Alloimmune Response.
  Magee CN, Riella LV, Shimizu T, Chandraker A, Najafian N
  Oral Presentation, Annual Fellows Conference: Advances in Organ Transplantation, Boston, May 2012

- The Role of Costimulatory Molecules in Directing the Functional Differentiation of Alloreactive T Helper Cells.
  Magee CN, Boenisch O, Najafian N
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<tr>
<td>ABMR</td>
<td>Antibody-mediated rejection</td>
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<tr>
<td>ABOi</td>
<td>ABO-incompatible</td>
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<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloprotease</td>
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<td>AECA</td>
<td>Anti-endothelial cell antibodies</td>
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<tr>
<td>aNotch-1</td>
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<td>aNotch-2</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>APRIL</td>
<td>A proliferation-inducing ligand</td>
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<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
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<td>AVMA</td>
<td>American Veterinary Medical Association</td>
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<td>BAFF</td>
<td>B cell activating factor</td>
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<td>BCMA</td>
<td>B cell maturation antigen</td>
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<td>B cell receptor</td>
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<tr>
<td>BLyS</td>
<td>B lymphocyte stimulator</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
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<td>Bronchiolitis obliterans</td>
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<tr>
<td>Breg</td>
<td>Regulatory B cell</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAA</td>
<td>Chronic allograft arteriopathy</td>
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<tr>
<td>CAN</td>
<td>Chronic allograft nephropathy</td>
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<tr>
<td>CAV</td>
<td>Cardiac allograft vasculopathy</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CLPs</td>
<td>Common lymphoid progenitors</td>
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<tr>
<td>CNIs</td>
<td>Calcineurin inhibitors</td>
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<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>DBD</td>
<td>Donor after brain death</td>
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<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>DCD</td>
<td>Donor after cardiac death</td>
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<tr>
<td>DI</td>
<td>Deionised (water)</td>
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<tr>
<td>DLL</td>
<td>Delta-like ligand</td>
</tr>
<tr>
<td>dLN</td>
<td>Draining lymph nodes</td>
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<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DNMAML</td>
<td>Dominant-negative mastermind-like protein</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
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<td>ELISA</td>
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<tr>
<td>ETPs</td>
<td>Early thymic progenitor cells</td>
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<tr>
<td>Flt3</td>
<td>Fms-like tyrosine kinase receptor 3</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
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<td>G</td>
<td>Gauge</td>
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<td>Green fluorescent protein</td>
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<tr>
<td>GSIs</td>
<td>γ-secretase inhibitors</td>
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<tr>
<td>HSCs</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<td>HLA</td>
<td>Human leucocyte antigen</td>
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<td>HMS</td>
<td>Harvard Medical School</td>
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<tr>
<td>ICCS</td>
<td>Intracellular cytokine staining</td>
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<td>IDO</td>
<td>Indoleamine 2, 3-dioxygenase</td>
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<tr>
<td>IF/TA</td>
<td>Interstitial fibrosis/tubular atrophy</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
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<td>Non-human primate</td>
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<td>NK cells</td>
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<td>nTreg</td>
<td>Natural regulatory T cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PDL1</td>
<td>Programmed death ligand 1</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>TACI</td>
<td>Transmembrane activator and calcium modulator ligand</td>
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<td>TALL-1</td>
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<td>TCMR</td>
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<td>T cell receptor</td>
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<td>TECs</td>
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<td>United Network for Organ Sharing</td>
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Summary of Thesis

Notch receptors are a family of highly conserved transmembrane receptors crucial to cell development and fate: Notch-1 plays a critical role in normal T cell development, and is further involved in T cell activation and differentiation, while Notch-2 is known to be important to B cell development. The ability to influence T and B cell fate is of great interest in the field of transplantation; however, limited data exist on the importance of Notch-1 and Notch-2 in immune regulation. The primary aim of this study was to investigate the role of Notch-1 and Notch-2 in the alloimmune response using an in vivo mouse model of solid organ transplantation and selective human anti-Notch-1 (aNotch-1) and anti-Notch-2 (aNotch-2) antibodies, with particular reference to their roles in T and B cell development and behaviour, respectively.

Inhibition of Notch-1 prolonged cardiac graft survival, primarily by expanding natural regulatory T cells (Tregs), but also by reducing effector T cells; use of aNotch-1 decreased Treg apoptosis whilst increasing Treg proliferation and suppressive function. The protective effect of aNotch-1 was abrogated by Treg depletion but not by prior thymectomy, indicating a principal effect on peripheral T cells. Furthermore, selective genetic deletion of Notch-1 on Tregs increased the proportion, proliferation and suppressive function of Tregs in vitro and in vivo. Lastly, transient Notch-1 inhibition combined with single-dose CTLA4-Ig induced long-term graft tolerance.

Notch-2 blockade also prolonged cardiac allograft survival, an effect that was associated both with a reduction in T effector cells and, most strikingly, marked changes in the B cell subsets. Near-complete loss of the marginal zone B cell subset and reduction in the plasma cell population resulted in a highly significant reduction in the levels of donor-specific antibodies.

These data reveal a promising, novel approach for immune modulation in transplantation by selectively targeting Notch-1 and Notch-2.
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Chapter One: Introduction

1.1 Solid Organ Transplantation

Transplantation is the treatment of choice for many patients with end-stage kidney, liver, lung or heart disease, and is associated with significant improvements in both the quality and quantity of life. It is, furthermore, associated with significant economic benefits. There remains, however, a considerable shortage in the availability of organs, such that many patients die each year on waiting lists. The most recent combined organ transplant data from the United Kingdom show that in the twelve months prior to 31 March 2014, 4,655 patients received an organ transplant, while 7,026 patients were listed as actively waiting for a transplant (1). Corresponding data from the United States show that in the 2012-2013 period, 28,953 organs were transplanted while 133,817 patients are currently listed as waiting for a transplant (2). It therefore becomes ever more important to optimise the outcome of clinical transplantation and to extend the lifespan of an existing transplant for as long as possible.

Although the advances in immunological screening and cross-matching techniques, combined with developments in immunosuppression achieved in the last several decades have resulted in significant reductions in the rates of early allograft loss, the rates of late allograft loss remain unacceptably high (3), with inexorable attrition due to chronic allograft dysfunction, a term encompassing both immunological and non-immunological factors (4). Indeed, a recent large series from the Mayo Clinic revealed that the majority of cases of late renal allograft loss were due to chronic antibody-mediated injury as a manifestation of the alloimmune response (5). This phenomenon, manifest in the kidney as a series of histological insults including chronic allograft arteriopathy (CAA), transplant glomerulopathy (TG) and interstitial fibrosis/tubular atrophy (IF/TA), descriptions encompassed by the umbrella term chronic allograft nephropathy (CAN), has parallels in other solid organ
transplants: cardiac allograft vasculopathy (CAV) in the heart and bronchiolitis obliterans (BO) in the lungs. Liver allografts, for reasons that are not fully understood, are subject to significantly less immune-mediated injury, and, as a consequence, are considered to be immune-privileged.

A wide variety of different strategies to manipulate the immune response have been trialed with only limited success, thought to be due to the multiple immune mechanisms simultaneously in play. It is now recognised that a multi-faceted approach is required.

1.2 Immunological Barriers To Transplantation
Normal immune homeostasis is characterised by tolerance to antigens expressed on the individual’s own cells, a process known as self-tolerance. Allografts, however, express non-self antigens, to which the recipient is not tolerant, and which are promptly recognised as foreign, thereby setting in motion a chain of immunological events which can lead to rejection of the allograft; this will be discussed in more detail later. Several types of antigens relevant to transplantation have been characterised, including the major histocompatibility complex (MHC) molecules, minor histocompatibility antigens, ABO blood group antigens, and monocyte and endothelial cell antigens. From a transplant perspective, the most important of these are the MHC antigens, also known in humans as human leucocyte antigens (HLA).

1.2.1 MHC or HLA Antigens
MHC molecules were first discovered in mice as the mediators of rejection of tumour transplants, and were subsequently shown to be responsible for rejection of other tissues (6). The ability to produce an efficient immune response to many antigens is inherited in a Mendelian autosomal dominant manner; the genes responsible for this are of the MHC. The function of the MHC is to bind peptide fragments derived from pathogens or, in the case of transplantation, foreign MHC antigens, and display them on their surface for
recognition and processing by the relevant T cells. Failure to mount an appropriate response to a peptide may be due to a genetically determined inability of the MHC to bind the antigenic peptide fragment (7). MHC antigens, initially known as H-2 in mice, were shown to initiate both a cytotoxic T cell response (8) and an antibody response (9) following an MHC-mismatched transplant. There is considerable homology between the MHC genes in mice, which are clustered on Chromosome 17 (8), and the human HLA genes, which are located on the short arm of Chromosome 6. The MHC is both polygenic, containing several different MHC class I and class II genes, and highly polymorphic, with multiple variants of the same gene within the population. The MHC consists of approximately 4 million base pairs of DNA, and contains more than 200 genes (10).

In humans, there are six identified MHC Class I isotypes and five identified Class II isotypes. Of the Class I isotypes, HLA-A, HLA-B and HLA-C are highly polymorphic and function both to present antigens to CD8 T cells and form ligands for Natural Killer (NK) cell receptors; of the remaining Class I isotypes, HLA-E and HLA-G are oligomorphic and also function to form ligands for NK cell receptors, while the function of the apparently monomorphic HLA-F is unknown (10). Each Class I MHC molecule consists of two polypeptide chains which are non-covalently associated: the heavy chain or α chain is inserted into the plasma membrane and contains the polymorphic residues which are located in the α1 and α2 domains; the invariant light chain is β2-microglobulin, the gene for which lies outside the MHC on Chromosome 15. The polymorphisms serve to define the shape of the peptide-binding groove, thereby determining which peptides will be bound and presented to T cells (11). Class I MHC molecules are expressed on all nucleated cells, including blood vessel endothelium.

The highly polymorphic Class II isotypes HLA-DR, HLA-DP and HLA-DQ function to present antigens to CD4 T cells; the remaining Class II isotypes, HLA-DM and HLA-DO, are oligomorphic and regulate the peptide loading of
HLA-DR, HLA-DP and HLA-DQ (10). Each Class II MHC molecule consists of two membrane-inserted and non-covalently associated glycosylated polypeptide chains, α and β, each of which has two domains. In contrast to Class I molecules, both the α and β chains contribute to Class II molecule polymorphism, with the exception of HLA-DR, in which the α chain is invariant. Class II molecules are expressed on antigen presenting cells, including dendritic cells, macrophages and B cells (12, 13), and may also be expressed on activated T cells (14).

MHC genes are co-dominantly expressed and are inherited from each parent as a linked set of alleles known as a haplotype. HLA-heterozygous individuals can therefore express up to six Class I isoforms and six or eight Class II isoforms that present antigen to T cells (an individual may have two functional HLA-DRB genes). The distribution of HLA antigens in the population is not random, with some alleles more common than others depending on geographical location and racial or ethnic origin. Furthermore, within a particular racial or ethnic group, certain haplotypes are found with greater frequency than would be predicted by random distribution such that these alleles are said to be in linkage disequilibrium. In some cases, the existence of linkage disequilibrium may be due to selective survival or inheritance, over many generations, of a combination of alleles that that provides defense against certain infectious diseases. From a transplant perspective, the distribution of HLA antigens in a particular population is clearly of concern when attempting to HLA-match donors to recipients. There is longstanding evidence that long-term graft survival is improved by avoiding, or at least minimising, HLA mismatches. Data from the United States show that the 5 year allograft survival is greater for 0-HLA–A, –B and –DR antigen mismatch compared to 6-HLA antigen mismatch kidneys, irrespective of whether they are living (88% vs 79%), deceased standard criteria (75% vs 66%) or deceased extended criteria donors (60% vs 55%). Indeed, current organ allocation policy in the United States, as directed by the United Network for Organ Sharing (UNOS), mandates that 0-HLA–A, –B
and –DR antigen mismatch deceased donor kidneys be shared amongst centres. There is also evidence that the HLA locus at which the match or mismatch exists exerts a relative influence (15): compared to graft survival rates where no HLA–A, –B or –DR mismatch exists, the presence of a single mismatch for HLA–A, –B or –DR increases the risk of graft loss two-, three- or five-fold, respectively (16). Unsurprisingly, the HLA-matching component of the current allocation policy in the United Kingdom is weighted accordingly to prioritise DR matching (17).

### 1.2.2 Non-HLA Antigens

In recent years, the immunogenic role of non-HLA antigens has increasingly been recognised. These antigens are expressed on both endothelial and epithelial allograft cells and include major histocompatibility complex class I chain-related gene A (MICA) or major histocompatibility complex class I chain-related gene B (MICB), or tissue-specific autoantigens such as angiotensin II receptor type I (AT1), vimentin, cardiac myosin (CM) and collagen V (Col V). MICA and MICB are highly polymorphic genes that are located near the HLA-B locus and bear some homology to MHC Class I. They are strongly implicated in innate immunity and act as ligands for the NK cell receptor NKG2D. They are expressed on the cell surface of a number of cell types, including endothelial cells (ECs), epithelial cells, fibroblasts, dendritic cells (DCs), and activated T and B cells. The presence or development of anti-MICA antibodies has been associated with poorer graft survival in both kidney and heart transplantation (18-20), although this association has not been borne out in all studies (21, 22).

Endothelial cells, resident in a critical location between intravascular and interstitial compartments, express tissue-specific antigens against which antibodies may also develop: tyrosine kinase with immunoglobulin-like and EGF-like domains 1 and 2 (TIE-1 and TIE-2) are cell surface proteins expressed by endothelial cells that bind and are activated by the angiopoietins. The presence of anti-endothelial cell antibodies (AECA) is associated with a
greater rate of kidney allograft rejection and lower graft function (23). The development of new, flow cytometry-based assays to detect anti-endothelial cell antibodies using isolated, TIE-2-enriched, donor endothelial cells will hopefully facilitate expansion of the conventional cross-match to include endothelial antigens, thereby allowing better risk stratification of patients (24, 25). Similarly, the presence of angiotensin II type-1 receptor (AT1R) antibodies, agonistic antibodies directed against 2 epitopes on the extracellular loops of the AT1 receptor, has been associated with a significant proportion of steroid-refractory antibody-mediated kidney graft rejection and, indeed, a significant minority of overall rejection episodes (26, 27). The use of Losartan, an AT1R antagonist, has been successfully employed to treat these rejection episodes (27).

1.2.3 ABO Blood Group Antigens
The ABO blood group antigens were initially identified in the late 1950s as the cause of transfusion reactions during red blood cell transfusions (28). The A and B groups are glycosylated differentially, whereas group O lacks the enzymes necessary for glycosylation. The A and B antigens are readily recognised by natural antibodies, termed haemagglutinins, that cause red cell agglutination. Individuals of blood group O have antibodies to both A and B antigens, those of blood group AB have antibodies to neither, while individuals of blood group A have antibodies directed against B antigens, and vice versa; as is the case in blood donation, individuals of blood group O are therefore universal donors, while those of blood group AB are universal recipients. From a transplant perspective, these antibodies are highly relevant, as, due to the expression of blood group antigens on endothelial cells, they may precipitate hyperacute rejection. Other red cell antigens, including rhesus (Rh) factor, are not pertinent, as they are not expressed on endothelial cells.

In last few decades, great progress has been made in desensitising potential recipients to A and B antigens to enable ABO-incompatible (ABOi) pairs to proceed with transplantation. A variety of desensitisation protocols exist, most
commonly involving a combination of plasma exchange, intravenous immunoglobulin (IVIG) and a B-cell depleting agent (e.g., rituximab).

Interestingly, ABOi transplants have been shown to display a phenomenon known as accommodation: following the temporary removal of circulating anti-A or anti-B antibodies in the peri-transplant period, there is subsequent evidence of return of circulating antibody (29), persistence of expression of A or B antigens on the graft endothelium (30) and antibody binding (31) to the relevant A or B antigens in the graft, but in the absence of any apparent allograft injury (31, 32). Proposed mechanisms have included a change in the binding characteristics of the antibody, alteration in the antigen, or some modification of the graft itself whereby it is capable of resisting antibody-mediated injury. The presence of complement deposition in these grafts argues against the first two, as it provides evidence of antibody binding to its cognate antigen, thereby indicating that it is the subsequent response that is altered (32). While the mechanism of accommodation indicates an alteration in immune reactivity that may, theoretically, either become injurious or facilitate the development of detrimental chronic immune responses at a later stage, the long-term outcomes of ABOi transplantation have been very encouraging, with graft survival equivalent to or exceeding that of ABO-compatible transplantation (33-35).

1.3 The Alloimmune Response
The alloimmune response is a complex interplay between pathogenic/inflammatory and regulatory/anti-inflammatory immune mechanisms; the supremacy of either process determines whether the ultimate fate of the allograft is rejection or tolerance, respectively (36-38). True transplant tolerance refers to selective and sustained hyporesponsiveness of the recipient’s immune system to donor alloantigens whilst maintaining an otherwise intact immune response; there is experimental data to support the achievement of true tolerance in animal models, and, although rare, there are isolated reports of the attainment of operational tolerance in the clinical setting, achieved through a variety of treatment regimens (39).
The alloimmune response is critically dependent on T cells, B cells, antigen-presenting cells (APCs) and the interaction thereof. The recognition of allogeneic MHC molecules by recipient T cells occurs in two ways: the first, termed direct allorecognition, occurs following the migration of local, antigen-loaded donor APCs, mainly DCs, out of the allograft towards secondary lymphoid organs where they encounter naïve recipient alloreactive T cells and initiate an immune response following the presentation of donor MHC antigens by donor APCs. The second, termed indirect allorecognition, involves the processing of donor MHC molecules by circulating recipient APCs, which initially endocytose the MHC molecules and process them into peptides, before later expressing them on their surface, from where they can be presented to naïve recipient alloreactive T cells.
Three distinct signals are required for activation of a naïve T cell. The first, Signal 1, is provided upon the presentation of an antigen to its cognate T cell receptor (TCR)/CD3 complex by MHC molecules on the surface of an APC; in transplantation, as discussed above, this can either be a donor or recipient APC. Signal 2 is provided by co-stimulatory molecules expressed by the APC. Co-stimulatory molecules are a heterogeneous group of cell surface molecules that act to amplify or counteract the initial activating signals provided to T cells from the TCR following its interaction with an antigen/MHC, thereby influencing T cell differentiation and fate. They belong to three major families, namely the immunoglobulin (Ig) superfamily, the tumor necrosis factor (TNF) – TNF receptor (TNFR) superfamily and the emerging T cell Ig and mucin (TIM) domain family; they may additionally be classified according to the nature of the signal they provide. They cannot activate T cells independently, but are critical to the functional naïve T cell response, the nature of which depends upon the outcome of integration of these stimulatory or inhibitory signals (40, 41).

In the absence of co-stimulation or Signal 2, TCR stimulation of naïve T cells induces T cell anergy in vitro, precluding an effective T cell response and promoting tolerance (42). Upon successful activation, however, the naïve T cell develops into an effector cell, and may subsequently develop into an effector or central memory cell (43); depending upon the nature and duration of antigenic stimulus, it may later display markers of exhaustion.

Successful activation of the T cell by Signal 1 and 2 leads to the initiation of Signal 3, achieved by the provision of various signals from the APC to the T cell that then determine proliferation and differentiation of the activated T cell. While the secretion of various inflammatory cytokines by APCs has long been known to influence T cell differentiation, it is increasingly recognised that there are a number of other signaling pathways whose activity may mediate this process. One such potential influence is the Notch signaling pathway; indeed, there is a growing body of data that supports its role as a Signal 3 mediator, with
evidence of involvement in a broad range of differentiation activities, the mechanisms of which will be discussed in more detail later (44, 45).

Knowledge of the different co-stimulatory pathways involved in the various stages of the immune response is crucial: it is now apparent that memory T cells resist many of the therapeutic strategies employed to target naïve alloimmune responses, while there is increasing interest in the feasibility of manipulating signals involved in T cell exhaustion for therapeutic benefit in chronic viral infections and malignancy. As with many immune mechanisms, there is a degree of functional overlap between certain co-stimulatory molecules, while some have diametrically opposite effects on different T cell subsets despite sharing common ligands. This is a critical point when considering these molecules as therapeutic targets in transplantation, as blockade of a particular co-stimulatory pathway, whilst desirable in itself, may prevent the ligation of an essential regulatory co-inhibitory molecule.

**Figure 1.2. Schematic representation of T cell activation**
Diagram author’s own.
1.3.1 T Cells

T cells originate from a variety of bone marrow progenitors: haematopoietic stem cells (HSCs) which are, by definition, long-lasting, self-renewing, and capable of generating all haematopoietic lineages; multipotent progenitors (MPPs) which, although no longer capable of self-renewal, retain the ability to differentiate into multiple lineages; lymphoid primed multipotent progenitors (LMPPs) which are characterised by high expression of the fms-like tyrosine kinase receptor-3 (Flt-3) and are predisposed towards lymphoid and granulocyte/macrophage lineages; and common lymphoid progenitors (CLPs) which comprise a heterogeneous cell population functionally distinguishable by their expression of the cell surface marker Ly6D: Ly6D− CLPs are capable of generating T cells, B cells, NK cells, DCs and myeloid cells, whereas Ly6D+ CLPs are predominantly restricted towards the B cell lineage (46, 47).

Similarly, in humans, multiple prethymic progenitor cells with T cell lineage potential have been identified; the most efficient, a population known as multitymphoid progenitors, has mixed myeloid and lymphoid potential, and can give rise to T cells, B cells, NK cells, DCs, monocytes and macrophages, but not granulocytes (48). Nevertheless, it is thought that the majority do not make a physiological contribution to the T cell lineage; unlike other blood cell types, T cells complete their differentiation and maturation within the thymus (46), and the thymus is only seeded by a small proportion of these precursors, termed thymic seeding progenitor cells (TSPs). For example, despite their ability to efficiently generate T cells if injected intrathymically, very few, if any, HSCs are evident within the thymus, while HSCs injected intravenously do not give rise to T cells for several weeks, indicating that HSCs cannot enter the thymus under physiological conditions. It is thought that this may be related to lack of expression of the chemokine receptor CCR9, which has been shown to confer the capacity to migrate to and enter the thymus (49), although CCR9-independent mechanisms of thymus-homing have been shown to exist (49, 50).

Maintenance of thymic T cell development requires intermittent or continuous influx of haematopoietic progenitors; these progenitors enter the thymus at the
cortico-medullary junction (51), and at least some descendants populate the perimedullary cortical zone for a protracted period of time before migrating outwards towards the subcapsular zone (52).

The earliest intrathymic T cell precursors are CD4−CD8− and are termed double-negative (DN) cells; they are further sub-divided into stages of DN development according to their expression of the cell surface markers CD44 and CD25. DN1 cells (CD44+CD25−) have been shown to proliferate extensively; they are heterogenous, and have the potential to generate αβ T cells, γδ T cells, B cells, dendritic cells, macrophages and NK cells (46, 53); within the DN1 population however, only the subset expressing c-kit or CD117, a tyrosine kinase receptor, efficiently give rise to T lineage cells (54). These cells are known as early thymic progenitor cells (ETPs) and comprise approximately 0.01% of total thymocytes; despite this, however, they are capable of efficiently repopulating thymocyte populations following intrathymic transfer (55). ETPs are also capable of giving rise to myeloid and NK lineages, although the majority do not yield B cells in vitro; this lineage potential is gradually lost as cells develop towards the DN2 (CD44+CD25+) stage, whereupon they migrate outwards from their initial position in the perimedullary cortex, trafficking to the inner cortex; T cell lineage commitment can only be considered definitive once the DN3 (CD44−CD25+) stage has been reached (46), which occurs upon migration of DN2 cells to the outer cortex (56). DN3 cells then undertake widespread TCRβ, γ and δ gene loci rearrangements: some differentiate into γδ T cells, while others undergo further proliferation to generate αβ T cells, which, in turn, give rise to CD4+CD8+ double positive (DP) cells (57), the most populous subset within the thymus. DP thymocytes initiate TCRα locus rearrangement, following which they undergo negative selection on the basis of their antigen receptor, developing finally into CD4 and CD8 single positive (SP) cells that can migrate out of the thymus to the periphery, where they circulate as naïve but immunocompetent T cells.
CD4⁺ T cells, or T helper cells, play a central role in the determination of the adaptive immune response in the settings of autoimmunity, allergy, and alloimmunity. Upon interaction with their cognate antigen via the T cell receptor (TCR) and their subsequent activation, as described earlier, naïve CD4⁺ T cells can differentiate into various lineages, including the classical effector T helper type 1 (Th1) and type 2 (Th2) subsets, and the more recently identified Th17, T follicular helper (Tfh), Th9 and induced regulatory (iTreg) T cell populations (58-62). These subsets are functionally distinct and may be identified by the production of characteristic cytokines (63).

The differentiation decision is influenced by a variety of factors, including the cytokine milieu in which this interaction occurs; the strength of the TCR signal (64); the affinity of the TCR for the antigen (65); and the nature of the co-stimulatory molecules providing the second signal to the TCR complex (41). CD4⁺ T cell differentiation was, until recently, considered to be a terminal event. However, considerable plasticity is now recognised to exist amongst many T helper subsets (66); indeed, Tregs and Th17 cells have been shown to be capable of reciprocal differentiation (67, 68).

As mentioned earlier, there is accumulating data to support the involvement of Notch signaling in the differentiation pathway, where it is thought to act as a Signal 3 mediator. Evidence for involvement of Notch signaling is provided by studies showing both increased expression of Notch ligands on APCs following exposure to TLR ligands (69) and increased Notch signaling following TCR ligation (70), while the Notch receptors have been shown to co-localise with CD4 in CD4⁺ T cells (70) and the E3 ligase Numb at the T cell – APC interface (71). These data will be discussed further in Section 1.5.1.
**Figure 1.3. CD4⁺ T helper cell differentiation**

The T helper 1 (Th1), Th2, Th17, Th9, Tfh and Treg lineages are shown, along with the transcription factors and cytokines required for their respective differentiation; their subsequent cytokine production is also indicated. Diagram author's own.
Natural regulatory T cells (nTregs) are formed in the thymus and are characterised by the expression of the transcription factor Foxp3. Adaptive or induced Tregs (iTregs) may also be generated in the periphery from CD4⁺CD25⁻ cells in the presence of TGFβ (58). Tregs are critical to the development and maintenance of self-tolerance (72), and, in transplantation, are indispensable to the induction and maintenance of allograft tolerance. While inducibly expressed on conventional T cells, cytotoxic T lymphocyte antigen 4 (CTLA-4) is constitutively expressed by Tregs, and is central to their normal homeostasis and function (42); indeed, mice deficient in CTLA-4 develop catastrophic lymphoproliferation and inflammation due to unchecked CD4⁺ T cell activation by environmental antigens. It remains controversial whether CTLA4 mediates its effects through cell-intrinsic or -extrinsic mechanisms, or both (42); there are data to indicate that it may involve inhibition of the Erk and Akt pathways; additionally, B7 ligation by CTLA-4 induces the enzyme indoleamine 2, 3-dioxygenase (IDO) which inhibits T cell proliferation and has been implicated in the maintenance of tolerance in a variety of settings (73). Furthermore, a recent study provided new insights into its immunoregulatory activity by demonstrating that CTLA-4 acts in a cell-extrinsic manner to capture its ligands B7-1 and B7-2 from other cells by trans-endocytosis, leading to their degradation inside CTLA-4 expressing cells, and preventing their ligation with CD28 (74); this was shown to be true for both activated effector cells and Tregs (74).

The development and approval of belatacept (75), a mutant form of CTLA4-Ig, for clinical use in transplantation renders these mechanisms particularly important: as CTLA-4-Ig functions by competing with CD28 for B7-1/2 binding, it would also block the CTLA-4/B7 interaction, and, consequently, inhibitory cell-intrinsic signaling. The recent identification of B7-1 as an additional ligand for PDL-1 is also of concern, as regulatory signaling mediated by PDL-1 through this pathway (76, 77) would also be blocked by CTLA4-Ig. Indeed, initial results from the Phase III belatacept trials reported an unexpected increase in early
acute rejection in the belatacept-treated group (78), causing significant concern. A recent paper from our group demonstrated that use of hCTLA4-Ig significantly inhibited the generation of nTregs in naive mice (79), and, as a corollary to data discussed above, prevented rejection in a fully MHC-mismatched cardiac transplant model, but accelerated cellular rejection in an MHC Class II mismatch model, in which long-term graft survival is dependent upon the emergence of Tregs (79).

1.3.2 B Cells

B cells, so named for their origin in bone marrow, or in the Bursa of Fabricius in birds, play a critical role in the adaptive immune response, with principal functions of antigen presentation, antibody formation and the generation of memory subsets; recently, a subset of B cells with suppressor functions has also been described (80, 81). Similar to T cell development, described above, B cells originate in the bone marrow from haematopoietic stem cells and progress through a series of precursors, the earliest of which is thought to be a subset of HSCs known as early lymphoid progenitors (ELPs); the appearance of this population from within the HSC colony appears to be dependent on the timely expression of the transcription factors Ikaros, PU.1, E2A and EBF1 (early B cell factor 1) (82). ELPs then progress to express CD127, the IL-7α receptor (IL-7Rα), a change that marks them as common lymphoid progenitors (CLPs). As discussed previously, CLPs are a heterogenous cell population functionally distinguishable by their expression of the cell surface marker Ly6D: Ly6D+ CLPs are capable of generating T cells, B cells, NK cells, DCs and myeloid cells, whereas Ly6D+ CLPs upregulate the B cell transcription factors EBF1 and Pax5, and are predominantly restricted towards the B cell lineage (46, 47).

Mice deficient in IL-7 or IL-7Rα show both a significant decrease in CLP numbers and a profound impairment in their ability to undergo further B cell differentiation and, moreover, impaired capacity to undergo cytokine-induced
expansion (83, 84). It is thought that this reliance on IL-7R signaling may be
due to involvement of IL-7R in modulating transcription factors necessary for
induction of the B cell specific transcription factor EBF1 (83, 84). EBF1
thereafter regulates its own expression both directly and indirectly: directly, by
induction of the EBF1α promoter; and indirectly, by upregulating the expression
of Pax5, which in turn leads to induction of the EBF1β promoter (85).
Interestingly, although many transcription factors act co-operatively to regulate
the expression of B cell genes, E2A and EBF1 are the only factors whose
synergistic activity has, to date, been shown to be necessary to achieve B cell
development, potentially due to modulation of Pax5, an essential B lineage
commitment factor (82). Pax5 is expressed at remarkably stable levels
throughout B cell development from the pro-B stage through to its
downregulation at the plasma cell stage and it is thought that Pax5 may
promote commitment to the B cell lineage by repressing the expression of non
B cell genes; indeed, pro-B cells deficient in Pax5 express many genes
associated with multi-potent progenitors or non-B lineage cells, and are,
furthermore, capable of differentiating into a broad spectrum of haematopoietic
cell types (86).

Pre-pro B cells are the first clearly identifiable committed B cell progenitors
generated from CLPs in the bone marrow; they are alternatively termed fraction
A or CLP-2 cells and can be identified by expression of the B cell marker B220
in the absence of cell surface immunoglobulin (82, 87). There are three further
B cell precursor subsets, termed Fraction B-D, respectively, which are also
positive for expression of B220 but negative for cell surface immunoglobulin;
these fractions can be distinguished by transition in cell surface phenotype,
differences in functional activity and by immunoglobulin gene rearrangements
(88-91). B cell precursors first assemble a heavy chain diversity and junction
(DJ) rearrangement, which is then followed by heavy chain variable DJ (VDJ)
rearrangements (87); most fraction A or pre-pro B cells lack such heavy chain
DJ rearrangements, suggesting that early B cell precursor development is
independent of immunoglobulin gene rearrangements (90). Fractions B and C, known as pro B cells, consist mainly of large mitotically active cells which have heavy chain DJ or VDJ rearrangements, while Fraction D is mainly comprised of small resting cells, termed pre B cells (88, 89). Immature B cells, which express surface IgM but not IgG, are generated in the bone marrow from the cells in Fraction D, a process that occurs throughout postnatal life in both mice and humans. Up to 70-75% of these cells are self-reactive (92, 93), wherein BCR signaling plays an important role in negative selection of self-reactive clones: cells that encounter self-antigen are either deleted, undergo receptor editing, or become anergic, depending on both the affinity and the physical form of the antigen encountered (94). It is thought that high-affinity interactions with membrane-bound antigens result in elimination and clonal deletion, whereas lower-affinity interactions and soluble antigens either permit BCR revision or result in anergy. Despite this, however, many self-reactive clones still emerge from the bone marrow into the immature peripheral B cell pool, although it is thought that only 10-30% of these immature B cells eventually contribute to the mature B cell pool (95). Following their emergence from the bone marrow, these immature B cells home to the spleen, where they progress through two transitional B cell stages, type 1 (T1) and type 2 (T2), before developing into peripheral mature B cells and plasma cells. Interestingly, plasma cells, a subset containing both short- and long-lived cells (96, 97) which develop following the activation of mature B cells by antigen in peripheral lymphoid organs, subsequently return to populate the bone marrow, a process dependent on the expression of CXC-chemokine receptor 4 (CXCR4) (98, 99).

Mature peripheral B cells include B1 cells, which predominantly reside in the pleural and peritoneal cavities, and B2 cells, which are considered the conventional subset (100). The B2 population is composed of follicular B cells (FO), which represent the largest peripheral B cell subset, and marginal zone B cells (MZ), so-called due to their location in the marginal zone of the spleen (101), the junction between the non-lymphoid red pulp and the lymphoid white
pulp, and which comprise approximately 5% of the total B cell population. FO and MZ B cells are both phenotypically and functionally distinct: FO B cells are IgD^{hi}CD21^{int}CD24^{hi}, whereas MZ B cells are IgM^{hi}CD21^{hi}CD24^{hi}. FO B cells actively recirculate through lymph nodes and splenic lymphoid follicles (102), where they encounter activated T cells and undergo germinal center reactions (103). MZ B cells are thought to arise from the T2 transitional B cell subset (104), and have been shown to mediate rapid antibody responses to blood-borne antigens in both a T cell-dependent (105) and -independent manner (106). Compared to FO B cells, they have been shown to be partially activated, express higher basal levels of costimulatory molecules and develop into plasma cells more rapidly following activation (107, 108); furthermore, they are far superior to FO B cells in inducing naïve CD4^{+} T cell expansion (109).

![Diagram of B cell maturation and development](https://via.placeholder.com/150)

**Figure 1.4. Schematic representation of peripheral B cell maturation and development**

Imm B, immature B cell emerging from bone marrow; T1 B, transitional B cells stage 1; T2B, transitional B cells stage 2; MZ, marginal zone B cells; MZ prog, marginal zone progenitors; FO, follicular B cells. Diagram author’s own.
While Notch signaling has been shown to be important in T cell development and differentiation, it is also centrally involved in normal B cell development: Notch-2 signaling has been shown to play a critical, non-redundant role in the development of marginal zone B cells (104), and has further been implicated in the development of B1 B cells (110). Later stages of B cell activity, including B cell activation and terminal differentiation into antibody-secreting cells (ASCs), have also been shown to be regulated by Notch signaling (111). The role of Notch signaling in B cell development will be discussed in detail later.

Figure 1.5. The mouse splenic marginal zone
A terminal arteriole branches from a central arteriole and opens into a sinus that is present at the margin of the B cell follicle, termed the marginal sinus. Gaps exist between cells lining the outer boundary of the sinus and blood that passes through these gaps filters by MZ B cells (purple), macrophages (pink) and DCs (green) on its way to the red pulp. Marginal metallophilic macrophages (blue) on the inner side of the sinus are also considered part of the MZ. FO B cells are in green and T zone T cells are in red. Blood flow is indicated by arrows. Adapted from Nature Immunology 1, 9 - 10 (2000).
In recent years, the discovery of a TNF superfamily axis, composed of B cell activating factor (BAFF), known also as B lymphocyte stimulator (BLyS) and TNF- and APOL-related leucocyte expressed ligand (TALL-1), and its homologue, A proliferation-inducing ligand (APRIL), has been heralded with great interest, as these molecules have been shown to be crucial for B cell development and survival, providing a further opportunity to pharmacologically manipulate B cell development and behaviour. BAFF is mainly produced by monocytes, dendritic cells and bone marrow stromal cells as a membrane-bound protein; it is subsequently cleaved to produce a soluble form that binds three different TNF receptors: BAFF receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA). The receptors, all of which bind BAFF with varying affinities, are differentially expressed on B cells at various stages; with the exception of BAFF-R, they are also shared with the cytokine APRIL, which limits its actions to plasmablasts and long-lived plasma cells (112). Ligation of these receptors initiates prosurvival signals, achieved both by signaling through nuclear factor-κB (NF-κB) pathways, and by activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, which results in the degradation of pro-apoptotic proteins, including the Bcl-2 family member Bim (113). BAFF-R expression begins at the late transitional stage and is present on all mature B cells, with the exception of plasmablasts and plasma cells; TACI is expressed following the late transitional cell stage and is also present on plasmablasts and plasma cells, while BCMA expression is entirely restricted to the latter subsets. BAFF signaling cross-talks with BCR signaling in preferentially promoting the survival of non self-reactive B cells: where BAFF is limited, BCR ligation by antigen leads to anergy and reduced immature transitional B cell lifespan, while BCR ligation by self-antigen leads to down-regulation of surface expression of BAFF-R (114); conversely, the presence of BAFF leads to promotion of transitional B cell survival and, furthermore, the survival of mature B cells; where BAFF is present in excess, autoreactive B cells can mature into follicular B cells, leading to development of B cell-
mediated autoimmune disease (115, 116). Recognition of its role therein has presented a new target for pharmacological manipulation; indeed, a BAFF inhibitor, in the form of a monoclonal antibody, has recently been introduced into clinical use for treatment of the autoimmune disease systemic lupus erythematosus (SLE).

However, regulatory B cell survival and function is also thought to depend on BAFF signaling (117), and as there is evidence that the existence of transplant tolerance may be characterised by expansion of the regulatory B cell subset, there is concern amongst the transplant community that use of an anti-BAFF agent may inhibit the development of allospecific B cell tolerance (112). Furthermore, it is also thought that inhibition of BAFF may negatively affect allospecific Treg development: BAFF-R and TACI can be expressed in select T lymphocyte subsets (118, 119), where they can function as costimulatory ligands (120, 121). Recent data indicates that an excess of BAFF, as found in BAFF transgenic (BAFF-tg) mice, is associated with prolongation of islet and skin allograft survival, an effect shown to be dependent upon increased frequencies of peripheral Tregs in these mice, and achieved through an indirect B cell dependent mechanism (122).

1.3.3 Rejection
Rejection of the allograft can occur at any time after transplantation, and may be driven either by cellular or antibody-mediated mechanisms. Irrespective of recipient characteristics, the immunological risk to the graft begins prior to transplantation due to systemic effects arising from donor brain death or ischaemia-reperfusion injury (IRI) (123). Although brain death has historically been considered to be less injurious to an organ than cardiac death, animal models of brain death have recently shown that it is associated with significant inflammatory signaling and downstream complement activation (124-127). IRI is a feature of both deceased and live transplants, although the period of cold ischaemia is usually significantly less in live transplants; ischaemia followed by
reperfusion upregulates the expression of MHC antigens by the graft and causes the release of an inflammatory cascade of chemokines, cytokines and adhesion molecules, which play roles in T cell trafficking to the graft and increase the risk of rejection (128, 129). Clinically, rejection is usually characterised by a rise in serum markers of graft function, may be accompanied by graft tenderness, and in the case of kidney transplantation, a decrease in urine output.

1.3.3.1 Cellular rejection

Cellular rejection occurs upon recognition of foreign or allogeneic MHC molecules by recipient T cells, in either a direct or indirect manner, as previously discussed, and is mediated by T lymphocytes (T cell mediated rejection; TCMR). Acute cellular rejection is defined as a decline in clinical function of the allograft, accompanied by well-defined histological changes on allograft biopsy; there may also be histological changes which are considered suspicious for, but not definitive of, acute rejection, and which occur in the absence of clinical dysfunction: this is considered borderline or subclinical rejection. In kidney allografts, TCMR is characterised by interstitial inflammation, with evidence of tubular infiltration by T cells and macrophages, secretion of IFNγ and TGFβ, tubular epithelial damage and subsequent degeneration (130).

It is thought that following the initial recognition of foreign MHC molecules, the responding T cell, which may be either a naïve T cell primed in a secondary lymphoid organ or a memory T cell responding either to a prior antigen exposure (e.g., pregnancy, blood transfusion or previous transplant) or in a cross-reactive heterologous manner following initial priming by infection or vaccination (131), activates a local inflammatory compartment that recruits both antigen-specific and non-specific T cells and macrophages; the response and downstream sequelae appear to be the same, irrespective of the mechanism of antigen presentation or, indeed, differences in the antigen itself. Leucocyte
recruitment by chemokines involves both positive and negative selection: T cells and macrophages are actively drafted while other cell types, including neutrophils and eosinophils, are excluded, potentially by inhibitory processes instigated by IFNγ (132). While both antigen-specific and non-specific CD4⁺ and CD8⁺ effector and effector memory cells are involved in the cellular infiltrate, the majority are thought to be non-specific (133); the infiltrating macrophage population is heterogenous and involves macrophage subsets involved in both initial allograft injury and subsequent repair responses (134). Small numbers of other cell types, including B cells, plasma cells, and, rarely, NK cells may be present; their recruitment is likely to be non-specific, and it is not thought that they play a significant role in TCMR (130). Once the inflammatory process has begun, the microcirculation becomes permeable, leading to localised oedema. Interestingly, responding effector T cells cross the capillary endothelium initially without causing endothelial cell death, thought to be due to impaired ability of the endothelium to present antigenic peptides, which renders them relatively resistant to effector T cell killing (135).

In kidney transplantation, local inflammation triggers dedifferentiation of the epithelium, which then loses transcripts and proteins necessary for its normal function, instead expressing embryonic, injury and cell cycle genes (136); mononuclear cells first accumulate in the interstitium before T cells traverse the basement membrane to enter the epithelium, an accumulation manifest as tubulitis, a classic lesion of TCMR. Interestingly, despite the significant tubular injury seen in renal TCMR, the main underlying process is not that of apoptosis or necrosis, but rather the dedifferentiation process described (137). It is thought that nephrons respond to adjacent epithelial cell injury in ways that determine the fate of all their component cells: the juxtaglomerular apparatus monitors the electrolyte content of distal convoluted tubule fluid as an indicator of epithelial function, and can induce afferent arteriole vasoconstriction and thereby shut down filtration (138), a suspension that could remain in place until it senses restoration of the integrity of the damaged epithelium; if injury persists,
this shutdown is likely to become irreversible, with nephrons undergoing atrophy, fibrosis and glomerulosclerosis.

TCMR uncomplicated by co-existent antibody-mediated damage is typically responsive to steroid therapy, usually given as methylprednisolone boosts at doses that are lytic to T cells; T cell depleting antibodies, either polyclonal or monoclonal as described in more detail below, can be used if necessary. While it was thought that late T cell rejection was associated with reduced graft survival, it is possible that this may be due to late, unrecognised antibody-mediated rejection, and it is now thought that the prognosis after treatment in a compliant patient is reasonably good (139).

1.3.3.2 Antibody-mediated rejection
Antibody-mediated rejection (ABMR) is defined by histological evidence of tissue injury in the setting of detectable donor-specific antibodies (HLA or other non-HLA antigens), with evidence of current or recent antibody interaction with the vascular endothelium, manifest as, for example, deposition of the complement split product C4d or the presence of microvascular inflammation. It is increasingly recognised that evidence of C4d deposition, previously thought to be pathognomonic of ABMR, may, in fact, be absent despite active rejection; a new entity of C4d-negative ABMR has lately been included in the revised Banff criteria for renal ABMR (140). As discussed earlier, antibodies that can mediate rejection include those directed against both HLA and non-HLA antigens, including ABO blood group antigens, the highly polymorphic MICA antigen and, as more recently described, endothelial cell antigens, smooth muscle antigens and the angiotensin II Type I receptor (27). The clinical phenotype of antibody-mediated rejection varies considerably according to the timing and acuity of the episode. The most devastating incarnation, hyperacute rejection, occurs within minutes of implantation: following cross-clamp release, the graft fails to perfuse and instead appears mottled, reflecting the immediate deposition of anti-HLA antibodies on the vascular endothelium. Intrgraft
activation of the classic complement cascade leads to endothelial necrosis, platelet deposition and local coagulation (141). The entity of hyperacute rejection is now, fortunately, vanishingly rare due to improvements in immunological cross-matching that facilitate the detection of the pre-existing donor-specific antibodies responsible.

Acute antibody-mediated rejection can occur within days to weeks post transplantation, and is characterised by rapid recall of previously formed antibodies directed against donor antigens, usually MHC antigens displayed on donor endothelium, which may have been present in undetectable or low levels prior to transplantation. Damaged endothelial cells then release various pro-inflammatory molecules including von Willebrand Factor (vWF) and P-selectin, which promote platelet aggregation, cytokines and chemokines, and chemoattractants, including C3a and C5a, which can trigger further complement activation and endothelial damage, potentially leading to microthrombi with haemorrhage, arterial wall necrosis and, finally, infarction (141). Treatment of ABMR is usually multi-faceted: antibody removal or neutralisation using plasma exchange, immunoadsorption or administration of IVIG, or a combination thereof; inhibition of further alloantibody production using B cell depleting agents (e.g., rituximab) or, more recently, proteasome inhibitors which can inhibit plasma cells (e.g., bortezomib); and attenuation of the immune response using high dose corticosteroids (142). Eculizumab, the new monoclonal antibody which acts as a terminal complement inhibitor, has shown promising results in preliminary studies; these remain to be confirmed by further investigation (142, 143). Antibody-mediated rejection also exists in a more chronic, indolent form, and, as previously discussed, is responsible for the majority of cases of late renal allograft loss (5).

1.4 Immunosuppressive Regimens
The majority of current immunosuppressive regimens are comprised of a biologic induction agent, followed by a combination of maintenance
immunosuppressive drugs, which may or may not include corticosteroids, depending on centre preference and patient characteristics. Induction regimens also vary from centre to centre, but commonly consist of either a monoclonal or polyclonal T cell depleting antibody (e.g., alemtuzumab or anti-thymocyte globulin, respectively) or a non-depleting agent, usually a monoclonal antibody directed against CD25, the α chain of the interleukin-2 (IL-2) receptor (e.g., basiliximab). T cell depleting antibodies deplete the majority of circulating peripheral alloreactive effector T cells and can also affect lymphocyte populations within secondary lymphoid organs, leading to lymphopaenia (144). Their use successfully prevents acute rejection immediately after transplantation; nonetheless, recipients ultimately develop subacute or chronic rejection (39, 145): despite near-complete naïve T cell depletion, a small fraction of memory T cells are capable of resisting depletion and tend to acquire an effector memory phenotype (146-148); furthermore, the ensuing lymphopaenia stimulates lymphocyte repopulation and homeostatic proliferation, which may occur at different rates within different lymphocyte subsets and may be influenced by the particular induction agent used. Interestingly, this discrepancy allows for the possibility that subset repopulation and proliferation could be manipulated to engineer a situation in which regulatory, anti-inflammatory cells outnumber pathogenic, pro-inflammatory cells, thereby leading to the establishment of a pro-regulatory or tolerogenic state; to date, two possible strategies have been shown to be effective therein: blockade of costimulation (40, 149, 150) and exploitation of the suppressive potential of Tregs (151).

The use of monoclonal anti-CD25 (IL-2R) antibodies is widespread, although not without some debate: CD25 is not expressed on most resting T cells but is instead upregulated upon T cell activation, whereupon the secretion of IL-2 from activated T cells leads to the proliferation of alloreactive T effector cells (152); it is, however, constitutively expressed on Tregs, whilst IL-2 signaling has been shown to be critical to Treg generation and function (153, 154) and,
furthermore, leads to suppression of Treg apoptosis by upregulation of the pro-
survival protein induced myeloid leukemia cell differentiation protein (Mcl-1), a
member of the Bcl-2 family (155, 156). The potential impact of targeted CD25
blockade on Treg survival and function is therefore a concern.

Calcineurin inhibitors (CNIs), originally introduced into clinical use in the 1980s
(4), are the mainstay of current maintenance immunosuppressive regimens.
Calcineurin dephosphorylates and activates cytoplasmic nuclear factor of
activated T cells (NFATc), thereby causing its translocation to the nucleus,
where it upregulates the expression of IL-2, a transcription factor important in
the growth and differentiation of T cells. Inhibition of calcineurin therefore
interferes with the cytokine milieu and lymphocyte function; as discussed
earlier, however, this mechanism can also conversely inhibit the survival and
function of Tregs (144). Although CNIs have had a major impact on early
allograft survival (4, 157), their use is not without significant adverse sequelae:
CNI-containing immunosuppressive regimens are associated with considerable
morbidity, including infection and neoplasia, whilst CNI-induced nephrotoxicity
remains a major barrier to long-term renal allograft survival, and the most
common cause of end-stage renal disease in recipients of other solid organ
transplants (39, 158). Furthermore, there is increasing recognition of their
association with non-immunological complications, including hypertension,
hyperlipidaemia and new onset diabetes after transplantation (NODAT) (39); in
view of the significant cardiovascular morbidity and mortality seen post-
transplant, now the most common reason for death with a functioning graft (5),
this is a significant cause for concern.

Therefore, the development of strategies to promote allograft survival whilst
eliminating the requirement for CNIs, and/or to induce antigen-specific allograft
tolerance, have become a major goal in the transplant community(39, 159-161).
Potential approaches include deletion of peripheral alloreactive effector T cells
with induction therapy, inhibition of T cell activation by costimulatory blockade,
interference with cytokine signaling, and promotion of active regulation by harnessing the regulatory potential of Tregs (36, 37, 159). However, as strategies to manipulate the T cell response have become more sophisticated, the role of B cells in the alloimmune response has been increasingly recognised, both in terms of antigen presentation and alloantibody production. The ability to influence T and B cell fate is of paramount interest in the field of transplantation as we seek therapeutic options that inhibit detrimental alloimmune responses whilst simultaneously promoting allograft tolerance.

1.5 Notch Receptors
The Notch signaling pathway is one of the seven identified major mammalian cell-cell signaling pathways, including Hedgehog, wingless-related (Wnt), transforming growth factor β (TGFβ), Janus kinase/signal transducer and activator of transcription (JAK/STAT), receptor tyrosine kinase and nuclear receptor pathways (111). A century ago, John S. Dexter, working at Olivet College in Michigan, described a notched defect on the wing blades of certain Drosophila melanogaster (162), due to what we now know to be a partial loss-of-function mutation in the Drosophila Notch gene; three years later, the geneticist Thomas Hunt Morgan and his group at Columbia University in New York identified the first allele of the gene (163).

Drosophila Notch was cloned in the mid-1980s (164, 165) and was shown to encode an unusual type 1 transmembrane receptor that is activated by two different membrane-bound ligands, known as Serrate (or Jagged) and Delta (166). In the intervening years, Notch receptors have been shown to be a family of transmembrane receptors that are highly conserved amongst species (167, 168): to date, four mammalian homologues of the Notch receptor have been identified, known as Notch 1-4, along with five ligands, Jagged-1 & -2 (Jagged1 & 2) and Delta-like-1, -3, & -4 (DLL1, 3, & 4).
Figure 1.6. Drosophila melanogaster
Photograph demonstrating the notched wing defect first noticed by John S. Dexter in 1914.

Notch receptors are first constitutively and proteolytically cleaved by a furin-like convertase in the Golgi apparatus (169), generating a heterodimeric receptor consisting of an extracellular subunit non-covalently linked to a second subunit containing the extracellular heterodimerisation domain (NECD) and the transmembrane domain, followed by the cytoplasmic region of the Notch receptor. Prior to its transport to the cell membrane, the extracellular domain is subject to glycosylation by members of the Fringe glycosyltransferase family, Manic, Radical and Lunatic fringe (170, 171), which add N-Acetylglucosamine to O-fucose residues contained within certain epidermal growth factor-like repeats of the receptors (172). The actions of the individual Fringe proteins can influence the subsequent interaction between a Notch receptor and its ligand (170, 173): all of the Fringe proteins promote Notch signaling by increasing Notch activation and binding of Notch to DLL; however, Manic and Radical fringe inhibit Notch-Jagged signaling without interfering with the binding affinity of Jagged (174, 175), thereby demonstrating that prior to their ligand encounter, Notch receptors can be primed to process signal from some ligands in preference to others (44).
Binding of a Notch receptor on the signal-receiving cell to one of its ligands expressed on a neighbouring, signal-sending cell induces conformational change in the negative regulatory region (NRR) of the receptor, thereby triggering two further proteolytic cleavages of the receptor: the first is achieved by ADAM10 and ADAM17, metalloproteases belonging to the ADAM family (A Disintegrin And Metalloproteinase), and occurs in the extracellular domain of the receptor approximately 12-13 amino acids external to the transmembrane domain at the S2 locus (166); this results in shedding of the extracellular domain and its subsequent endocytosis, along with its ligand, by the signal-sending cell (176), a process dependent on monoubiquitinylation of the cytoplasmic tails of the ligands by E3-ubiquitin ligases of the Neuralized (Neur) and Mindbomb (Mib) families (45). The remaining transmembrane receptor is then cleaved by a γ-secretase complex (S3 cleavage) that contains Presenilin (177), thereby releasing the intracellular domain of the Notch receptor (NICD).

Initiation of this third cleavage step is absolutely dependent on endocytosis of the Notch ligands (178), the exact purpose of which was, until recently, unknown. Two models had been proposed to explain the underlying mechanism: Wang et al proposed the Recycling Model, in which “inactive” Notch ligands on the signal-sending cell are subject to endocytosis and unknown post-translational modifications to become “activated”, following which they are recycled back to the cell surface better able to interact with Notch receptors (179). Nichols et al offered an alternative hypothesis, known as the Mechano-transduction Model, in which pulling forces generated by endocytosis of the ligand into the signal-sending cell following receptor-ligand binding expose the S2/ADAM cleavage site, thereby facilitating the S2 cleavage and receptor activation in the signal-receiving cell, followed by endocytosis of the NECD into the signal-sending cell (180). This hypothesis has recently received further support from a study that identified a distinct mode of clathrin-mediated endocytosis which generated a mechanical pulling force that induced a conformational change in Notch, thereby facilitating proteolysis and activating
Notch signaling (181); this was shown to be dependent on ligand ubiquitylation, epsins (a family of membrane proteins important in generating membrane curvature), dynamin (a GTPase), and actin (a globular multi-function protein that forms microfilaments) (181).

Release of the NICD achieved by S3 cleavage is followed by its translocation to the nucleus, where it forms a nuclear transcription complex with the DNA binding transcription factor recombination signal binding protein for immunoglobulin κJ region (RBPJ; also known as CSL in humans, Suppressor of Hairless in Drosophila melanogaster, or Lag1 in Caenorhabditis elegans); it then recruits the co-activators Mastermind-like 1 (MAML1) and p300 to promote target gene transcription and downstream signaling (45, 168, 182-184). In the absence of NICD, CSL complexes with various co-repressors to inhibit Notch target gene expression (168, 185, 186).

Figure 1.7. The Notch receptor-ligand interaction
Diagram author’s own.
Blockade of Notch-1 or Notch-2, but not Notch-3 or Notch-4, results in death during embryogenesis, highlighting the pivotal and non-redundant roles these receptors play during development: the Notch pathway has been shown to be involved in neurogenesis and the adoption of neural cell fates, angiogenesis and haematopoiesis (45, 168). Indeed, signaling through the Notch-1 receptor is critical to T cell development, while Notch-2 is crucial to normal B cell development and differentiation, as will be discussed in the sections below.

1.5.1 The Role of Notch Signaling in T cell Development

Multiple gene targets downstream of Notch-1 have been identified, all shown to play different but crucial roles in T cell development: the genes encoding the HES (hairy and enhancer of split) family of basic helix-loop-helix proteins, in addition to NRARP (Notch-regulated ankyrin-repeat protein), PTCRA (pre-T-cell receptor-α), and the genes encoding the Deltex family of E3 ligases; Notch signaling is also known to regulate the HEY (HES with YRPW motif) family of transcription factors (44). More recently, Notch receptors have been shown to target and regulate the NF-κB pathway (187-189); NF-κB in turn regulates the expression of genes encoding IL-2, CD25 (the IL-2 receptor α chain) and IFN-γ, all of which are essential for T cell activation and proliferation (44). Multiple studies investigating the effect of loss- or gain-of-function of Notch-1 have confirmed its importance in T cell lineage commitment. Inducible inactivation of Notch-1, achieved by the use of mice in which an essential portion of the Notch1 gene was floxed and deleted inducibly through the activation of an IFN-responsive Cre recombinase transgene, resulted in transient growth inhibition and severe deficiency in thymocyte development (190); moreover, these mice demonstrated ectopic B cell development within the thymus (191), indicating that the Notch-1-driven T cell lineage commitment involves a simultaneous block or inhibition of B cell development. Conversely, an excess of Notch-1 signaling, as seen in gain-of-function studies investigating the overexpression of a constitutively active form of Notch-1 (192, 193), DLL1 (194) or DLL4 (194, 195), results in increased T cell development in the bone marrow at the
expense of B cells. In the clinical setting, unchecked Notch-1 signaling has been shown to be oncogenic: activating Notch-1 mutations have been reported in more than 50% of cases of human acute T cell lymphoblastic leukaemia (T-ALL) (196, 197).

Once commitment to the T cell lineage has been made, which, as discussed earlier, can only be considered definitive once the DN3 (CD44–CD25+) stage has been reached (46), thymocytes then undergo widespread TCRβ, γ and δ gene loci rearrangements: some differentiate into γδ T cells, while others undergo further proliferation to generate αβ T cells, which in turn give rise to CD4+CD8+ DP cells (57), the most populous subset within the thymus. While the development of γδ T cells appears to be Notch independent (57), the generation of αβ T cells, in contrast, is dependent on continuous Notch signaling up to the DN3 stage, at which point β-selection, a critical step or checkpoint to further αβ T cell development, occurs (198). Successful β-selection is known to depend on signaling through the pre-TCR, but cannot proceed in the absence of co-operative Notch-1 signaling (199). Interestingly, Notch-1 itself, together with the transcription factor E2A, directly increases the transcription of the Notch1 gene in pre-β-selection thymocytes (200). Thymocytes that pass β-selection then immediately downregulate Notch1 expression, an effect achieved by pre-TCR-mediated upregulation of the E2A inhibitor Id3 (200).

The individual contribution of the Notch ligands to T cell lineage commitment has also been evaluated. Neither of the Jagged family ligands have been shown to be essential: conditional inhibition of Jagged1 does not prevent normal haematopoiesis (201), while mice deficient in Jagged2 display a minor decrease in γδ T cells but normal αβ T cell development (202). In contrast, members of the Delta family appear to play a critical role: expression of DLL1 or DLL4 on stromal cells can induce T cell development from mouse or human
haematopoietic progenitor cells in vitro (203, 204), while DLL4 expressing fibroblasts have also been shown to support T cell development from HSCs (205). However, the absence of DLL1 does not interfere with T cell lineage commitment or maturation (206), indicating that its role therein is dispensable and suggesting that signaling through DLL4 may compensate for the lack of DLL1 function in vivo (207). In support of this, there is evidence that a hierarchy of Notch-Delta interactions exist. Indeed, Delta ligands have been shown to bind to Notch-1 with differing affinities: Notch-1 preferentially interacts with DLL4, while the binding of Notch-1 to DLL1 is weaker (207, 208); furthermore, the Notch-1-DLL4 interaction appears to be less dependent on Lunatic fringe than other Notch-Delta interactions. DLL1 can signal through either Notch-1 or Notch-2, and, interestingly, can maintain early commitment to the T cell lineage in vitro by signaling through Notch-2 in the absence of Notch-1, although the cells do not progress beyond the DN stage (207). In contrast, DLL4 induces and maintains T cell lineage commitment and maturation both in vitro and in vivo by exclusively signaling through Notch-1 (207). Upon maturation of thymocytes to the DP stage, expression of DLL4 is downregulated on cortical thymic epithelial cells (TECs) (209), providing further evidence of cross-talk between developing thymocytes and TECs (210).

Downstream of the role the Notch-ligand interactions play in T cell lineage commitment, they are further involved in T cell survival, the differentiation of naïve CD4+ T cells into various T helper cell subsets and peripheral T cell signaling. Notch-1 has been shown to protect T cells from TCR-induced cell death by a number of mechanisms, including inactivation of the pro-apoptotic protein Nur77 (211) and upregulation of inhibitors of apoptosis, including BCL-2 (B cell lymphoma 2) and FLIP (FLICE-like inhibitor protein) (212). Notch-1 further acts to protect T cells from apoptosis by interacting with LCK and PI3K (phosphatidylinositol 3 kinase) to activate Akt, a protein kinase involved in inhibition of apoptosis (212).
As discussed earlier, the differentiation of naïve T cells into the various T helper cell lineages is consequent to their interaction with activating APCs, predominantly DCs, which tailor their differentiation signals according to the nature of the encountered antigen and the interaction of that antigen with its cognate TCR. The ability of APCs to direct T cell differentiation has been shown to be dependent on direct TLR-mediated activation of the APCs themselves, rather than by secreted inflammatory mediators (213). Evidence for involvement of Notch signaling in the differentiation pathway is provided by studies showing both increased expression of Notch ligands on APCs following exposure to TLR ligands (69) and increased Notch signaling following TCR ligation (70), while the Notch receptors have been shown to co-localise with CD4 in CD4+ T cells (70) and the E3 ligase Numb at the T cell – APC interface (71). Earlier work indicated that Notch-1 was primarily involved in the differentiation of naïve T cells to the Th1 lineage: incubation of CD4+ T cells with a DLL1-Fc fusion protein induced expression of the Th1-associated transcription factor Tbet, while decreased Notch activity has been shown to correlate with decreased IFNγ secretion by CD4+ T cells (214). More recently, however, there has been evidence for a role of Notch-1 in Th2, Th17 and Th9 differentiation (45, 166, 215-217). Notch has been shown to directly regulate the expression of the transcription factor Gata3; it then acts synergistically with Gata3 to promote IL-4 secretion, and, in turn, Th2 differentiation (215). Notch signaling through DLL4 promotes the development of Th17 cells via a mechanism shown to rely on the interaction of Notch with CSL-binding sites on the promoter regions of the transcription factor RORγT and IL-17 (216). In contrast, Notch-1 signaling via Jagged2 directly activated IL-9 transcription: NICD1 interacts with Smad3, a protein involved in the mediation of TGFβ signals, to activate the Il9 promoter and generate Th9 cells (217).

There is also evidence to support the involvement of Notch signaling in the development and maintenance of Tregs. Presentation of the house dust mite antigen Der p1 to naïve CD4+ cells by DCs modified to overexpress Jagged1
led to the development of antigen-specific Tregs that, when injected into naïve mice, were capable of conferring tolerance (218). Further evidence in support of this mechanism is provided by a study showing that overexpression of Jagged1, achieved by adenovirus vector transduction of allogeneic Epstein Barr virus lymphoblastoid B cells, led to upregulation of TGFβ with reduced production of IFNγ, IL-2 and IL-5 when incubated with CD45RA+ naïve T cells; transfer of these T cells to fresh cultures led to inhibition of proliferation and cytotoxicity in response to a new challenge by the alloantigen (219); further studies by the same group, instead utilising human T cells, yielded similar results (220). In an experimental autoimmune encephalomyelitis (EAE) model, inhibition of DLL4, achieved by use of a blocking anti-DLL4 mAb, resulted in expansion of the peripheral Treg population, and, consequently, reduction in the severity of disease (221). However, Notch-1 has been shown to co-operate with both Smad3 (as discussed earlier) and RBP-J to activate the transcription of Foxp3 (222, 223). Conversely, the use of γ-secretase inhibitors in vivo reduced the development and maintenance of peripheral Tregs with consequent development of autoimmune disease (222). However, as will be discussed later, inhibition of Notch signaling in a graft-versus-host disease mouse model, achieved by conditional expression of DNMAML in donor alloreactive T cells, resulted in accumulation of pre-existing nTregs (224).

1.5.2 The Role of Notch Signaling in B Cell Development

While Notch-1 signaling has been shown to be pivotal in T cell development and differentiation, as discussed above, it is Notch-2 that plays a critical, non-redundant role in normal B cell development. Expression of Notch-2 has been demonstrated on B cells at varying stages of development: using quantitative PCR, Notch-2 transcripts were detected in pro-B, pre-B and immature B cells, with subsequent upregulation on circulating peripheral B cells (104).

Determination of the expression of Notch ligands, as they pertain to developing B cells, has, however, been more challenging. Delta-like and jagged ligands are
expressed on many bone marrow stromal cells (111), although this has been shown to depend on the specific microenvironment or niche occupied: the early B cell progenitors, pre-pro B cells, reside in niches that do not have Notch ligand expression, while later progenitors, pro-B and pre-B cells, reside in niches that contain IL-7-expressing stromal cells, some of which express DLL1 and Jagged1 (98). Within the spleen, DLL1, DLL4 and Jagged1 are expressed on vascular stromal cells with partial overlap, while DLL1 is selectively expressed on endothelial cells located in the red pulp and MZ (225).

The development of marginal zone B cells has been shown to critically depend on Notch-2 signaling: mice conditionally deficient in Notch-2 displayed a severely diminished MZB population (104), and, furthermore, a significant reduction in the T2 population, providing evidence that the MZB precursors lie within this population (104). Deletion or absence of other elements of the Notch signaling pathway, including RBP-J (226), MAML1 (227, 228) and ADAM10 (229), have yielded similar results. The ligand involved has convincingly been shown to be DLL1: mice conditionally deficient in DLL1 display the same MZB-deficient phenotype as that seen in Notch-2-deficient mice (206), as do mice lacking Mib-1, an E3 ligase known to regulate DLL1 endocytosis (230). The interaction between DLL1 and Notch-2 is thought to be inherently weak in vivo (111, 225), and has been shown to be regulated by the co-operative signaling of Lunatic and Manic Fringe, which function to enhance MZB cell precursor competition for DLL1 in red pulp endothelial niches, and, in doing so, regulate the size of the MZB cell population (225).

Notch-2 signaling has further been implicated in the development of B1 B cells: mice expressing only one functional notch2 allele displayed reduced numbers of peritoneal B1 B cells (110). Later stages of B cell activity, including B cell activation and terminal differentiation into antibody-secreting cells (ASCs), have also been shown to be regulated by Notch signaling (111). Activation of FO B cells ex vivo was enhanced in the presence of DLL1, while the production of
IgG1+ cells was also increased (231). Interestingly, the interaction of Notch-1 and DLL1 was separately shown to both enhance the FO B cell proliferation response following stimulation with anti-CD40 mAb and to promote the differentiation of B cells into ASCs (232), suggesting that while Notch-2 may be critical for normal B cell development, Notch-1 is important for later stages of B cell activity (232).

1.5.3 The Role of Notch Signaling in the Alloimmune Response

1.5.3.1 Solid Organ Transplantation

Previous work in our laboratory has investigated the role of both DLL1 and Jagged2 in the alloimmune response (233, 234). The expression of DLL1 on APCs was upregulated in vivo upon cardiac transplantation in a full-MHC mismatch transplant model. Isolated inhibition of DLL1 using a blocking mAb produced a modest, but significant, prolongation of graft survival, an effect that was greatly enhanced when anti-DLL1 mAb was combined with a single dose of CTLA4-Ig or when CD28KO mice were used as recipients. Mice treated with anti-DLL1 demonstrated reduced frequencies of CD4+ and CD8+ effector memory cells, although, interestingly, frequencies of MZ B cells that were not significantly different than those in control-treated mice; furthermore, the use of anti-DLL1 inhibited alloreactive Th1 cells and promoted polarisation towards a Th2-mediated phenotype (233).

In contrast to this data, constitutive expression of DLL1 on cells bearing allogeneic MHC Class I or Class II antigens was shown to render these antigens non-immunogenic in an in vivo assay, and further induced antigen-specific hyporesponsiveness to a subsequent re-challenge in the absence of DLL1 transfection, although this was shown to be true only when these antigens were presented through the direct pathway (235). This study was extended to evaluate the effect of pre-treatment with DLL1-transfected allogeneic MHC Class I or II-bearing cells in a mouse model of cardiac transplantation, wherein
mice were injected with the cells 14 days prior to receiving a heterotopic vascularised cardiac transplant. Indeed, pre-treatment effected prolongation of graft survival in an antigen-specific manner, which was, moreover, dependent on the presence of CD8+ T cells (235).

The expression of Jagged2 on recipient APCs was modestly increased in a fully-MHC mismatched cardiac transplant model but significantly increased in a Class II MHC-mismatched model. Use of a signaling Jagged2 mAb precipitated rejection in both the full MHC-mismatched model where recipients were lacking CD28 and in the Class II MHC-mismatched model (234). Surprisingly, despite the accelerated tempo of rejection seen in the latter model following the use of Jagged2 mAb, these mice displayed increased frequencies of Tregs (234); however, they also displayed increased IL-6 production, an effect previously shown to be consequent to Jagged2-Notch signaling. While the combination of IL-6 and TGFβ is known to promote differentiation towards a pro-inflammatory Th17 phenotype (236), we did not see increased production of IL-17 in our model, and suggest that it is possible that the increased secretion of IL-6 rendered T effector cells resistant to suppression by Tregs (237), irrespective of their increased frequency.

### 1.5.3.2 Bone Marrow Transplantation

Notch signaling has been shown to have a critical role in the development and maintenance of graft-versus-host-disease (GVHD) following allogeneic bone marrow transplantation (BMT) (224, 238, 239). Pan-Notch inhibition of donor alloreACTIVE T cells, achieved by conditional expression of DNMAML in either or both CD4+ and CD8+ T cells, demonstrated that the transplanted cells proliferated in response to alloantigens in vivo but failed to produce a wide range of effector cytokines. Interestingly, despite preserved Tbet expression, the secretion of IFNγ was significantly decreased, an effect most marked when the CD4+ T cells expressed DNMAML in addition to the transplanted CD8+ T cells (224). Importantly, however, both the transplanted CD4+ or CD8+ T cells
retained graft-versus-tumour activity, but failed to generate GVHD (224, 239). Furthermore, inhibition of Notch signaling in this model also resulted in accumulation of pre-existing nTregs (224), although the mechanisms underlying this were not explored.

Use of γ-secretase inhibitors (GSIs) similarly achieved reduced production of inflammatory cytokines by alloreactive T cells following allogeneic BMT, but resulted in severe gastrointestinal toxicity which was rapidly fatal (238). The combination of Notch-1 and Notch-2 blockade has been shown to cause severe intestinal toxicity due to interference with the normal architecture of intestinal crypts (240, 241); however, it is thought that this toxicity may be aggravated and accelerated by intestinal injury following total body irradiation (238).

1.5.4 Rationale for Further Investigation
As discussed above, the vast majority of earlier work investigating the role of the Notch pathway in transplantation has either been achieved by blockade of individual Notch ligands or by pan-Notch receptor inhibition, achieved pharmacologically by the use of γ-secretase inhibitors (GSIs), or genetically, by the use of mice expressing the pan-Notch inhibitor DNMAML. However, despite inferences that may be drawn from the solid organ transplantation ligand data, due to the known hierarchy of Notch receptor-ligand binding, there is no definitive evidence demonstrating the role of the individual receptors therein.

Similarly, although GSIs are very effective, and indeed have progressed to clinical trials in haemato-oncological spheres, they fail to distinguish between the individual Notch receptors and therefore effect pan-Notch inhibition; furthermore, the γ-secretase complex is not exclusive to the Notch signaling pathway, such that inhibition of γ-secretase leads to a variety of undesired on-target effects (242).
The role of the individual Notch receptors Notch-1 and Notch-2 in the alloimmune response following solid organ transplantation is, therefore, unknown. The recent development of antibodies directed against the individual Notch receptors Notch-1 and Notch-2 by Genentech, Inc. (243), has provided an opportunity to specifically antagonise their cognate receptors and determine their individual effects.
1.6 Aims of this Study

The overall objective of this project was to define the role of Notch-1 and Notch-2 in alloimmunity, and to understand their mechanism of action in regulating alloimmune responses *in vivo* using murine transplant models, with the ultimate goal of achieving sustainable and reproducible allograft tolerance.

The specific aims were:

1. To investigate the use of GSIs in solid organ transplantation and to determine the effect and tolerability of the novel antagonistic Notch-1 (aNotch-1) and Notch-2 (aNotch-2) antibodies on naïve mice prior to testing them in the alloimmune setting.

2. To investigate the role of Notch-1 in the alloimmune response in solid organ transplantation by using both aNotch-1 in a fully MHC-mismatched, heterotopic cardiac transplantation model and a selective, conditional Notch-1 KO mouse model; to examine the effect of Notch-1 inhibition on allograft survival, and furthermore, to perform *ex vivo* mechanistic studies to evaluate T cell phenotype and function, cytokine milieu and alloreactivity in aNotch-1-treated animals compared to controls.

3. To investigate the role of Notch-2 in the alloimmune response using the same heterotopic cardiac transplantation model, with particular focus on its contribution to the B cell component; to examine the effect of Notch-2 blockade on allograft survival and to perform *ex vivo* mechanistic studies to evaluate B cell phenotype, alloantibody production, cytokine milieu and alloreactivity in aNotch-2-treated animals compared to controls.
Chapter Two: Materials and Methods

2.1 Methodology
This chapter contains a full description of the materials and methods used for each experiment and will complement detail provided in subsequent chapters.

2.2 Antibodies
All antibodies were administered as an intraperitoneal injection (technique described below).

2.2.1 Anti-Notch-1 & anti-Notch-2
Anti-Notch-1 (aNotch-1) and anti-Notch-2 (aNotch-2) antibodies were generously provided by Dr. Christian Siebel of Genentech, Inc. (South San Francisco, CA, USA). These antibodies were generated using phage display technology, and are formulated to target the NRR of each receptor; this strategy was based on previous studies which had suggested that antibody targeting of the NRR might stabilise its ‘off’ conformation (244, 245). Full details of the methods employed in antibody generation can be found in the online version of the paper (243). They are antagonistic, fully human IgG1 antibodies which potently inhibit their respective paralogues but not other Notch receptors, and which bind with similarly high affinity to both the mouse and human orthologues (243). Both aNotch-1 and aNotch-2 inhibit signaling induced through their respective receptors and the ligands DLL1, DLL4, Jagged1 and Jagged2, demonstrating efficacy irrespective of the receptor/ligand combination. Despite approximately 45% sequence identity between NRR1 and NRR2, only 29% of the epitope residues are identical, demonstrating the basis for the specificity of each antibody for its respective NRR (243).

Following a series of experiments to determine the optimal dose and dosing schedule, both antibodies were administered at a dose of 5mg/kg; aNotch-1
was given on day 0, 2, 4, 6, 8 & 10, while aNotch-2 was given on day 0, 3, 5, 7, 9, & 11. Control IgG, also obtained from Genentech, Inc., was administered as a control antibody at the same dose and on the same schedule as aNotch-1 or aNotch-2, according to the respective experiments.

2.2.2 CTLA4-Ig
hCTLA4-Ig is a fusion protein composed of a human IgG1 Fc fused to the extracellular domain of CTLA4. It was purchased from Bristol Myers Squibb (BMS; New York, NY, USA) and was administered as a single dose (250 µg) on day 2 post transplantation.

2.2.3 Anti-CD25
Rat anti-mouse CD25 mAb (PC61 clone; aCD25) was manufactured and purified from an original hybridoma by a commercial source Bioexpress Cell Culture (West Lebanon, NH, USA) and was administered in two separate doses of 250 µg on days -6 and -1 prior to transplantation.

2.2.4 Anti-CD20
The aCD20 clone 5D2 (murine IgG2a) was purchased from Genentech, Inc. It was initially administered at a dose of 200 µg on day -1 before transplantation; the protocol was subsequently amended to dose 200 µg on days -4 and -1.

2.2.5 γ-secretase inhibitors (GSIs)
The GSI Dibenzazepine (DBZ; (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)propionamide) was purchased from Calbiochem (EMD Millipore, Billerica, MA), and was prepared as following: the lipid soluble compound was suspended finely in 0.5% (w/v) hydroxypropylmethylcellulose (Methocel E4M) and 0.1% (w/v) Tween 80 in water, as previously described (241). DBZ was administered daily at a dose of 500 µg/100 g body weight (246), translating into a usual dose of 125 µg, for a planned regimen of 10 days.
2.3 Mice

All animals were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Harvard Medical School (HMS) Animal Management Committee approved all animal experiments (Animal Experimentation Protocols #02943 and #05050; see Appendix 1). Euthanasia was performed using CO$_2$ overdose, consistent with the recommendations of the American Veterinary Medical Association (AVMA) and in accordance with HMS IACUC guidelines; every effort was made to minimise suffering.

2.3.1

6 – 8 week old C57BL/6 (B6; Thy1.1$^+$), C57BL/6 (WT B6; Thy1.2$^+$), BALB/c (H-2$^d$), Rag1$^{-/-}$ (on C57BL/6 background) and B6.C-H2$^{bm12}$/KhEg (bm12) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in our facility.

2.3.2 Foxp3-GFP mice

Foxp3-GFP mice were maintained as a breeding colony in our laboratory, having originally been obtained from the Kuchroo laboratory at Harvard Medical School (236). These mice were generated using a gene-targeting approach, in which a bicistronic enhanced green fluorescent protein (EGFP) reporter was introduced into the endogenous Foxp3 locus (Foxp3–GFP ‘knockin’ mice), allowing accurate identification and tracking of Foxp3-expressing regulatory cells in vivo, and, furthermore, facilitating the study of factors that influence Foxp3 expression and Treg generation. The mice were generated in the Kuchroo laboratory as follows: an IRES-EGFP derived from pMSCV-IRES-EGFP was subcloned into the Clal site and BamHI site of the TKPbs-LoxP-Neo cassette. An SV40 polyadenylation sequence derived from pTRE vector (Clontech) was subsequently cloned into the BamHI site. A BAC clone (RP23-54C14) containing C57BL/6 Foxp3 genomic DNA was purchased from the
BACPAC (CHORI) and was used as a template for PCR amplification to generate 5.3 kb and 1.7 kb arms. The 5.3 kb arm containing the 3’ un-translated region of the Foxp3 gene was generated using the primers 5’-GTCGACCAAGAAAAGACCAGACTGAG-3’ and 5’-GCGGCCGCGTCCTCGCTTCCTCCCTATCTACT-3’. The 1.7 kb arm containing the exons 11, 12 and part of the exon 13 of the Foxp3 gene was generated using the primers 5’-ATCGATAGCGCTTTGCTGCATCGTAG-3’ and 5’-ATCGATAAGTTCATCTTGGGGCAGATTG-3’, sequenced and sub-cloned into a ClaI site. The 5.3 kb arm was sub-cloned into the Sall/Notl site of the EGFP containing TKPbs-LoxP-Neo cassette. The targeting construct was electroporated into Bruce4 ES cells. PCR amplification and southern blotting were performed for the identification of homologously recombined ES cells. Targeted ES cells were injected into BALB/c blastocysts and male chimeras were bred with female C57BL/6.

2.3.3 ABM-tg mice
The ABM-tg model, established and published by our group (247, 248), has been used to study mechanisms of tolerance by positive T cell costimulatory blockade, as well as mechanisms of immuno-regulation by negative T cell costimulatory pathways (249, 250). In brief, this is a B6 TCR-tg mouse with CD4+ T cells that express a TCR with defined specificity against the bm12 antigen (ABM-tg). These TCR-tg T cells are adoptively transferred into congenic WT B6 mice. Recipients simultaneously receive skin or heart transplants from the relevant donor strain (bm12). The behavior of the alloantigen-specific TCR CD4+ T cells in the lymph nodes and spleen are then monitored at different time points post-transplantation. In the ABM-tg model, allospecific cells can be tracked using the cell surface marker Thy1.2 (CD90.2), as they are injected into B6 recipients that express Thy1.1 (CD90.1). The ABM-tg mice were further bred with the Foxp3-GFP mice described above to produce ABMtg.Foxp3GFP mice, which were both generated and maintained as breeding colonies in our facility. The generation of the ABMtg.Foxp3GFP mouse facilitated identification and
tracking of allospecific Foxp3-expressing regulatory T cells, in addition to permitting study of the influence of various factors on this population.

2.3.4 Notch-1/2-DKO mice

Notch-1/2 DKO mice were generated using flox-cre technology (Notch1/2\textsuperscript{f/f}CD4\textsuperscript{Cre}), and are characterised by conditional deletion of both Notch-1 and Notch-2 in CD4\textsuperscript{+} cells following injection with Poly-IC (5 doses). These mice were a generous gift from Dr. Wassim Elyaman, Ann Romney Center for Neurologic Disease, Brigham & Women’s Hospital, Harvard Medical School, Boston, MA.

2.3.5 Notch-1 conditional knock-out mice

Notch-1 conditional knock-out mice (N1cKO on a B6 background) are characterised by deletion of Notch-1 in cells upon their expression of Foxp3 (Notch1\textsuperscript{f/f}Foxp3\textsuperscript{Cre}), leading to selective absence of Notch-1 in Tregs. These mice were a generous gift from Dr. Talal Chatila, Division of Immunology, Children’s Hospital Boston, Harvard Medical School, Boston, MA.

2.4 Surgical Techniques

2.4.1 Murine Cardiac transplantation

Vascularised intra-abdominal heterotopic cardiac allograft transplants were performed by Tetsunosuke Shimizu (T.S.) or Shunuske Ohori (S.O.), both surgical fellows in our laboratory. Where the transplants were performed as part of survival experiments, all procedures were performed by a single surgeon (T.S.), with the exception of one additional survival set for both aNotch-1 and aNotch-2; the results of these, however, were entirely consistent with previous T.S. data, and were therefore included. Where the transplants were performed for mechanistic experiments (wherein mice were sacrificed on day 7 or 8, according to experimental design), procedures were performed by either T.S. or S.O., although all procedures pertaining to a particular experiment were performed by a single surgeon.
All cardiac transplants were performed in a sterile manner, according to institutional guidelines: surgical instruments were autoclaved prior to the initial transplant of each experiment, following which they were sterilised using a glass bead steriliser (to sterilise the tips of each instrument in between animals); the surgical area was first cleaned with a topical disinfectant, and then prepared with sterile, non-fenestrated drapes; the surgeons wore protective barrier equipment (surgical scrubs, cap and mask), and performed standard surgical handwashing using Betadine scrub, followed by the use of sterile gloves.

General anaesthesia was initially achieved using intraperitoneal injection of a combination of ketamine and xylazine. Briefly, a master solution containing 8 mls of sterile water or saline, 1 ml of ketamine (100 mg/ml) and 1 ml of xylazine (20 mg/ml) was prepared; the amount administered to each mouse depended on their individual weight: 0.13 ml for a 20 g mouse, 0.16 ml for a 25 g mouse and 0.20 ml for a 30g mouse. Approximately one year following the start of the project, the laboratory anaesthetic practice changed such that general anaesthesia was then achieved using inhaled sevoflurane: the mice were first placed in an induction chamber, and following the achievement of general anaesthesia, were removed; anaesthesia was maintained by the administration of sevoflurane throughout surgery via individual masks secured to each mouse. The mice were assessed at intervals throughout the procedure to ensure a sufficient depth of anaesthesia was maintained.

The donor procedure was performed first. Following the achievement of general anaesthesia, the mouse was shaved (away from the surgical area to prevent contamination) and its skin sterilised with 70% alcohol; the mask was once again secured and it was draped with a sterile fenestrated drape. A midline abdominal incision was made and 1.0 ml of heparin solution (100U/ml) was injected into the inferior vena cava (IVC) using a 1ml syringe with a 27 gauge (G) needle. A midline sternotomy was then performed. The thymus and
surrounding adipose tissue were identified and resected, following which the right superior vena cava (SVC) was ligated close to the right atrium using 6-0 silk suture material. The ascending aorta and the main pulmonary artery were then transected as distally as possible. Finally, the pulmonary vein and left superior vena cava were ligated en bloc using 6-0 silk suture material. The vessels were flushed and the harvested heart was placed in preservation solution (heparin solution 100Uml⁻¹) until the recipient animal was ready.

The recipient mouse was prepared in a similar manner as the donor and positioned as described above. A midline abdominal incision was made, following which the intestine, bladder and testes (if male) were identified and covered with sterile wet gauze. The tissue surrounding the abdominal aorta and IVC below the renal vessels was carefully dissected, and a vascular clamp was placed so as to simultaneously clamp the infrarenal abdominal aorta and IVC. The donor heart was then removed from the preservation solution and placed into the right side of the abdomen. To prepare the anastomosis, a puncture was made in the abdominal aorta and the IVC using a small micro-forceps. The donor aorta was anastomosed to the recipient abdominal aorta and the donor main pulmonary artery to the recipient IVC in an end-to-side fashion using 10-0 nylon with approximately 10 sutures each in total. Once the anastomosis was complete, the abdomen was closed two-layer using 6-0 silk suture material.

Where a second heart transplant was performed, the previous allograft was explanted and the new allograft implanted as described above.
Figure 2.1. The vascularised heterotopic cardiac transplant model
Demonstration of the vascular anastomosis performed in the heterotopic cardiac transplant model: pulmonary artery (PA) to inferior vena cava (IVC); aorta (A) to aorta (left panel); a transplanted graft shown in situ (right panel).

Following surgery, animals were allowed to recover from anaesthesia in cages placed on electrical heating pads prior to their return to usual housing; post-operative analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg, q.12 hourly for 48 hours).

Graft survival was assessed by daily palpation; rejection was defined as complete cessation of cardiac contractility and was confirmed by direct visualisation at laparotomy; further confirmation, and gradation of severity, was provided by the subsequent histological examination. Following harvesting of the allograft, it was typically divided into three sections: the first was placed in 10% formalin for later histological examination (see below); the second was placed in a cryomold with optimised cutting temperature (OCT) compound, a formulation of water-soluble glycols and resins, to facilitate future cryostat sectioning, and was stored at -80C; the third was placed in sterile PBS to permit cellular isolation and examination.
2.4.2 Murine Thymectomy

All thymectomies described herein were performed by a single surgeon (S.O.). The surgical area was first cleaned with a topical disinfectant, and then prepared with sterile, non-fenestrated drapes; the surgeon wore protective barrier equipment (surgical scrubs, cap and mask), and performed standard surgical handwashing using Betadine scrub, followed by the use of sterile gloves. General anaesthesia was achieved and maintained using inhaled sevoflurane, as described in the murine cardiac transplantation section. A midline sternotomy was performed; the thymus and surrounding adipose tissue were identified and resected using a suction technique, as described by Lurie et al (251). Once haemostasis was achieved, the thorax was closed with 6-0 silk suture material.

Following surgery, animals were allowed to recover from anaesthesia in cages placed on electrical heating pads prior to their return to usual housing; post-operative analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg, q.12 hourly for 48 hours). The wound was inspected daily. Approximately two weeks following thymectomy, these mice underwent a vascularised intra-abdominal heterotopic cardiac allograft transplant, as described above.

2.4.3 Murine Skin Transplantation

The surgical area was first cleaned with a topical disinfectant, and then prepared with sterile, non-fenestrated drapes. All surgical instruments had been autoclaved prior to the procedure according to laboratory protocol; between transplants, they were sterilised using a glass bead steriliser.

The donor mouse was euthanised and then shaved away from the surgical area. The mouse was fixed to a styrofoam board covered in a sterile drape using 27G needles. A vertical midline incision was made in the skin overlying
the inferior aspect of its anterior trunk and extended to the base of its neck. A horizontal incision was then made in both directions at each end of the first incision, and extended laterally to the posterior trunk; the mouse was repositioned and the incisions extended to achieve circumferential excision of the skin. The harvested skin was then pinned with tension to the draped styrofoam board with the dermis facing; careful but thorough dissection of the adherent connective tissue was performed using a sterile forceps. Once removed, the dermis was covered with sterile filter paper, and the skin was cut into rectangular pieces approximately 2 cm x 3 cm; to prevent dessication of the dermis, the individual pieces were placed in a petrie dish filled with sterile saline until ready for use.

The recipient animal was then prepared. General anaesthesia was achieved using intraperitoneal injection of a combination of ketamine and xylazine, as described in the murine cardiac transplantation protocol; a circumferential area on the trunk was shaved. An incision was made on the flank with a scissors, and an area of skin slightly bigger than the graft was cut; a piece of skin from the donor animal was removed from the saline and placed on the prepared area with a sterile forceps. Using 3-0 suture material, the graft was sutured to the edges of the recipient skin with approximately 8 sutures in total. The graft was bandaged with gauze; a band-aid was then secured around the torso.

Following surgery, animals were allowed to recover from anaesthesia in cages placed on electrical heating pads prior to their return to usual housing; post-operative analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg, q.12 hourly for 24 hours). The bandages were maintained in place for 5 days approximately, following which they were removed and survival of the graft assessed visually on a daily basis.
2.4.4 Intraperitoneal Injections
The mice were allowed to gain purchase on the ridged lid of their cage with their forefeet and were then picked up with one hand using the scruff of the neck; the hind legs and tail were held behind the little finger of the same hand. Once safely restrained, the mice were turned over, and their abdomen exposed. Injections were administered to their lower left quadrant (avoiding the bladder and bowel), using a sterile tuberculin 1 cc syringe with a 25G needle.

2.5 Histological and Immunohistochemical Assessment
Samples obtained from the mice were initially stored in 10% formalin prior to their transport to a specialised animal pathology laboratory at Harvard Medical School, where all specimens underwent further processing. Five-micron-thick, formalin-fixed, paraffin-embedded sections were stained with standard Haematoxylin/Eosin stain (H&E). Sections from cardiac allografts were also stained with an Elastin Van Gieson stain. Acute cellular rejection was semi-quantitatively graded (0R-3R) according to the revised International Society of Heart and Lung Transplantation (ISHLT) guidelines (252). The extent of cellular infiltration and myocyte loss was also assessed.

2.6 Sample preparation
2.6.1 Spleen
Mice were euthanised on day 7 or 8, according to experimental design, using inhaled CO₂. Following this, spleens were retrieved through a flank incision and were placed directly into individual sterile containers containing sterile phosphate-buffered saline (PBS). The sample-containing tubes were transferred on ice to a laminar flow hood, in which all subsequent sterile steps were performed. Each spleen was transferred to a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA) placed into a 50 ml Falcon tube and mechanically dissociated using a flat sterile instrument while being rinsed with sterile PBS to a total volume of approximately 45 ml. These tubes were then centrifuged at 1800 rpm for 6 minutes. The effluent was carefully decanted and
approximately 2 ml of ACK (ammonium chloride potassium) red cell lysis buffer (catalogue # R7757, Sigma-Aldrich, St. Louis, MO) was added to each pellet; manual dissociation was performed by repeat pipetting for approximately 10 – 15 seconds, following which the solution was maintained at room temperature for 3 minutes; the reaction was terminated by the addition of sterile PBS to a total volume of approximately 45 ml. The solution was then carefully poured through a sterile 70 µm cell strainer into another 50 ml Falcon tube and centrifuged once more at 1800 rpm for 6 minutes. The effluent was decanted and each pellet was resuspended in 10 ml of sterile PBS for counting; thereafter, each sample was centrifuged once more and then resuspended in the appropriate volume of complete sterile RMPI medium (stock solution: 450 ml sterile RPMI supplemented with 10% (50 ml) foetal calf serum, 1% (5 ml) L-glutamine and 1% (5 ml) penicillin/streptomycin) to yield the desired concentration of x million cells per ml.

2.6.2 Thymus & Lymph Nodes
Mice were euthanised on day 7 or 8, according to experimental design, using inhaled CO₂. The thymus was retrieved via thoracotomy, while the draining lymph nodes were retrieved via laparotomy, with the nodes most proximal to the graft identified and removed. Each sample was placed directly into individual sterile containers containing sterile phosphate-buffered saline (PBS) and then transferred on ice to a laminar flow hood, in which all subsequent sterile steps were performed. Each thymus or LN was transferred to a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA) placed into a 50 ml Falcon tube and mechanically dissociated using a flat sterile instrument while being rinsed with sterile PBS to a total volume of approximately 45 ml. These tubes were then centrifuged at 1800 rpm for 6 minutes. The effluent was decanted and each thymus pellet was resuspended in 10 ml of sterile PBS for counting, while the lymph node pellet was resuspended in a maximum of 5 ml. Occasionally, if there appeared to be a significant red cell fraction in the thymic pellets, they were treated with ACK red cell lysis buffer, as described in the splenic
preparation section above. Following their count, the samples were centrifuged once more and resuspended in the appropriate volume of sterile complete RPMI, as described above.

2.6.3 Hearts
To isolate cells from the allografts, the heart samples (usually 1/3 – 1/2 of each excised graft) were finely minced in a petrie dish using a sterile blade; the fragments were then covered in sterile RPMI medium supplemented with 500 U collagenase (Worthington Biochemical, Lakewood, NJ, USA) per ml of medium and incubated for 30 min at 37°C. The collagenase-digested fragments were then washed through a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA) placed in a 50 ml sterile Falcon tube and centrifuged. The cell pellet was treated with ACK red cell lysis buffer and processed as a single cell suspension, as detailed above. The cells were resuspended in sterile complete RPMI medium and plated into a 96 well round bottomed plate; the cells were then restimulated with a 5-fold concentration of a stimulation solution containing PMA (5 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) plus ionomycin (500 ng/ml; Sigma-Aldrich); and brefeldin A (10 mg/ml; Sigma-Aldrich), which was slowly pipetted in against the wall of each well; the plate was returned to the incubator for 4 hours following which the cells were harvested and underwent intracellular cytokine staining as detailed later.

2.6.4 Cell counting
To obtain a cell count, 20 µl of each 10 ml cell solution was added to individual eppendorf tubes containing 980 µl of a 1:5 dilution of trypan blue. Each sample was then counted using a haemocytometer: all cells in the four outer quadrants were counted; each sample was counted in duplicate and the total divided by 16 and then multiplied by 10 to give a result of x million cells.
2.6.5 Serum
Whole blood was obtained by puncture of the right subclavian artery under sevoflurane anaesthesia (described above) just prior to euthanasia; the sample volume, on average, was approximately 1 ml. These whole blood samples were placed in 2 ml eppendorf tubes, allowed to stand at room temperature for at least 90 minutes, following which they were centrifuged at 14000 rpm for 20 minutes. The serum fraction was then pipetted off and transferred to a clean eppendorf tube; the samples were frozen at -80C until use.

2.7 Flow Cytometry (FC) Analysis

2.7.1 FC Antibodies
Anti-mouse antibodies against CD4, CD8, CD25, CD44, CD62L, ICOS, PD-1, CXCR5, B220 (CD45R), CD5, CD1d, CD21/35, CD24, CD138 (Syndecan) H2Kb and Thy1.2 (CD90.2) were purchased from BD Biosciences (San Jose, CA, USA) and used according to optimum dilutions determined in our laboratory. Where the Genentech IgG, aNotch-1 and aNotch-2 antibodies were used for flow cytometry, a concentration of 4 µg/1 x 10^6 cells/200 µl of each antibody was used as the primary antibody, as per the manufacturer’s instructions; a secondary goat anti-human fluorochrome AF488 (Invitrogen # A11013) was used for staining.

Intracellular Foxp3 staining was performed following cell permeabilisation using a FixPerm Kit (eBioscience, San Diego, CA, USA); anti-Foxp3 antibodies were purchased from eBioscience. Frequencies of T effector or regulatory cells were reported as the percentage of the total parent CD4^+ or CD8^+ cells; where specified, absolute counts were also calculated.

Evaluation of apoptotic cells was performed using the Annexin V Apoptosis Detection Kit (BD Pharmingen), according to the manufacturer’s instructions, with the exception of an alteration in the staining concentration of Annexin V
and 7AAD required. Previous dose titration experiments in our laboratory had indicated that the optimum concentration was 1 µl of stain per 100 µl.

Cells isolated from splenic, thymic and peripheral lymphoid tissues were analysed by flow cytometry using a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA) or a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). Further analysis was performed using FlowJo software version 9.6.2 (Treestar, Ashland, OR).

2.7.2 Expression of Notch-1 and Notch-2 by lymphocyte subsets
The cells were prepared as described in earlier to the point of counting. A proportion was removed for naïve (time 0) staining, while the remainder were stimulated in vitro for 1 or 3 hours. Once harvested and washed, the cells were re-suspended in 4% Formalin-PBS and placed on ice for 15 minutes. Now fixed, they were spun down as previously described and resuspended in PBS. The solution was then split: half was retained for surface staining, while the remaining half was permeabilised. An equal volume of ice-cold 100% methanol was added to the PBS solution to achieve a final concentration of 50% methanol; the solution was placed on ice for 30 minutes, after which the cells were considered fixed and permeabilised, and ready for staining.

For surface staining, an aliquot of 1 x 10^6 cells was removed and twice washed with 2 – 3 mls of PBS, and following centrifugation, resuspended in 100 µl of incubation buffer (0.5g BSA in 100 ml PBS) and plated in a 96 well v-bottomed plate. The Genentech primary antibodies with secondary fluorochrome goat anti-human AF488 (Invitrogen # A11013), along with a combination of other cell surface markers, were then used: as per the manufacturer’s instructions, a concentration of 4 µg/1 x 10^6 cells/200 µl of each antibody was used as the primary antibody.
2.8 Enzyme-linked immunospot (ELISPOT) assays

The frequencies of alloreactive IFN$_\gamma$-, Granzyme B-, IL-4- and IL-6-producing splenocytes were measured by ELISPOT assay (ELISPOT kits, BD Bioscience, San Jose, CA, USA for IFN$_\gamma$, IL-4 and IL-6; ELISPOT Development Module, R&D Systems, Minneapolis, MN, USA for Granzyme B). All kits were stored at 4°C. In a laminar flow hood, the primary or capture antibody for each cytokine was diluted in sterile PBS as follows: 1:200 for IFN$_\gamma$, IL-4 and IL-6; 1:60 for Granzyme B; each solution was carefully pipetted to ensure it was sufficiently mixed. Using a multi-channel pipette, 100 µl of this diluted antibody solution was added to each microwell on a sterile 96 well ELISPOT plate, and the plates were stored at 4°C overnight. The primary antibody was then discarded and the plates washed twice with 200 µl/well of sterile PBS; to prevent non-specific binding, 200 µl of a blocking protein solution, in the form of 1% bovine serum albumin (BSA), was then added to each microwell and left to incubate for 2 hours at room temperature. This was discarded and two further washes with sterile PBS 200 µl/well were performed; to prevent the filter membranes from dessicating, the second wash was not discarded until the final cell solutions were ready to plate.

To prepare the cell solutions, splenocytes from either IgG- or aNotch-1-treated mice recipient of a BALB/c cardiac allograft were isolated, counted and resuspended in complete sterile RPMI as described earlier; cells from each mouse were prepared separately. A cell concentration of $5 \times 10^6$ cells per ml for each solution was prepared; 100 µl, thereby containing $0.5 \times 10^6$ cells, was then added to the wells of an individual row (one animal per row). A further solution of allogeneic BALB/c splenocytes was prepared, and again diluted in sterile complete RPMI medium to achieve a cell concentration of $5 \times 10^6$ cells per ml; this sample was then irradiated (30 Gy) to ensure that these splenocytes remained capable of stimulation but were not a source of cytokine production. To determine alloreactivity, as our ELISPOT assays were designed to do, 100 µl of this allogeneic cell solution was added to the previously plated responder
(recipient) cells; each sample was plated in triplicate. For negative controls, 100 µl of medium was added to the previously plated responder cells; each negative control was plated in duplicate. For a positive control, the mitogen Concanavalin A (ConA) was used as stimulation; a master solution of 2 µl of ConA per ml of RPMI was prepared and 100 µl of this solution was added to the final well in each row. Each plate was covered with a sterile lid and then transferred to an incubator, where it was maintained in sterile humidified conditions at 37°C with 5% CO₂ for 16 hours (Granzyme B), 24 hours (IFNγ) or 48 hours (IL-4/IL-6).

Following their respective incubation periods, the plates were removed from the incubator; at this point, aseptic conditions were no longer required, therefore all subsequent steps were performed outside a laminar flow hood. The well contents were discarded and the plates washed twice with 200 µl/well of PBS, and then twice with 200 µl/well of PBS-Tween (0.1% Tween; 0.5 ml Tween 20 per 500 ml PBS). To prevent dessication of the filter membranes, the second wash was allowed to remain in the wells until the secondary antibody was ready to plate. The secondary or detection antibody for each cytokine was diluted in dilution buffer (PBS with 1% BSA) as follows: 1:100 for IFNγ, IL-4 and IL-6; 1:60 for Granzyme B. 100 µl of diluted antibody was added to each well, and the plates incubated overnight at 4°C. The next morning, the well contents were discarded and the plate was washed twice with 200 µl/well of PBS-Tween; Streptavidin-Horseradish Peroxidase (HRP) was diluted 1:100 in dilution buffer, and 100 µl/well of this was added to each well and allowed to incubate in the dark at room temperature for 2 hours. The well contents were then discarded and the plate was washed twice with 200 µl/well of PBS-Tween, and then twice with 200 µl/well of PBS. Finally, the visualisation solution was generated: for IFNγ, IL-4 and IL-6, 1 drop (20 µl) of AEC chromogen was added to each 1 ml of AEC substrate solution (BD Bioscience, San Jose, CA, USA) and vortexed briefly to ensure equal distribution. For the Granzyme B assay, a visualisation solution containing acetate buffer, chromogen and hydrogen peroxide was
created. 100 µl of the appropriate visualisation solution was added to each well and the plates incubated in the dark at room temperature until spot development was seen (usually 5-20 minutes). The reaction was then terminated by washing the plates thoroughly with deionised (DI) water; the plates were dried overnight in the dark before an automated count was performed using a computer-assisted ELISPOT image analyzer (Cellular Technology). The results were reported as number of spots per 0.5 x 10⁶ splenocytes.

2.9 Donor-specific antibody assay
To determine the presence of donor-specific antibodies (DSA), serum samples were obtained from aNotch-1-treated recipients on day 7 post-transplant, and from aNotch-2-treated recipients on day 8 post-transplant, according to their respective dosing regimens; samples were taken from the correlate IgG-treated controls simultaneously. The serum samples were processed as previously described and frozen at -80°C until the day of use, at which point they were gently thawed. Incremental dilutions of the individual serum samples (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024) were made: 180 µl of FACS buffer was added to each well in the first column of a 96 well round-bottomed plate, while 120 µl was added to each well in the remaining columns; 60 µl of each individual serum sample was then added to an individual well in the first column to make the first (1:4) dilution; using a multi-channel pipette, the contents of each well in the first column were then mixed thoroughly; the tips were changed and 120 µl of each sample in the first column was added to the corresponding well in the second column and mixed well; the pipette tips were changed and 120 µl of each sample in the second column was then added to the wells of the third column; this step was repeated for all remaining columns so that the last column (1:1024) contained 240 µl.

A cell solution of naïve BALB/c splenocytes was then prepared with a final cell solution of 10 x 10⁶ cells per ml; 100 µl of this cell solution (1 x 10⁶ cells) was
added to each well of a 96 well v-bottomed plate, which was then centrifuged at 2000 rpm for 3 minutes; the effluent was discarded and 100 µl of each diluted serum sample was then transferred to the corresponding wells of the v-bottomed plate; using a multi-channel pipette, the contents of each well were thoroughly pipetted to ensure disruption of the cell pellet and sufficient mixing of each well. The plate was incubated for 30 minutes at 4C, and then centrifuged at 2000 rpm for 3 minutes; the effluent was discarded and each well was washed with 150 µl FACS buffer. This step was repeated, and following the discard of the effluent, 100 µl of a master staining mix, containing a combination of fluorochrome-conjugated antibodies directed against IgG1, IgG2a, CD4 and B220 (CD45R), was added to each cell pellet; using a multi-channel pipette, the contents of each well were thoroughly pipetted and incubated for 30 minutes at 4C. The centrifuge and washing steps described above were performed twice, and the cell pellets resuspended in 100 µl of FACS buffer; the samples were then analysed by flow cytometry using a FACSCanto™ II or a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). If it was not possible to run the plate within a few hours, the cell pellets were instead suspended in 100 µl of 1% FACS-Formalin, and run within 48 hours. Further analysis was performed using FlowJo software version 9.6.2 (Tree Star, Inc., Ashland, OR, USA): DSA were calculated as the percentage of IgG1-positive or IgG2a-positive cells of CD4⁺ (Class I MHC) or B220⁺ (Class II MHC) lymphocytes.

2.10 Mixed lymphocyte reaction (MLR) assay
Mice recipient of an allogeneic cardiac transplant were treated with aNotch-1 or aNotch-2, according to dosing protocol, and euthanised on day 7 or 8, respectively. Splenocytes were isolated and processed as a single cell suspension in a laminar flow hood, as detailed above, and resuspended in sterile complete RPMI to a concentration of 10 x 10⁶ splenocytes/ml. The cell suspensions were maintained on ice until ready for use. A naïve donor strain mouse was euthanised, the spleen harvested and processed as above, and a cell suspension with a final concentration of 10 x 10⁶ splenocytes/ml generated.
100 µl of each cell suspension was added to each well. If, however, an additional *in vitro* condition was being tested, e.g., the effect of aNotch-1 or aNotch-2 *in vitro*, the donor splenocytes were instead resuspended at a concentration of 20 x 10^6 cells/ml; 50 µl of this was then added, with the final 50 µl composed of the treatment. The plate was then transferred to an incubator where it was maintained in sterile humidified conditions at 37°C with 5% CO₂ for 72 hours.

Thereafter, supernatants were collected from the wells, taking care not to disturb the cell pellet, and immediately frozen at -80°C until subsequent analysis by Luminex; the volume collected depended on whether or not the plate was additionally going to be used to examine the rate of cell proliferation. If so, this was achieved by measurement of the rate of incorporation of tritiated thymidine (3H-TdR), which was added 16 hours before the end of the culture period, used in accordance with laboratory protocols on the use of radioactive material. At the end of the culture period, the plate was either analysed immediately using a liquid scintillation counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter) or was frozen in its entirety at -80°C until thawed for later analysis.

### 2.11 Treg suppression assay (non-allospecific)

Spleens harvested from four naïve BALB/c mice were pooled and prepared as described earlier, and were resuspended at a concentration of 100 x 10^6 cells/ml sterile PBS. 10 Foxp3-GFP mice were each sensitised by intraperitoneal injection of 150 µl (15 x 10^6 splenocytes) of the splenocyte solution on day 0. 5 were treated with 5 mg/kg IgG on days 0, 2, 4, 6, 8, 10 & 12, while the remaining 5 were treated with 5 mg/kg aNotch-1 on days 0, 2, 4, 6, 8, 10 & 12. The mice were sacrificed on day 14; the spleen and two lymph nodes were harvested from each mouse; samples obtained from the mice within each group were combined; all samples were prepared as single cell suspensions in a laminar flow hood, as earlier described.
The cell suspensions from the IgG group and the aNotch-1 group were then subject to MACS separation (Miltenyi Biotec, Cambridge, MA, USA). Following determination of the cell count for each sample, the samples were centrifuged again at 1800 rpm for 8 minutes; the effluent was discarded and the samples resuspended in 90 µl of sterile MACS buffer per 10 x 10^6 cells; 8 µl of LT34 beads per 10 x 10^6 cells was then added; the sample was thoroughly pipetted to ensure sufficient mixing and was then incubated on ice for 15 minutes. Following this, the reaction was terminated by the addition of MACS buffer to a total volume of 45 ml. The sample was once again centrifuged as described above before resuspending in 2 ml of MACS buffer.

Meanwhile, two LS columns were placed in a MACS separator and 4 x 15 ml sterile Falcon tubes were labeled as follows:

1) IgG CD4^-
2) IgG CD4^+
3) aNotch-1 CD4^-
4) aNotch-1 CD4^+

Tubes 1 and 3 were placed in a tube rack that was then positioned underneath the LS columns. Each column was prepared by the addition of 3 ml of sterile MACS buffer; the buffer was rinsed through and collected in the tubes below. The IgG cell suspension was added to the column above tube 1, and the aNotch-1 suspension to the column above tube 3. Once each suspension had passed through its respective column, and the unlabeled cell fraction collected in the tube below, the columns were rinsed again by the addition of 3 x 3 ml of MACS buffer; each 3 ml wash was added only once the column reservoir was empty. Following the third wash, the columns were removed from the magnetic separator and placed into tubes 2 and 4. 5 µl of sterile MACS buffer was placed into each column and the labeled cell fraction (CD4^+) was flushed out using the supplied plunger. The CD4^- fractions were kept on ice for later use.
The CD4\(^+\) fraction of each group was centrifuged as before and then resuspended in 5 ml of sterile MACS buffer; 2 \(\mu\)l of anti-CD4 fluorochrome (usually APC) was added to each cell suspension and thoroughly mixed; each sample was incubated at 4C for 20 minutes, following which the sample was washed with sterile MACS, centrifuged and then resuspended in sterile MACS buffer to a maximum concentration of 20 x 10\(^6\) cells/ml. Each sample was then flow-sorted into CD4\(^+\)GFP(Foxp3)\(^+\) and CD4\(^+\)GFP(Foxp3)\(^-\) fractions using a FACS Aria™ cell sorter, and then stored on ice until ready for use.

A proportion (50 x 10\(^6\) cells) of the CD4\(^-\) fraction isolated from the IgG-treated mice was stained with anti-CD3 PE, and then magnetically sorted, as described above, using anti-PE microbeads (Miltenyi Biotec, Cambridge, MA, USA) to generate a CD3\(^+\) and CD3\(^-\) fraction; the CD3\(^-\) fraction was considered to be the APC population. Once counted, the APCs were resuspended in 0.9 ml of sterile RPMI, to which 0.1 ml of mitomycin was then added; the cell solution was incubated at 37C for 20 minutes, was then washed twice with sterile PBS and finally resuspended in sterile RPMI supplemented with 1 \(\mu\)l of 2-mercaptoethanol (2-ME) per ml of medium at a concentration of 3 x 10\(^6\) cells/ml.

Following flow-sorting, the sorted cell populations were centrifuged once at 1800 rpm for 8 minutes. The CD4\(^+\)GFP(Foxp3)\(^-\) cells (Teff) isolated from the IgG-treated mice were resuspended in sterile RPMI supplemented with 1 \(\mu\)l of 2-ME per ml of medium at a concentration of 2 x 10\(^6\) cells/ml. This Teff suspension was then mixed with the APC suspension in a 1:1 ratio, so that the concentration was 1Teff + 1.5 APC x 10\(^6\) cells/ml. Finally, \(\alpha\)CD3 was added to the suspension at a concentration of 4 \(\mu\)g/ml. 100 \(\mu\)l (0.1 x 10\(^6\) cells) of this suspension was added to each well of a 96 well round bottomed plate.

The flow-sorted CD4\(^+\)GFP(Foxp3)\(^+\) cells (Tregs) were resuspended in 2-ME-supplemented sterile RPMI at a concentration of 1 x 10\(^6\) cells/ml; serial dilutions of this suspension were then made, and 100 \(\mu\)l of each CD4\(^+\)GFP(Foxp3)\(^+\)
dilution was co-cultured with the Teff+APC fraction to yield varying ratios (Treg:Teff+APC 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) for 96 hours. If sufficient cells had been yielded, IgG or aNotch-1 were also added in vitro at a concentration of 10 µg/ml to a quarter of the plate each, to assess any potential additive effect. As a positive control, the Teff+APC suspension was plated with medium alone, while the Treg suspension was plated alone as a negative control. The plate was covered with a sterile lid and placed in an incubator, where it was maintained in sterile humidified conditions at 37°C with 5% CO₂ for 96 hours.

Cell proliferation was visually assessed daily by microscopy of the sealed plate, but was formally measured by the rate of incorporation of tritiated thymidine (³H-TdR), which was added 16 hours before the end of the culture period, and was used in accordance with laboratory protocols on the use of radioactive material. At the end of the culture period, the plate was either analysed immediately using a liquid scintillation counter or was frozen in its entirety at 80°C until thawed for later analysis.

2.12 Treg suppression assay (allospecific)
The experiment was performed exactly as described for the non-allospecific Treg suppression assay above. However, ABMtg.Foxp3-GFP mice were used instead of Foxp3-GFP, and these mice were sensitised by intraperitoneal injection of a single cell suspension of bm12 splenocytes instead of BALB/c splenocytes.

2.13 In vivo Treg conversion assay
Splenocytes were isolated from 3 – 5 naïve ABMtg-Foxp3-GFP mice and processed as described in the Treg suppression assay above to the point of flow sorting into CD4⁺GFP(Foxp3)⁺ and CD4⁺GFP(Foxp3)⁻ fractions. The CD4⁺GFP(Foxp3)⁻ fraction was centrifuged and resuspended in sterile PBS at a concentration of 20 x 10⁶ cells/ml. Naïve 6 – 8 week old Thy 1.1⁺ B6 mice received a skin transplant, as detailed above, from a bm12 donor, which carries
the Thy 1.2 (CD90.2) cell surface antigen. Following the transplant, but on the same day, the mice received an intraperitoneal injection of 200 µl of the CD4\(^+\)GFP(Foxp3)\(^-\) cell suspension (4 x 10\(^6\) CD4\(^+\)Foxp3\(^-\) cells). They were further treated with either IgG, aNotch-1 or aNotch-2 at a dose of 5 mg/kg on days 0, 2, 4 and 6. The mice were sacrificed on day 7; the spleen, thymus and two abdominal lymph nodes were harvested from each mouse and were processed as single cell suspensions, according to our protocol. The samples were then stained for a combination of surface and intracellular markers, including Thy 1.2, and analysed by flow cytometry on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). Further analysis was performed using FlowJo software version 9.6.2 (Tree Star, Inc., Ashland, OR, USA): the proportion of allospecific Tregs was calculated by determining the percentage of CD4\(^+\) lymphocytes expressing Thy1.2 and Foxp3.

2.14 In vitro Treg generation assay
Using a 96-well round bottom tissue culture plate, 1×10\(^5\) CD4\(^+\)Foxp3\(^-\) T cells and 1×10\(^5\) CD3\(^-\) cells (APCs) isolated from naïve B6 GFP.Foxp3 mice were cultured in the presence of \(\alpha\)CD3 (2 µg/ml; BD Biosciences, San Jose, CA, USA), hTGF-β (3 ng/ml; R&D Systems, Minneapolis, MN, USA) ± mIL-2 (10ng/ml; R&D Systems), as described (253). APCs were isolated by depleting T cells from total splenocytes with \(\alpha\)CD3-PE and \(\alpha\)PE microbeads (Miltenyi Biotec), and were briefly treated with mitomycin C (50 µg/ml, Sigma-Aldrich), as described earlier. To determine the effect of Notch-1 inhibition, either IgG or aNotch-1 was added to each well; two concentrations for each antibody were examined in each experiment, 1 µg/ml and 10 µg/ml. The plates were incubated at 37°C for 4 days prior to cell harvesting, staining and analysis by flow cytometry.
2.15 T cell differentiation assay

Splenocytes were isolated from naïve Foxp3-GFP mice and processed as described in the Treg suppression assay above to the point of flow sorting into CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ fractions. The CD4⁻ fraction (collected during the first MACS separation) was again subject to magnetic separation into CD3⁺ and CD3⁻ fractions, but this time using the MACS CD3 biotin kit (Miltenyi Biotec, Cambridge, MA, USA) and LD columns. CD4⁺GFP(Foxp3)⁻ cells were resuspended in X-VIVO medium supplemented with 1 µl of 2-ME per ml of medium at a concentration of 2 x 10⁶ cells/ml. The CD3⁻ or APC fraction was resuspended in 2-ME-supplemented X-VIVO at a concentration of 2 x 10⁶ cells/ml. The first time this experiment was performed, the APC fraction was treated with mitomycin, as described in the Treg suppression assay; however, the degrees of polarisation achieved for each condition were poor, which was thought to be potentially due to the treatment of the APCs. In subsequent experiments, I used untreated APCs with better results, and hence modified the protocol to represent this.

A four-fold concentration of six different differentiation conditions were prepared in sterile X-VIVO medium:

1) Th0: 8 µl of αCD3 [1 mg/ml]
2) Th1: 8 µl of αCD3 [1 mg/ml] + 4 µl of IL12 [10 µg/ml] + 40 µl of αIL4 [1 mg/ml]
3) Th2: 8 µl of αCD3 [1 mg/ml] + 4 µl of IL4 [10 µg/ml] + 40 µl of αIL12 [1 mg/ml]
4) Th17: 8 µl of αCD3 [1 mg/ml] + 12 µl of IL6 [10 µg/ml] + 1.2 µl of TGFβ [5 µg/ml]
5) Th9: 8 µl of αCD3 [1 mg/ml] + 4 µl of IL4 [10 µg/ml] + 2.4 µl of TGFβ [5 µg/ml] ± 20 µl of αIFNγ
6) Treg: 8 µl of αCD3 [1 mg/ml] + 2.4 µl of TGFβ [5 µg/ml] ± 4 µl of IL2 [1 mg/ml]
The effect of IgG, aNotch-1 or aNotch-2 on each of the different polarising conditions was evaluated; two different concentrations (1 and 10 µg/ml) of each antibody were tested. Firstly, a 1 mg/ml concentration of each antibody was prepared in sterile X-VIVO medium. To prepare a solution that would provide a final (well) concentration of 10 µg/ml, a four-fold concentration was made: 2 µl of the 1 mg/ml solution was added per each 48 µl of X-VIVO medium; to prepare the 1 µg/ml (final) solution, a 1:10 dilution was made: 100 µl of each 10 µg/ml solution was added to an individual sterile eppendorf containing 900 µl of sterile X-VIVO and vortexed briefly to ensure sufficient mixing.

50 µl of each of the above solutions and suspensions was added to each well of a 96 well round bottomed plate, to a total well volume of 200 µl. Each well therefore contained 0.1 x 10^6 CD4^+ cells and 0.1 x 10^6 APCs. Each condition (i.e., each differentiation condition with each concentration of the three antibodies) was plated in duplicate. The plate was covered with a sterile lid, placed in an incubator and maintained in sterile humidified conditions at 37°C with 5% CO_2 for 72 hours. At the end of the incubation period, the plate was removed from the incubator, and in a laminar flow hood, 40 µl of the supernatant was carefully removed using a multi-channel pipette without disturbing the cell pellet. The supernatant collected was plated in the corresponding wells of a fresh 96 well round-bottomed plate and immediately frozen at -80°C. The remaining wells were then pulsed with a stimulation solution: 40 µl of a 5-fold concentration of a stimulation solution containing Golgi-stop, PMA and ionomycin was carefully added (slowly pipetted in against the wall of each well) and the plate was returned to the incubator for 4 hours.

At the end of the stimulation period, the plate was removed from the incubator, and using a multi-channel pipette, the wells were thoroughly resuspended and transferred to their corresponding well on a fresh v-bottomed plate. The plate was centrifuged at 2000 rpm for 3 minutes, the effluent discarded, and the wells washed with 150 µl of PBS. The samples were first stained with a fluorochrome-
conjugated anti-CD4 antibody appropriately diluted in FACS buffer. Following the usual incubation period, the plate was centrifuged and washed, according to protocol. To facilitate intracellular cytokine staining, the cells were then permeabilised using the BD Cytofix/Cytoperm™ Kit (BD Bioscience, San Jose, CA, USA; catalogue # 554714), which was used for all conditions except Tregs; for the wells undergoing Treg polarisation, the Foxp3 Transcription Factor Fixation/Permeabilisation kit (eBioscience, San Diego, CA, USA; catalogue # 00-5521-00) was used. Following permeabilisation, the cells were washed with the appropriate buffer and stained with a combination of fluorochrome-conjugated antibodies for each differentiation condition (listed below); the antibodies were diluted in the wash buffer provided with each permeabilisation kit.

The following staining protocols were used:

1) Th0: CD4/B220/Granzyme B/IL-2/IFNγ
2) Th1: CD4/B220/Granzyme B/IL-2/IFNγ
3) Th2: CD4/B220/IL-13/IL-4/IL-10
4) Th17: CD4/B220/IL-21/IL-17
5) Th9: CD4/B220/IL-10/IL-9
6) Treg: CD4/CD25/IL-10/CTLA-4/Foxp3

The plates were then run on a FACSCanto™ II flow cytometer (BD Bioscience, San Jose, CA, USA). Further analysis was performed using FlowJo software version 9.6.2 (Tree Star, Inc., Ashland, OR, USA): each differentiation population was classified as the proportion of CD4+ lymphocytes secreting the relevant combination of cytokines.

2.16 Enzyme-linked immunosorbent assay (ELISA)

2.16.1 BAFF ELISA

To measure levels of BAFF in the serum, the Quantikine Mouse BAFF/BLyS/TNFSF13B Immunoassay kit (R&D Systems, Minneapolis, MN,
USA; catalogue # MBLYS0) was used. The kit was stored at 4C and all reagents allowed to return to room temperature before use. The Control was reconstituted with 1 ml of (deionised) DI water, vortexed to ensure sufficient mixing and allowed to stand. The wash buffer was prepared by adding 20 ml of wash buffer concentrate to 480 ml of DI water and allowed to stand.

To prepare the standards, 7 eppendorf tubes were first labeled as follows:

1) Standard 0 (0 pg/ml)
2) Standard 1 (46.9 pg/ml)
3) Standard 2 (93.8 pg/ml)
4) Standard 3 (188 pg/ml)
5) Standard 4 (375 pg/ml)
6) Standard 5 (750 pg/ml)
7) Standard 6 (1500 pg/ml)

The BAFF/BLyS Standard was reconstituted with Calibrator Diluent RD6-12, to produce a stock solution of 3000 pg/ml, and allowed to stand for at least 5 minutes. Meanwhile, 200 µl of Calibrator Diluent RD6-12 was added to tubes 1 – 7. The stock solution was gently vortexed, following which 200 µl was removed and added to tube 7 to produce a 2-fold dilution and a standard of 1500 pg/ml (Standard 6). This standard was then vortexed to ensure sufficient mixing and a fresh pipette tip was used to remove 200 µl and add it to tube 6, thereby generating a standard of 750 pg/ml. The above steps were repeated as far as tube 2 (Standard 1); tube 1 (Standard 0) contained only Calibrator Diluent RD6-12 and served as the zero standard while the stock solution (3000 pg/ml) served as the high standard.

A 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse BAFF/BLyS was provided with the kit; any wells not designated for use in the experiment were removed prior to the assay. 80 µl of Assay Diluent RD1N was added to each well. The Standards were first plated in
duplicate, followed by the Control, all at a volume of 40 µl. The serum samples were diluted 3-fold (40 µl of sample + 80 µl of Calibrator Diluent RD6-12), and 40 µl of each diluted sample was plated in duplicate, according to the experimental layout. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. Following the incubation period, the plate was removed from the shaker and each well aspirated and washed five times using a multichannel pipette; a volume of 400 µl of Wash Buffer was used to wash each well; after the last wash, any remaining Wash Buffer was removed by aspirating or decanting; finally, the plate was inverted and blotted against clean paper towels. 120 µl of Mouse BAFF/BLyS Conjugate was added to each well, and the plate covered with a new adhesive strip; it was once again incubated for 2 hours at room temperature on the shaker.

Towards the end of this incubation period, the Substrate Solution was prepared by mixing Colour Reagents A (stabilised hydrogen peroxide) and B (stabilised chromogen) together in equal volumes (mixed within 15 minutes of use) and was stored in the dark at room temperature. Following incubation, the five aspiration/wash steps were repeated and 120 µl of Substrate Solution added to each well. The plate was covered with aluminium foil and was incubated (without shaking) in the dark at room temperature for 30 minutes. 120 µl of the Stop Solution (diluted hydrochloric acid) was added to each well and the plate gently tapped to ensure sufficient mixing of the well contents.

The plate was then analysed within 15 minutes using a microplate reader set to 450 nm with wavelength correction set at 540 nm. A standard curve was generated and the concentration multiplied by the dilution factor of each sample. The duplicate readings for each standard, control and sample were averaged.
All kits were obtained from Life Diagnostics, Inc. (West Chester, PA, USA), and were stored at 4°C; reagents were allowed to return to room temperature before use. Each kit provided a 96 well plate pre-coated with its respective antibody. The Diluent for each kit was provided as a 10x stock and was diluted to 1x with DI water; the Wash Solution was provided as a 20x stock and was also diluted to 1x with DI water. Once prepared (detailed below), 100 µl of the standards and diluted samples were plated in duplicate and incubated on an orbital micro-plate shaker at room temperature for 45 minutes. The contents of each well were aspirated and washed five times with the 1x Wash Solution (400 µl/well) using a plate washer. The plate was inverted, struck sharply and blotted against clean paper towels. 100 µl of the appropriate Enzyme Conjugate Reagent was added to each well; the plate was again incubated on an orbital micro-plate shaker for 45 minutes (30 minutes for IgG1, IgG2a, IgG2b & IgG3). The plate was again aspirated and washed as described earlier. 100 µl of TMB reagent was added to each well; the plate was incubated on the plate shaker for a further 20 minutes at room temperature. 100 µl of Stop Solution (diluted hydrochloric acid) was added to each well and the plate gently tapped to ensure sufficient mixing of the well contents. The optical density of each well was read within 5 minutes at 450 nm using a microplate reader. A standard curve was generated and the concentration multiplied by the dilution factor of each sample (all reported as ng/ml). The duplicate readings for each standard and sample were averaged. The standards, sample dilutions and enzyme conjugate reagent for each kit varied and were prepared as follows:

2.16.2.1 IgM (catalogue # 5015-1)
Anti-mouse IgM coated 96 well plate.
Mouse IgM standards: 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 0 ng/ml, prepared as detailed in the BAFF ELISA section.
HRP Conjugate Reagent
Sample dilution: 10,000 fold. To prepare, 247.5 µl of 1x Diluent was pipetted into 2 eppendorf tubes. 2.5 µl of sample was added to the first tube to generate a 100 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2.5 µl of this dilution was added to the second eppendorf tube to provide a 10,000 fold dilution of the sample.

2.16.2.2 IgG (catalogue # 5010-1)
Anti-mouse IgG coated 96 well plate.
Mouse IgG standards: 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 0 ng/ml, prepared as detailed in the BAFF ELISA section.
HRP Conjugate Reagent
Sample dilution: 200,000 fold. To prepare, 998 µl and 798 µl of 1x Diluent were pipetted into 2 separate eppendorf tubes. 2 µl of sample was added to the first tube (998 µl) to generate a 500 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2 µl of this dilution was added to the second eppendorf tube to provide a 200,000 fold dilution of the sample.

2.16.2.3 IgG1 (catalogue # 5010-1A)
Anti-mouse IgG coated 96 well plate.
Mouse IgG1 standards: 50, 25, 12.5, 6.25, 3.13, 1.56 ng/ml, prepared as detailed in the BAFF ELISA section.
Anti mouse IgG1-HRP Conjugate Reagent
Sample dilution: 100,000 fold. To prepare, 498 µl and 798 µl of 1x Diluent were pipetted into 2 separate eppendorf tubes. 2 µl of sample was added to the first tube (498 µl) to generate a 250 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2 µl of this dilution was added to the second eppendorf tube to provide a 100,000 fold dilution of the sample.
2.16.2.4 IgG2a (catalogue # 5010-1B)
Anti-mouse IgG coated 96 well plate.
Mouse IgG2a standards: 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 ng/ml, prepared as detailed in the BAFF ELISA section.
Anti mouse IgG2a-HRP Conjugate Reagent
Sample dilution: 100,000 fold. To prepare, 498 µl and 798 µl of 1x Diluent were pipetted into 2 separate eppendorf tubes. 2 µl of sample was added to the first tube (498 µl) to generate a 250 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2 µl of this dilution was added to the second eppendorf tube to provide a 100,000 fold dilution of the sample.

2.16.2.5 IgG2b (catalogue # 5010-1C)
Anti-mouse IgG coated 96 well plate.
Mouse IgG2b standards: 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 ng/ml, prepared as detailed in the BAFF ELISA section.
Anti mouse IgG2b-HRP Conjugate Reagent
Sample dilution: 100,000 fold. To prepare, 498 µl and 798 µl of 1x Diluent were pipetted into 2 separate eppendorf tubes. 2 µl of sample was added to the first tube (498 µl) to generate a 250 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2 µl of this dilution was added to the second eppendorf tube to provide a 100,000 fold dilution of the sample.

2.16.2.6 IgG3 (catalogue # 5010-1D)
Anti-mouse IgG coated 96 well plate.
Mouse IgG3 standards: 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml, prepared as detailed in the BAFF ELISA section.
Anti mouse IgG3-HRP Conjugate Reagent
Sample dilution: 50,000 fold. To prepare, 498 µl and 796 µl of 1x Diluent were pipetted into 2 separate eppendorf tubes. 2 µl of sample was added to the first tube (498 µl) to generate a 250 fold diluted sample; the tube was vortexed to
ensure sufficient mixing. 4 µl of this dilution was added to the second eppendorf tube to provide a 50,000 fold dilution of the sample.

2.16.2.7 IgA (catalogue # 5016-1)

Anti-mouse IgA coated 96 well plate.

Mouse IgGA standards: 30, 15, 7.5, 3.75, 1.88 and 0.93 ng/ml, prepared as detailed in the BAFF ELISA section.

HRP Conjugate Reagent

Sample dilution: 10,000 fold. To prepare, 247.5 µl of 1x Diluent was pipetted into 2 eppendorf tubes. 2.5 µl of sample was added to the first tube to generate a 100 fold diluted sample; the tube was vortexed to ensure sufficient mixing. 2.5 µl of this dilution was added to the second eppendorf tube to provide a 10,000 fold dilution of the sample.

2.17 Luminex

Luminex/XMAP is a high throughput multiplexed-bead based technology, where beads are internally dyed with fluorescent dyes to produce a specific spectral signature. Biomolecules (such as an antibody or receptor) can be conjugated to the surface of beads to capture analytes of interest; a secondary antibody, conjugated to a fluorochrome (phycoerythrin) is then added. Inside the Luminex analyzer, a light source excites the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. This technology was based on flow cytometric analysis and indeed shares common components with general flow cytometry instruments such as lasers, fluidics, and optics. However, unlike other flow cytometer microsphere-based assays which use a combination of different sizes and color intensities to identify an individual microsphere, patented xMAP Technology uses a single 5.6 micron size microsphere and a proprietary dying process to create 500 unique dye mixtures which are used to identify an individual microsphere.
2.17.1 Chemokine/Cytokine Luminex

All kits were purchased from Millipore (Billerica, MA, USA) and stored at 4°C; all reagents were allowed to return to room temperature before use. To prepare the kit, the 10X Wash Buffer was diluted to a 1X solution using DI water; the Serum Matrix was reconstituted with 2.0 ml of Assay Buffer, vortexed thoroughly and allowed to stand for at least 10 minutes at room temperature. 90 µl of each analyte bead solution were combined in an opaque bottle and placed in the dark at room temperature. The Controls (QC1 & 2) were reconstituted with 250 µl of DI water, vortexed to ensure sufficient mixing and allowed to stand at room temperature. Meanwhile, the Standard was reconstituted with 250 µl of Assay Buffer to provide a stock solution of 10000 pg/ml; serial 1:5 dilutions (10000, 2000, 400, 80, 16, 3.2 and 0 pg/ml) were made as described in the BAFF ELISA section using Assay Buffer as the diluent.

200 µl of diluted Wash Buffer was added to each well of a 96 well microplate and placed on an orbital microshaker for 5 minutes. The plate was then placed on a micro-plate vaccum and the contents suctioned out carefully, ensuring a slow but steady rate of removal to avoid damage to the filter. 15 µl of each standard and control was plated (the standards were plated in duplicate, while QC1 and QC2 were plated singly). The samples, either serum or supernatant, were then plated in either duplicate or triplicate. Finally, 15 µl of Assay Buffer was added to the wells containing the samples, while 15 µl of Serum Matrix was added to the wells containing the Standards and Controls. If supernatants were being analysed, 15 µl of the relevant culture medium (used in the original experiment) was added to the wells containing the Standards and Controls instead of Serum Matrix. The mixing bottle (containing the bead solution) was vortexed thoroughly and 15 µl of the bead solution was added to each well. The plate was covered in aluminium foil and incubated on an orbital plate shaker overnight at 4°C.
The next morning, the plate was vacuumed as described above and underwent two further wash/vacuum steps (washed with 150 µl of Wash Buffer per well). 15 µl of the Detection Antibody was then added to each well and the plate once more covered with foil and incubated at on a plate shaker for 60 minutes at room temperature. Following this incubation step, 15 µl of Streptavidin-Phycoerythrin (PE) was added directly to the wells containing the Detection Antibody, and the plate again covered with foil and incubated on a plate shaker for 30 minutes at room temperature. The plate was then vacuumed as described earlier, and underwent two further wash/vacuum steps (washed with 150 µl of Wash Buffer per well). 150 µl of Sheath Fluid was added to each well; the plate was covered with foil and placed on the shaker for a further five minutes. It was then analysed on a Luminex® 200™ system using XPONENT software with a minimum cut-off read of 50 beads per well. The Luminex® 200™ system was fully calibrated on a weekly basis, while the probe was sonicated and then cleaned with a 70% alcohol and DI water rinse prior to the start of each experiment.

2.18 Statistics
Kaplan-Meier survival graphs were constructed, and a log-rank comparison of the groups was used to calculate $p$ values. The unpaired $t$-test was used for comparison of 2 experimental groups. The one-way analysis of variance (ANOVA) was used for comparison of three or more groups. Differences were considered to be significant where $p \leq 0.05$.

Prism software was used for data analysis and drawing graphs (GraphPad Software, Inc., San-Diego, CA, USA). Data represent mean ± SEM. Images were prepared using Adobe Illustrator.
Chapter Three: Investigation of the tolerability and effect of γ-secretase inhibitors compared to more selective Notch receptor inhibition

3.1 Introduction
The alloimmune response is critically dependent on T cells, B cells, antigen-presenting cells (APCs) and the interaction thereof: both CD4+ and CD8+ T cells play a dominant role in cell-mediated alloimmunity and have been shown to be of key importance in allograft rejection (254-256), while the role of B cells has gained increasing recognition, both in terms of antigen presentation and alloantibody production. The ability to influence T and B cell fate is clearly of great interest in the field of transplantation; the involvement of the Notch receptors in both T and B cell development and differentiation therefore makes them a very attractive potential target for therapeutic manipulation.

As discussed earlier, the pharmacological achievement of Notch receptor inhibition has, to date, been realised by γ-secretase inhibitors (GSIs), which were originally developed to block the production of the amyloid-β peptide from amyloid precursor protein in efforts to treat Alzheimer's Disease (257). Their progression into clinical use, however, has been stymied by a range of undesirable on-target effects. As the γ-secretase cleavage step is common to all Notch receptors, GSIs do not distinguish between the individual receptors and therefore effect pan-Notch inhibition; furthermore, the γ-secretase complex is not exclusive to the Notch signaling pathway, such that inhibition of γ-secretase leads to a variety of undesired on-target effects (242). Use of GSIs in a bone marrow transplant model achieved reduced production of inflammatory cytokines by alloreactive T cells following allogeneic BMT, but resulted in severe gastrointestinal toxicity which was rapidly fatal (238). While the combination of Notch-1 and Notch-2 blockade has been shown to cause intestinal toxicity due to interference with the normal architecture of intestinal
crypts (240, 241), it was thought that this may have been aggravated and accelerated by intestinal injury following total body irradiation (238). There is no published data regarding the use of GSIs in solid organ transplantation, however, and both their effect on the alloimmune response therein, and the extent to which the reported associated intestinal toxicity would compromise their use, remain unclear.

In an expansion of the arsenal of available Notch inhibitors, Genentech, Inc. have recently developed antibodies directed against the individual Notch receptors Notch-1 and Notch-2 (243), providing an opportunity to specifically antagonise their cognate receptors and determine their individual effects.

The aims of this chapter were to investigate the use of GSIs in solid organ transplantation and to determine the effect and tolerability of the novel antagonistic Notch-1 and Notch-2 antibodies in naïve mice prior to testing them in the alloimmune setting.
3.2 Methods
All experimental methods are described in detail in Chapter 2, so for the purposes of this chapter will be only briefly described.

3.2.1 Mice
6 – 8 week old C57BL/6 (B6; Thy1.1+) and BALB/c (H-2d) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in our facility. Naïve B6 mice were used for all experiments investigating the effects of the Notch-1 and Notch-2 antibodies, and were also used as recipients of a BALB/c cardiac allograft in the transplantation experiments.

Notch-1/-2 DKO (N1N2 DKO) mice were generated using flox-cre technology (Notch1/2\(^{f/f}\)CD4\(^{Cre}\)), and are characterised by conditional deletion of both Notch-1 and Notch-2 in CD4\(^{+}\) cells following injection with Poly-IC, as detailed in section 2.3.4; CD4\(^{Cre}\) mice were used as controls.

3.2.2 Transplant Model
Vascularised intra-abdominal heterotopic cardiac allograft transplants were performed using sterile microsurgical techniques (258) by Tetsunosuke Shimizu (T.S.), a surgical fellow in our laboratory, as described in detail in Chapter 2.4.1. General anaesthesia was achieved by intraperitoneal injection of a combination of ketamine and xylazine.

3.2.3 Antibodies

3.2.3.1 GSIs
The GSI Dibenzazepine (DBZ) was purchased from Calbiochem (EMD Millipore, Billerica, MA), and was prepared as previously described in Section 2.2.5. DBZ was administered at a dose of 500 \(\mu\)g/100 g body weight/day, translating into a usual dose of 125ug daily, for 10 days.
3.2.3.2 Notch receptor antibodies
Anti-Notch-1 (aNotch-1) and anti-Notch-2 (aNotch-2) antibodies were generously provided by Dr. Christian Siebel of Genentech, Inc. (South San Francisco, CA, USA). They are antagonistic, fully human IgG1 antibodies which potently inhibit their respective paralogues but not other Notch receptors, and which bind with similarly high affinity to both the mouse and human orthologues (243). Control IgG, also obtained from Genentech, Inc., was administered as a control antibody at the same dose and on the same schedule as aNotch-1 or aNotch-2, according to the respective experiments.

3.2.3.3 Flow cytometry antibodies for expression of Notch receptors
The Genentech IgG, aNotch-1 and aNotch-2 antibodies used for the in vivo experiments were also used to detect expression of Notch-1 and Notch-2 by flow cytometry; a secondary goat anti-human fluorochrome AF488 (Invitrogen # A11013) was used for staining.
3.3 Results

3.3.1 Effect of γ-secretase inhibitors on allograft survival

This experiment was undertaken by my colleague, Dr. L.V. Riella, following on from his work investigating the role of both DLL1 and Jagged2 in the alloimmune response (233, 234). Inhibition of DLL1 led to modest prolongation of graft survival, reduced frequencies of CD4\(^+\) and CD8\(^+\) effector memory cells, inhibition of alloreactive Th1 cells and polarisation towards a Th2-mediated phenotype (233), while use of a signaling Jagged2 mAb increased IL-6 production and precipitated graft rejection, notwithstanding increased frequencies of Tregs (234). The hypothesis, therefore, was that employment of a technique to inhibit Notch signaling in the setting of transplantation would engender further graft survival with polarisation towards a more favourable Th2 phenotype.

To investigate this, 6 – 8 week old WT B6 mice underwent transplantation of a BALB/c cardiac allograft, as previously described, and were treated with either DBZ according to a dosing protocol of 125ug daily for 10 days, or control IgG given at the same dose and on the same schedule. However, the use of DBZ in these mice was quickly found to be catastrophic, with death occurring universally amongst DBZ-treated mice within 3 – 5 days of transplantation (graph not shown). All grafts were beating normally at the time of death.

As a result, no further investigation or experiments with DBZ were undertaken, and the focus of the project was directed exclusively towards the tolerability and effect of the novel antagonistic Notch-1 and Notch-2 antibodies, firstly in naïve mice, and thereafter in the alloimmune setting.
3.3.2 Expression of Notch-1 by lymphocyte subsets

Within the immune system, Notch receptors are predominantly expressed by T cells, and their ligands by APCs (259). The expression of Notch-1, as detected by the use of the aNotch-1 antibody, was first investigated on various naïve lymphocyte subsets. As shown in Figure 3.1, Notch-1 was expressed on both CD4$^+$ and CD8$^+$ T cells; further examination of the CD4$^+$ subsets revealed expression on the T follicular helper cell (Tfh) and CD4$^+$CD25$^+$ T cell populations, and, particularly, on CD4$^+$Foxp3$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ T cells. In contrast, no expression was seen on B220$^+$ B cells.

![Figure 3.1. The expression of Notch-1 on various lymphocyte subsets](image)

The expression of Notch-1 on naïve splenocyte subsets: CD4$^+$ (A) and CD8$^+$ (B) T cells; splenic T follicular helper cells (C; CD4$^+$PD1$^+$ICOS$^+$); CD4$^+$CD25$^+$ T cells (D); splenic regulatory T cells (E; CD4$^+$Foxp3$^+$) and (F; CD4$^+$CD25$^+$Foxp3$^+$); B220$^+$ B cells (G).
The effect of T cell activation on the expression of Notch-1 was next examined. Using αCD3 and αCD28 stimulation for 1 hour in vitro, both CD4⁺ and CD8⁺ T cells were shown to significantly upregulate the expression of Notch-1 upon activation (Figure 3.2).

**Figure 3.2.** Both CD4⁺ and CD8⁺ T cells upregulate Notch-1 expression upon stimulation in vitro
3.3.3 Expression of Notch-2 by lymphocyte subsets

The expression of Notch-2, as detected by the use of the aNotch-2 antibody, was similarly investigated on various naïve lymphocyte subsets. As shown in Figure 3.3, Notch-2 was also expressed on both CD4+ and CD8+ T cells; further examination of the CD4+ subsets revealed expression on the T follicular helper cell (Tfh) and CD4+CD25+ T cell populations, and on CD4+CD25+Foxp3+ T cells. In contrast to Notch-1, Notch-2 was expressed on B220+ B cells.

![Figure 3.3. The expression of Notch-2 on various lymphocyte subsets](image)

The expression of Notch-2 on naïve splenocyte subsets: CD4+ (A) and CD8+ (B) T cells; splenic T follicular helper cells (C; CD4+PD1+ICOS+); CD4+CD25+ T cells (D); splenic regulatory T cells (E; CD4+Foxp3+) and (F; CD4+CD25+Foxp3+); B220+ cells (G).
3.3.4 Expression of Notch genes on T helper cell subsets

This experiment was undertaken by a collaborator, Dr. W. Elyaman, as part of his work investigating the role of Notch in Th9 cell differentiation (217).

As shown in Table 3.1 and Figure 3.4, Notch1 and Notch2 show high levels of mRNA expression across all T helper cell subtypes, reinforcing the decision to investigate their contribution to the adaptive immune response.

Table 3.1. mRNA expression of Notch 1 – 4 on T helper cell subsets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Th1</th>
<th>Th2</th>
<th>Th9</th>
<th>Th17</th>
<th>Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_008714 Notch1</td>
<td>23.029485</td>
<td>17.161965</td>
<td>13.736061</td>
<td>19.454182</td>
<td>12.667874</td>
</tr>
<tr>
<td>NM_010928 Notch2</td>
<td>16.432908</td>
<td>11.353716</td>
<td>20.315311</td>
<td>5.565426</td>
<td>11.527335</td>
</tr>
<tr>
<td>NM_008716 Notch3</td>
<td>0.186790</td>
<td>0.122137</td>
<td>0.302605</td>
<td>0.057212</td>
<td>0.074740</td>
</tr>
<tr>
<td>NM_010929 Notch4</td>
<td>0.025275</td>
<td>0.040064</td>
<td>0.023035</td>
<td>0.114686</td>
<td>0.074395</td>
</tr>
</tbody>
</table>

Figure 3.4. mRNA expression of Notch-1 and Notch-2 on various T helper cell subsets
3.3.5 Conditional deletion of Notch-1 and Notch-2 on CD4+ cells results in decreased proliferation and reduced production of inflammatory cytokines following allostimulation \textit{in vitro}.

Investigation of the potential benefits of Notch-1 and Notch-1 inhibition was initially undertaken in a pilot experiment using the conditional Notch-1/2 DKO (N1N2 DKO; Notch1/2\textsuperscript{f/f} CD4\textsuperscript{Cre}) mouse model.

CD4\textsuperscript{+} splenocytes were isolated from control (CD4\textsuperscript{Cre}) and N1N2 DKO (Notch1/2\textsuperscript{f/f} CD4\textsuperscript{Cre}) mice and were incubated with αCD3/αCD28 or irradiated naïve BALB/c splenocytes for 72 hours; supernatants were collected at approximately 56 hours, and tritiated thymidine (3H-TdR) added for the remainder of the culture to assess the rate of cell proliferation, as detailed in section 2.10. As shown in Figure 3.5 A & B, the proliferation of cells isolated from N1N2 DKO mice was significantly lower than those isolated from controls, whether stimulated with αCD3/αCD28 or irradiated allo-splenocytes (p=0.001 and p=0.024, respectively).

\textbf{Figure 3.5.} N1N2 DKO splenocytes are less proliferative \textit{in vitro}

(A) Proliferation following αCD3 and αCD28 stimulation \textit{in vitro} (B) Proliferation following allo-stimulation with irradiated BALB/c splenocytes \textit{in vitro}.
Supernatants harvested from these cultures just prior to the addition of thymidine were then examined for their concentration of individual cytokines using Luminex. Full details are provided in Chapter 2, Materials & Methods, sections 2.10 and 2.17.1.

As shown in Figure 3.6, supernatants collected from cultures containing N1N2 DKO cells stimulated with αCD3 and αCD28 demonstrated significantly lower levels of pro-inflammatory cytokines compared to controls, including TNFα (321 ± 100 vs 1007 ± 37 pg/ml, respectively; p=0.02), IL-1β (3.175 ± 0.08 vs 209.7 ± 16.09 pg/ml, respectively; p=0.006), and IL-17 (1805 ± 112 vs 8007 ± 129.3 pg/ml, respectively; p=0.0008); the levels of IFNγ were also reduced, although this did not reach statistical significance (7015 ± 665 vs 9977 ± 357 pg/ml, respectively; p=0.059). There was no difference in the levels of IL-6 detected in either group (4951 ± 564 vs 5851 ± 315 pg/ml, respectively; p=0.298), although the levels of IL-5 were significantly lower in supernatants collected from N1N2 DKO cultures compared to controls (279 ± 52.62 vs 5339 ± 199 pg/ml, respectively; p=0.0016).

The levels of IL-10 (887 ± 141 vs 5419 ± 494 pg/ml, respectively; p=0.013) and IL-13 (92.4 ± 27.74 vs 3221 ± 150, respectively; p=0.0024) were also reduced. There was no significant difference in the levels of IL-4 between groups (7791 ± 1726 vs 5057 ± 573 pg/ml, respectively; p=0.27).

The levels of IL-2 were also examined but the results were excluded due to an error with the QC beads for the analyte.
Figure 3.6. Conditional deletion of Notch-1 and Notch-2 on CD4$^+$ cells results in reduced production of inflammatory cytokines following $\alpha$CD3 and $\alpha$CD28 stimulation in vitro.
3.3.6 Effects of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 to naïve mice

To determine the safety and tolerability of in vivo administration of the antibodies, IgG, aNotch-1, aNotch-2 and a combination of aNotch-1 + aNotch-2 were administered at doses detailed below to 6 – 8 week old naïve B6 mice:

1. IgG: 5 mg/kg on days 0, 3, 7 and 10
2. aNotch-1: 5 mg/kg on days 0, 3, 7 and 10
3. aNotch-2: 20 mg/kg on day 0, 5 mg/kg on days 3, 7 and 10
4. aNotch-1 + aNotch-2: 2.5 mg/kg (each) on days 0, 3, 7 and 10.

The mice recipient of IgG or aNotch-1 had no significant adverse outcomes; the mice recipient of aNotch-2 and the combination of aNotch-1 + aNotch-2, however, all died within 14 days of commencement of their regimen, the majority between day 8 and day 10. Mice in the combination group, in particular, were noted to have lost a significant amount of weight and to be less mobile prior to their demise. Examination of these mice post-mortem revealed significant bowel oedema indicating severe intestinal toxicity (Figure 3.7).

![Bowel oedema](image)

**Figure 3.7. Administration of a combination of Notch-1 and Notch-2 leads to significant intestinal toxicity that is ultimately fatal**
A further trial of aNotch-1 and aNotch-2 in naïve B6 mice was then undertaken according to the dosing schedule below with modification of the aNotch-2 regimen to omit the loading dose:

1. aNotch-1: 5 mg/kg on days 0, 3, 7 and 10
2. aNotch-2: 5 mg/kg on days 0, 3, 7 and 10

There were no significant adverse outcomes in either group following the use of these protocols; indeed, the mice in each group survived for a further 5 months following completion of the antibody course.

3.3.7 Examination of the effects of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on naïve lymphocyte subsets

The effects of these regimens on the percentages and absolute number of cells in different lymphocyte subsets in naïve WT B6 mice were then investigated. Mice were treated either with IgG, aNotch-1 or aNotch-2 at 5mg/kg; a further group were treated with the combination regimen of aNotch-1/aNotch-2 at 2.5mg/kg (each); all groups were sacrificed on day 7, which meant that only two doses of each drug regimen were administered. In keeping with the previous data showing that all deaths in the combination group occurred after day 8, all mice treated with the combination regimen remained alive until day 7. The mice used in the previous safety/tolerability experiment, now approximately 6 months old, were also sacrificed on the same day, and the effect of remote administration of aNotch-1 and aNotch-2 investigated.

We investigated the effect of the respective antibody regimens on total splenic CD4$^+$ and CD8$^+$ T cells, splenic CD4$^+$ Tregs, thymocyte subsets, total splenic B cells and B cell subsets; both percentages and absolute counts were calculated.
As shown in Figure 3.8 and Table 3.1, there was no significant difference in either the percentage or absolute number of splenic CD4\(^+\) T cells between groups; the percentage of CD4\(^+\) T cells in both the aNotch-1 and aNotch-2-treated 6-month-old mice was also comparable.

**Figure 3.8.** The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on naïve CD4\(^+\) splenocytes

(A & B) The percentage and absolute number, respectively, of CD4\(^+\) splenocytes isolated from mice treated with the regimens indicated.

**Table 3.1.** The percentage and absolute number of splenic CD4\(^+\) cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>19.53</td>
<td>19.77</td>
<td>19.60</td>
<td>18.13</td>
<td>23.03</td>
<td>20.27</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2.479</td>
<td>2.641</td>
<td>2.740</td>
<td>1.634</td>
<td>4.406</td>
<td>1.168</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.348</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>10690000</td>
<td>6052000</td>
<td>5838000</td>
<td>6459000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>4677000</td>
<td>2131000</td>
<td>1002000</td>
<td>2124000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.167</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Similarly, there was no significant difference in either the percentage or number of splenic CD8$^+$ T cells between groups, including the 6-month-old mice (Figure 3.9 and Table 3.2).

**Figure 3.9. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on naïve CD8$^+$ splenocytes**  
(A & B) The percentage and absolute number, respectively, of CD8$^+$ splenocytes isolated from mice treated with the regimens indicated.

**Table 3.2. The percentage and absolute number of splenic CD8$^+$ cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2**

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>12.57</td>
<td>11.78</td>
<td>11.98</td>
<td>10.57</td>
<td>15.13</td>
<td>14.33</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.429</td>
<td>1.835</td>
<td>1.913</td>
<td>1.489</td>
<td>3.612</td>
<td>1.159</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.1018</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>6810000</td>
<td>3605000</td>
<td>3551000</td>
<td>3731000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2749000</td>
<td>1274000</td>
<td>537540</td>
<td>1100000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0824</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
As shown in Figure 3.10 and Table 3.3, there were significant differences in the proportion of CD4$^+$Foxp3$^+$ cells between groups, with both the aNotch-1 treated and combination-treated mice displaying higher percentages than IgG-treated controls; the absolute numbers of CD4$^+$Foxp3$^+$ cells were equivalent.

Figure 3.10. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on naïve CD4$^+$Foxp3$^+$ splenocytes
(A & B) The percentage and absolute number, respectively, of CD4$^+$Foxp3$^+$ splenocytes isolated from mice treated with the regimens indicated.

Table 3.3. The percentage and absolute number of splenic CD4$^+$Foxp3$^+$ cells in mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>7.245</td>
<td>12.48</td>
<td>7.680</td>
<td>12.30</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.4455</td>
<td>3.250</td>
<td>0.3934</td>
<td>1.388</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0139</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Mean n cells | 586202 | 771436 | 449615 | 814590 |
| Std. deviation | 132788 | 421957 | 88166  | 353380 |
| ANOVA        | p=0.4585 |        |        |          |
As will be discussed in more detail later, the thymus was noted to be significantly reduced in size in mice recipient of treatment with aNotch-1, and, to a lesser extent, combination treatment with aNotch-1 and aNotch-2. In keeping with this, the number of thymocytes isolated from both aNotch-1-treated and combination-treated mice was significantly lower than from IgG- or aNotch-2-treated mice (Figure 3.11 and Table 3.4). Interestingly, however, the 6-month-old mice, which had been treated with the usual limited course of aNotch-1 at approximately 6-8 weeks of age, showed complete recovery of their thymocyte counts.

Figure 3.11. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on the absolute number of naïve thymocytes

Table 3.4. The absolute number of thymocytes in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean n cells</td>
<td>43330000</td>
<td>5667000</td>
<td>57670000</td>
<td>14580000</td>
<td>58330000</td>
<td>47670000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>25030000</td>
<td>1528000</td>
<td>7638000</td>
<td>9899000</td>
<td>11550000</td>
<td>11590000</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0005</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IgG Control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean n cells</td>
<td>43330000</td>
<td>5667000</td>
<td>57670000</td>
<td>14580000</td>
<td>58330000</td>
<td>47670000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>25030000</td>
<td>1528000</td>
<td>7638000</td>
<td>9899000</td>
<td>11550000</td>
<td>11590000</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Given the marked reduction in thymocyte number following aNotch-1 treatment, the effect on individual thymic subsets was then investigated. As discussed previously in the introduction, the earliest intrathymic T cell precursors are CD4⁻CD8⁻ and are termed double-negative (DN) cells; they are further sub-divided into stages of DN development according to their expression of the cell surface markers CD44 and CD25: DN Stage I, CD4⁺CD8⁻CD44⁺CD25⁻; DN Stage II, CD4⁺CD8⁻CD44⁺CD25⁺; DN Stage III, CD4⁺CD8⁻CD44⁻CD25⁺; a representative schematic is shown in Figure 3.12. T cell lineage commitment can only be considered definitive once the DN3 stage has been reached (46), which occurs upon migration of DN2 cells to the outer cortex (56). DN3 cells then undertake widespread TCRβ, γ and δ gene loci rearrangements: some differentiate into γδ T cells, while others undergo further proliferation to generate αβ T cells, which, in turn, give rise to CD4⁺CD8⁺ double positive (DP) cells (57), the most populous subset within the thymus. DP thymocytes initiate TCRα locus rearrangement, following which they undergo negative selection on the basis of their antigen receptor, developing finally into CD4 and CD8 single positive (SP) cells that can migrate out of the thymus to the periphery, where they circulate as naïve but immunocompetent T cells.

Figure 3.12. Stages of normal thymocyte development
Schematic courtesy of Dr. L.V. Riella.
As shown in Figure 3.13 and Table 3.5, the proportion of DN thymocytes was significantly increased in mice treated with aNotch-1, and, to a lesser extent, those treated with the combination of aNotch-1 and aNotch-2; however, the absolute number of DN thymocytes remained lower than in IgG- or aNotch-2-treated mice.

**Figure 3.13. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on CD4^+CD8^- DN thymocytes**

(A & B) The percentage and absolute number, respectively, of DN thymocytes isolated from mice treated with the regimens indicated.

**Table 3.5. The percentage and absolute number of DN thymocytes in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2**

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean %</strong></td>
<td>2.473</td>
<td>4.363</td>
<td>2.300</td>
<td>3.635</td>
<td>2.413</td>
<td>3.127</td>
</tr>
<tr>
<td><strong>Std. deviation</strong></td>
<td>0.3204</td>
<td>1.108</td>
<td>0.1819</td>
<td>1.128</td>
<td>0.2706</td>
<td>0.2055</td>
</tr>
<tr>
<td><strong>ANOVA</strong></td>
<td>p=0.0202</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean n cells</strong></td>
<td>1055000</td>
<td>243400</td>
<td>1319000</td>
<td>573130</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Std. deviation</strong></td>
<td>548645</td>
<td>82068</td>
<td>111935</td>
<td>139041</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANOVA</strong></td>
<td>p=0.008</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Conversely, both the proportion and absolute number of DP thymocytes were profoundly reduced in aNotch-1-treated mice, indicating arrest of normal thymocyte development at the DN stage (Figure 3.14 and Table 3.6).

![Graphs and Tables]

**Figure 3.14.** The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on CD4⁺CD8⁺ DP thymocytes
(A & B) The percentage and absolute number, respectively, of DP thymocytes isolated from mice treated with the regimens indicated.

**Table 3.6.** The percentage and absolute number of DP thymocytes in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>85.83</td>
<td>6.025</td>
<td>85.80</td>
<td>71.57</td>
<td>83.77</td>
<td>81.43</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2.272</td>
<td>3.486</td>
<td>0.4583</td>
<td>11.97</td>
<td>2.401</td>
<td>1.201</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>37070000</td>
<td>368350</td>
<td>49470000</td>
<td>13930000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>21460000</td>
<td>319542</td>
<td>6405000</td>
<td>6578000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0115</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Interestingly, the proportion of both CD4\(^+\) SP and CD8\(^+\) SP thymocytes was increased in aNotch-1- and combination-treated mice (Figure 3.15 A and Table 3.7; Figure 3.16 A and Table 3.8, respectively), while the absolute numbers of these subsets were comparable to IgG- and aNotch-2-treated mice, indicating preservation of these populations despite the disruption to their precedent subset (Figure 3.15 B and Table 3.7; Figure 3.16 B and Table 3.8, respectively).

**Figure 3.15. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on CD4\(^+\) SP thymocytes**

(A & B) The percentage and absolute number, respectively, of CD4\(^+\) SP thymocytes isolated from mice treated with the regimens indicated.

**Table 3.7. The percentage and absolute number of CD4\(^+\) SP thymocytes in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2**

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>6.633</td>
<td>46.53</td>
<td>5.990</td>
<td>31.17</td>
<td>6.133</td>
<td>7.083</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.333</td>
<td>7.868</td>
<td>0.2166</td>
<td>22.92</td>
<td>0.8334</td>
<td>0.5292</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>2990000</td>
<td>2600000</td>
<td>3462000</td>
<td>2499000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1849000</td>
<td>531037</td>
<td>553440</td>
<td>544328</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.16. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on CD8⁺ SP thymocytes

(A & B) The percentage and absolute number, respectively, of CD8⁺ SP thymocytes isolated from mice treated with the regimens indicated.

Table 3.8. The percentage and absolute number of CD8⁺ SP thymocytes in mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>2.480</td>
<td>20.77</td>
<td>2.087</td>
<td>13.77</td>
<td>1.733</td>
<td>1.997</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.6587</td>
<td>6.268</td>
<td>0.1589</td>
<td>12.23</td>
<td>0.1415</td>
<td>0.2237</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0043</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>1101000</td>
<td>1128000</td>
<td>1202000</td>
<td>957123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>646723</td>
<td>237770</td>
<td>163889</td>
<td>163916</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.8667</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Again, as seen in the recovery of thymic size and thymocyte number, thymic subsets in the aNotch-1-treated 6-month-old mice were seen to be comparable in proportion to those in IgG- or aNotch-2-treated mice, indicating restoration of the previous derangement.
Having demonstrated an increase in the DN population and a decrease in the DP population in aNotch-1-treated mice, indicating arrest prior to the DP stage, further investigation of the DN population was undertaken.

As shown in Figure 3.17, mice treated with aNotch-1 displayed an increase in the proportion of cells in DN Stage I (CD4⁻CD8⁻CD44⁺CD25⁻) compared to mice treated with IgG or aNotch-2. In contrast, the DN Stage II cell population (CD4⁻CD8⁻CD44⁺CD25⁺) was absent in aNotch-1-treated mice and markedly reduced in combination-treated mice. The proportion of thymocytes in DN Stage III (CD4⁻CD8⁻CD44⁻CD25⁺) was markedly reduced in mice treated with aNotch-1, and, to a lesser extent, mice treated with the combination of aNotch-1 and aNotch-2.

![Figure 3.17](image-url)  
*Figure 3.17. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on stages of naïve DN thymocyte development*
Table 3.9. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on stages of naïve DN thymocyte development

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DN I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean %</td>
<td>20.87</td>
<td>40.85</td>
<td>19.37</td>
<td>25.03</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.665</td>
<td>10.82</td>
<td>1.922</td>
<td>2.538</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0054</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DN II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean %</td>
<td>5.730</td>
<td>0.0</td>
<td>5.847</td>
<td>0.4910</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.321</td>
<td>0.0</td>
<td>0.6293</td>
<td>0.2998</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DN III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean %</td>
<td>29.77</td>
<td>0.2700</td>
<td>28.53</td>
<td>4.867</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.2082</td>
<td>0.4331</td>
<td>1.747</td>
<td>4.670</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Examination of the B220$^+$ splenocyte population failed to detect any significant difference in the various treatment groups; the percentage of B220$^+$ cells in both the aNotch-1 and aNotch-2-treated 6-month-old mice was also comparable (Figure 3.18 A and Table 3.10). While the absolute number of B220$^+$ cells was lower in each of the aNotch-1, aNotch-2 and aNotch-1&2 treatment groups compared to the IgG control group, this did not reach statistical significance (Figure 3.18 B and Table 3.10).

**Figure 3.18. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on B220$^+$ splenocytes**

The proportion (A) and absolute number (B) of B220$^+$ splenocytes seen in mice treated with the regimens indicated.

**Table 3.10. The percentage and absolute number of B220$^+$ splenocytes in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2**

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>48.73</td>
<td>44.43</td>
<td>46.57</td>
<td>49.20</td>
<td>44.20</td>
<td>52.17</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>5.843</td>
<td>3.691</td>
<td>5.905</td>
<td>3.582</td>
<td>9.329</td>
<td>2.730</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.478</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>26200000</td>
<td>14060000</td>
<td>14660000</td>
<td>17820000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>8732000</td>
<td>6875000</td>
<td>6384000</td>
<td>6763000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.2145</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The B220$^+$ population was then further examined for the proportions of B cell subsets of interest, including T1, T2, FO and MZ B cells; the relevance of these will be discussed in more detail in Chapter Five, sections 5.3.6 and 5.3.8.

T1, T2, FO cells were identified by the expression of the surface marker B220, and thereafter according to their relative expression of CD21 and CD24 (260): T1 cells were classified as B220$^+$CD24$^{hi}$CD21$^{lo/int}$, T2 cells as B220$^+$CD24$^{hi}$CD21$^{int/hi}$, and mature FO B cells as B220$^+$CD24$^{int}$CD21$^{int}$.

As shown in Figure 3.19 and Table 3.11, there was no significant difference in either the proportion or absolute number of T1 B cells among mice treated with aNotch-1, aNotch-2 or aNotch-1 + aNotch-2. In the aNotch-2-treated mice examined at 6 months, however, there was a reduction in the proportion of T1 B cells compared to mice treated with IgG control (15.53 ± 2.71 vs 20.87 ± 1.32%, respectively; p=0.0375).

In contrast, however, there was a highly significant reduction in both the proportion and absolute numbers of T2 B cells in both aNotch-2- and aNotch-1 + aNotch-2-treated mice compared to both IgG- and aNotch-1-treated mice (Figure 3.20 and Table 3.12). At 6 months, the proportion of T2 B cells in aNotch-2 treated mice was seen to be equivalent to aNotch-1-treated mice (Figure 3.20 A and Table 3.12).

Furthermore, there was a significant increase in the proportion of FO B cells in both aNotch-2- and aNotch-1 + aNotch-2-treated mice compared to both IgG- and aNotch-1-treated mice, an effect that was sustained in the 6 month mice (Figure 3.21 A and Table 3.13). There was, however, an overall decrease in the absolute numbers of these cells in each of the groups treated with aNotch-1, aNotch-2 or aNotch-1 + aNotch-2, although this did not reach significance (Figure 3.21 B and Table 3.13).
Figure 3.19. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on the T1 B cell subset

The proportion (A) and absolute number (B) of B220⁺ CD24^hi CD21^- splenocytes seen in mice treated with the regimens indicated.

Table 3.11. The percentage and absolute number of T1 B cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>20.87</td>
<td>21.33</td>
<td>23.97</td>
<td>22.58</td>
<td>21.40</td>
<td>15.53</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.320</td>
<td>2.765</td>
<td>2.173</td>
<td>2.885</td>
<td>2.787</td>
<td>2.710</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0212</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>5537000</td>
<td>2907000</td>
<td>3594000</td>
<td>3962000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2123000</td>
<td>1160000</td>
<td>1810000</td>
<td>1459000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.316</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.20. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on the T2 B cell subset

The proportion (A) and absolute number (B) of B220⁺ CD24^hiCD21⁺ splenocytes seen in mice treated with the regimens indicated.

Table 3.12. The percentage and absolute number of T2 B cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>17.27</td>
<td>15.40</td>
<td>6.730</td>
<td>7.520</td>
<td>12.27</td>
<td>12.22</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.2082</td>
<td>1.572</td>
<td>2.958</td>
<td>1.446</td>
<td>1.790</td>
<td>1.967</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>4514000</td>
<td>2189000</td>
<td>916694</td>
<td>1283000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1470000</td>
<td>1130000</td>
<td>357319</td>
<td>316017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0034</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.21. The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on the FO B cell subset
The proportion (A) and absolute number (B) of B220⁺ CD24<sup>int</sup>CD21<sup>int</sup> splenocytes seen in mice treated with the regimens indicated.

Table 3.13. The percentage and absolute number of FO B cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>58.43</td>
<td>57.60</td>
<td>65.30</td>
<td>64.98</td>
<td>58.83</td>
<td>65.60</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.779</td>
<td>1.386</td>
<td>1.682</td>
<td>3.887</td>
<td>2.413</td>
<td>3.161</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>15230000</td>
<td>8139000</td>
<td>9544000</td>
<td>11690000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>4756000</td>
<td>4095000</td>
<td>4023000</td>
<td>4813000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.298</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The MZ B cell subset was identified by first gating on B220$^+$ splenocytes, and then according to their expression of CD21 and CD1d (B220$^+$CD21$^{hi}$CD1d$^{hi}$).

As shown in Figure 3.22 and Table 3.14, there was a marked, highly significant reduction in both the proportion and absolute number of MZ B cells in aNotch-2- and aNotch-1 + aNotch-2-treated mice compared to both IgG- and aNotch-1-treated mice. Again, this was shown to have recovered in the 6 month mice (Figure 3.22 A).

![Graph showing the effect of aNotch-1, aNotch-2, or aNotch-1 + aNotch-2 on MZ B cell subset](image)

**Figure 3.22.** The effect of administration of aNotch-1, aNotch-2 or aNotch-1 + aNotch-2 on the MZ B cell subset

The proportion (A) and absolute number (B) of B220$^+$ CD1d$^{hi}$CD21$^+$ splenocytes seen in mice treated with the regimens indicated.

**Table 3.14.** The percentage and absolute number of MZ B cells in naïve mice treated with IgG, aNotch-1, aNotch-2 or aNotch-1 + aNotch-2

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>aNotch-1</th>
<th>aNotch-2</th>
<th>aNotch-1&amp;2</th>
<th>aNotch-1 (6mo)</th>
<th>aNotch-2 (6mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>9.583</td>
<td>8.507</td>
<td>2.256</td>
<td>2.046</td>
<td>8.387</td>
<td>8.793</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.1955</td>
<td>2.028</td>
<td>2.827</td>
<td>1.512</td>
<td>0.7184</td>
<td>1.244</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>2504000</td>
<td>1147000</td>
<td>108201</td>
<td>314864</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. deviation</td>
<td>814168</td>
<td>415447</td>
<td>29974</td>
<td>205336</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0011</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
3.4 Discussion

The contribution of animal models of transplantation to our current understanding of the alloimmune response cannot be overestimated. From the original kidney and heart transplant experiments performed in dogs by the French surgeon Alexis Carrel over a century ago, to the latest genetically engineered mouse models of transplantation, a wealth of knowledge, both technical and immunological, has been gained from the use of small and large animal models. The use of mice, in particular, has been of great utility, due partly to the precise characterisation of their genetics and immune system, and latterly, the ability to genetically manipulate strains of mice to isolate different aspects of the alloimmune response; the wide availability of reagents has enhanced the efficacy of these models.

There are various in vivo mouse models available for the study of the alloimmune response, including cellular, non-solid organ and solid organ transplant models. Cellular and non-solid organ transplant models include islet cell, trachea, skin, and, more recently, bowel transplants, while solid organ transplant models include heart, liver, lung and kidney transplantation; the choice of model is determined by the aims of the experiment. In mice, the skin transplant model is considered the most stringent, consistently provoking the most rigorous rejection response, attributed to the large number of skin resident lymphocytes and APCs, which reflects the skin’s function as a primary defense system; the skin transplant model is also not vascularised, which renders it vulnerable to necrosis, and, potentially, more susceptible to immune-mediated injury. This does not accurately mirror the situation in solid organ transplantation, although has the advantage of being a technically easier procedure. Heart, kidney and islet allografts are thereafter ranked in order of decreasing immunogenicity, while liver allografts, a much more recent addition to the repertoire of mouse transplant models, are subject to the same immunoprivilege seen in humans, with a high rate of spontaneous acceptance.
reported (261). While kidneys remain the most commonly transplanted solid organ in humans, the success of murine models of kidney transplants is mitigated by both technical and immunological factors: the average diameter of the mouse renal artery is only 0.3 mm, making transplant anastomoses extremely challenging; furthermore, rates of spontaneous acceptance of kidney allografts in mice are reported to approach 30%, depending on the donor/recipient strain combination employed (262), which renders the model less representative of the human alloimmune experience.

Murine cardiac allografts, in contrast, have a low rate of spontaneous acceptance, are technically relatively straightforward, and have the marked advantage of an easily identifiable, external method of monitoring survival. The model employed in this study, which remains in wide use, was first described by Corry in 1973 (258, 263), and involves the anastomosis of the donor aorta to the recipient abdominal aorta and the donor main pulmonary artery to the recipient IVC in an end-to-side fashion; the heterotopic design of this model allows palpation of the recipient abdomen to determine the strength and persistence of beating of the transplant heart as a means of monitoring survival, although this has been criticised as being both crude and subjective. On a histological level, murine cardiac grafts display evidence of both T and B cell involvement, and demonstrate cardiac allograft vasculopathy, the classic lesion of chronic antibody-mediated injury in human cardiac transplantation. The degree of MHC-mismatch in the cardiac model has been shown to influence the histological pattern of injury, allowing careful dissection of the contribution of the various cellular components of the immune system to the end response.

All of the immunosuppressive agents currently in clinical use in transplantation were initially trialed in rodent or non-human primate models before investigation in humans. There have been several notable examples of failure to translate animal model success to humans, however, which serve to remind us that despite the many similarities between animal and human physiology, there
are critical differences. Interruption of the CD40-CD40L pathway, achieved by use of anti-CD40L monoclonal antibodies or genetic knock-out mice, initially showed great promise in transplantation, significantly enhancing allograft survival and preventing acute rejection in rodents (264, 265) and primates (266, 267), an effect associated with inhibition of Th1 responses and deviation to a Th2 phenotype (268). Furthermore, anti-CD40L mAbs were shown to act synergistically with other therapies including CTLA4-Ig (265), and, when given in concert with donor-specific transfusion, to generate chimerism in the absence of bone marrow conditioning, leading to donor-specific tolerance in mice (269). While these results have clear therapeutic implications, efforts to develop anti-CD40L antibodies for human clinical use were hindered by the unexpectedly high incidence of thrombotic complications in both non-human primate (NHP) studies and phase I clinical trials (270), thought to be due to the interruption of CD40L signaling on platelets (271). Use of a superagonistic anti-CD28 mAb in rodent and NHP models was shown to preferentially activate and expand functional Tregs both in vitro and in vivo (272, 273); unfortunately, attempts to translate this therapy into clinical use were disastrous: in Phase I clinical trials, six healthy volunteers developed a massive cytokine storm upon administration of a superagonistic anti-CD28 mAb, resulting in severe angioedema, lymphopaenia and multi-organ failure (274).

Conversely, it is also possible that agents that fail in pre-clinical models and do not progress to further trials would be both safe and effective in humans. In today's stringent climate, however, it would be virtually impossible to test such an agent in humans in the absence of a clear and unequivocal physiological justification. Nevertheless, there are numerous examples of agents successfully translated from rodent and NHP studies to human use, and this therapeutic algorithm is considered the best possible means of ensuring both safety and efficacy.
Following on from previous studies performed in our laboratory that investigated the role of the Notch ligands DLL1 and Jagged2 in the alloimmune response (233, 234), one of the first aims of this study was to investigate the effect of GSIs on the alloimmune response; this was based on the hypothesis that employment of a technique to inhibit Notch signaling in the setting of transplantation would promote graft survival with polarisation towards a more favourable Th2 phenotype. While GSIs are known to be highly effective inhibitors of Notch signaling, concerns regarding their side-effects have limited their therapeutic potential. Indeed, as was rapidly and clearly borne out, use of the GSI DBZ in this transplant model was catastrophic, with death occurring universally amongst DBZ-treated mice within 3 – 5 days of transplantation. While all grafts were beating strongly at the time of death, it is impossible to comment further on their efficacy in transplantation other than to conclude that their use is not therapeutically viable in this setting. It was therefore decided to investigate the effect of selective Notch receptor inhibition, a decision facilitated by the availability of the newly developed aNotch-1 and aNotch-2 antibodies.

Further data provided by a collaborator, Dr Wassim Elyaman, indicated high levels of Notch1 and Notch2 mRNA expression across all T helper cell subtypes, adding weight to the decision to focus the investigation on the contribution of the Notch-1 and Notch-2 receptors to the adaptive immune response.

The expression of both Notch-1 and Notch-2 on various lymphocyte and APC subsets was then investigated, and revealed expression patterns consistent with previous data. In keeping with its role in T cell development, Notch-1 was shown to be expressed on T cell subsets, particularly Tregs, with higher levels of expression seen in CD4⁺ and CD8⁺ T cells upon activation. In contrast, no expression was seen on B220⁺ B cells. These results reinforced the belief that manipulation of both Notch-1 and Notch-2 signaling in the alloimmune setting could be of therapeutic benefit.
However, on trialing these antibodies *in vivo* in naïve mice, it became quickly apparent that concurrent Notch-1 and Notch-2 blockade was not a feasible therapeutic option, with universal fatality observed in mice treated with this combination. The significant associated weight loss noted in this group indicated possible gastrointestinal toxicity, which indeed was borne out by post-mortem examination. In keeping with previous data (240), this suggests that the majority of the gastrointestinal side-effects noted following treatment with GSIs in our model are due to combined Notch-1 and Notch-2 inhibition, and not as a consequence of Notch-3 or Notch-4 blockade. While the initial dose of aNotch-2 trialed was also lethal, a simple modification to the regimen to avoid the high loading dose resulted in universal survival.

Examination of the effects of aNotch-1, aNotch-2 and a combination of aNotch-1 & aNotch-2 provided both evidence of the efficacy of the antibodies and the rationale to pursue investigation of their effects in solid organ transplantation.

Briefly, the use of aNotch-1 was shown to preserve the splenic CD4$^+$ and CD8$^+$ populations while resulting in an increase in the proportion of CD4$^+$Foxp3$^+$ cells. Most striking, however, were its effects on the thymus, with marked reduction in thymic size and evidence of a block in thymocyte development at the DN I stage; as discussed earlier, Notch-1 signaling has been shown to play an important role in T cell maturation within the thymus (275).

Inhibition of Notch-2, as expected, was shown to have a primary effect on B cell development (111): while the proportion of B220$^+$ splenocytes was not affected, use of aNotch-2 exerted significant influence on the B220$^+$ subsets, with a marked decrease in the T2 B cell subset, an increase in the FO B cell population, and most strikingly, near-complete absence of the MZ B cell population, in keeping with the critical role Notch-2 has been shown to play in the development thereof (104).
It can also be observed from the above experiments that these effects are dose-dependent, as each change effected by use of the individual antibodies was achieved on a lesser scale by the combination regimen, wherein 50% of the standard, single-agent dose of aNotch-1 + aNotch-2 was administered.

The consistency of the effects on thymocyte subsets and MZ B cells achieved by use of aNotch-1 and aNotch-2, respectively, provided further verification of the selective inhibition realised by use of these antibodies (243), and served as a useful marker of their successful delivery in all future experiments.

The effect of selective Notch-1 and Notch-2 blockade in an *in vivo* cardiac transplant model will be discussed in Chapter Four and Five, respectively.
Chapter Four: Investigation of the effects of inhibition of Notch-1 in solid organ transplantation.

4.1 Introduction

T cells play a central role in the determination of the adaptive immune response in the settings of autoimmunity, allergy, and alloimmunity. Indeed, the balance of effector versus regulatory T cells has been shown to influence the outcome of transplantation. The ability to selectively influence T cell fate is therefore of paramount interest: potential approaches include deletion of peripheral alloreactive effector T cells with induction therapy, inhibition of T cell activation by costimulatory blockade, interference with cytokine signaling, and promotion of active regulation by harnessing the regulatory potential of Tregs (36, 37, 159). Calcineurin inhibitors (CNIs), the mainstay of current maintenance immunosuppressive regimens, act by inhibiting the production of IL-2, thereby interfering with the cytokine milieu and lymphocyte function; however, this mechanism of action conversely inhibits the survival and function of Tregs (144).

Signaling through Notch-1 is critical to T cell development, with multiple identified downstream gene targets, all shown to play different but crucial roles in T cell lineage commitment (44). There is accumulating data to support the involvement of Notch signaling in the differentiation pathway, where it is thought to act as a Signal 3 mediator (44, 45). An increasing body of evidence indicates that Notch is further involved in T cell survival (211, 212) and the differentiation of naïve CD4⁺ T cells into various T helper cell subsets (69-71).

Initial work on Notch signaling in adaptive immunity focused on the role of the various Notch ligands on antigen-presenting cells in the differentiation of CD4⁺ T cells. As has been reviewed in detail elsewhere (44, 45, 276), Delta ligands were shown to favour the development of Th1 cells, while Jagged ligands preferentially induced Th2 cells. Our group previously explored an antibody
approach to inhibition of DLL1 in solid organ transplantation, demonstrating a beneficial effect in graft survival achieved both by enhancing polarisation to Th2 cells and by reducing Th1 cells and cytotoxic CD8$^+$ T cells (233). Meanwhile, use of a signalling Jagged2 mAb precipitated rejection in a MHC class II-mismatched cardiac transplant model dependent on Tregs for graft survival. The accelerated rejection was driven by upregulation of IL-6 production and consequent resistance to Treg suppression (234). In a graft-versus-host-disease (GVHD) model, combined DLL1 and DLL4 inhibition protected against GVHD, while anti-cancer activity was preserved (238). Collectively, these findings indicated an important role for the Notch ligands in alloimmunity, though the contribution of the individual Notch receptors was unclear.

First attempts to target the Notch receptors were performed using GSIs, which inhibit Notch signaling in a non-selective manner by blocking a cleavage step critical to activation of the Notch receptors. In vitro, inhibition of Notch activation with GSIs significantly decreased T cell proliferation and IFNγ production following TCR stimulation (277). In a GVHD model, GSIs achieved reduced production of inflammatory cytokines by alloreactive T cells following allogeneic BMT, but resulted in rapidly fatal gastrointestinal toxicity (238), a known complication of combined Notch-1 and Notch-2 blockade which occurs due to interference with the normal architecture of intestinal crypts (240, 241). As discussed in Chapter 3, the use of GSIs was investigated in a murine cardiac transplant model: administration was unfortunately rapidly and uniformly fatal despite multiple dose titrations, precluding further investigation of the underlying mechanisms. A genetic approach using conditional expression of a pan-Notch receptor inhibitor (DNMAML) in T cells resulted in significant reduction in inflammatory cytokine secretion and an inability to generate GVHD (224, 238). It is therefore clear that pan-Notch receptor inhibition significantly inhibits T cell activation, though the severe side effects associated with the pharmacological achievement of this limit its translational potential in solid organ transplantation.
Having determined tolerability and preliminary cellular effects of the novel, selective, antagonistic Notch-1 antibody in Chapter 3, the aims of this chapter were to investigate the effect of selective Notch-1 inhibition in solid organ transplantation using both aNotch-1 and a selective, conditional Notch-1 KO mouse model.
4.2 Methods

All experimental methods are described in detail in Chapter 2, so for the purposes of this chapter will be only briefly described.

4.2.1 Mice

6 – 8 week old C57BL/6 (WT; Thy1.2\(^+\)) C57BL/6 (Thy1.1\(^+\)), BALB/c (H-2\(^d\)), Rag1\(^{-/-}\) (on C57BL/6 background) and B6.C-H2\(^{bm12}\)/KhEg (bm12) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Foxp3-GFP mice were maintained and ABMtg.Foxp3-GFP mice were both generated and maintained as breeding colonies in our facility. Notch1\(^{f/f}\)Foxp3\(^{Cre}\) mice (kindly provided by Dr. Talal Chatila, Harvard Medical School, Boston, MA) allowed efficient, selective deletion of Notch-1 on Tregs; Foxp3\(^{Cre}\) mice were used as their controls.

4.2.2 Surgical procedures

Vascularised intra-abdominal heterotopic cardiac allograft transplants were performed using sterile microsurgical techniques (258) by Tetsunosuke Shimizu (T.S.) or Shunsuke Ohori (S.O), surgical fellows in our laboratory, as described in detail in Chapter 2.4.1. General anaesthesia was achieved either by intraperitoneal injection of a combination of ketamine and xylazine or by inhaled isoflurane. Graft survival was assessed by daily palpation; rejection was defined as complete cessation of cardiac contractility, and was confirmed by direct visualisation.

Thymectomy was performed via a retrosternal approach using an aspiration technique, as described by Lurie et al (251).
4.2.3 Antibodies

4.2.3.1 Notch receptor antibody
The anti-Notch-1 (aNotch-1) antibody was generously provided by Dr. Christian Siebel of Genentech, Inc. (South San Francisco, CA, USA). As described earlier, it is an antagonistic, fully human IgG1 antibody which potently inhibits Notch-1 but not other Notch receptors, and which binds with similarly high affinity to both the mouse and human orthologues (243). Control IgG, also obtained from Genentech, Inc., was administered as a control antibody at the same dose and on the same schedule as aNotch-1, according to the respective experiments.

4.2.3.2 Anti-CD25
Rat anti-mouse CD25 mAb (PC61 clone; aCD25) was manufactured and purified from an original hybridoma by a commercial source (Bioexpress Cell Culture, West Lebanon, NH, USA) and was administered in two separate doses of 250 µg on days -6 and -1 prior to transplantation.

4.2.3.3 CTLA4-Ig
hCTLA4-Ig is a fusion protein composed of a human IgG1 Fc fused to the extracellular domain of CTLA4. It was purchased from Bristol Myers Squibb (BMS; New York, NY, USA) and was administered as a single dose (250 µg) on day 2 post transplantation.
4.3 Results

4.3.1 Use of aNotch 1 significantly prolongs cardiac allograft survival

Following on from the trial of aNotch-1 in naïve mice, it was next administered, according to the dosing schedule below, to B6 mice recipient of a heterotopic, vascularised, full MHC-mismatched cardiac transplant, as detailed earlier in Section 2.4.1; allograft survival was compared to that of recipients treated with control IgG at the same dose and on the same schedule:

1. IgG: 5 mg/kg on days 0, 3, 7 and 10
2. aNotch-1: 5 mg/kg on days 0, 3, 7 and 10

Mice treated with IgG had a median survival time (MST) of 7 days, while those treated with aNotch-1 had an MST of 10 days ($p<0.0001$; see Figure 4.1 A) While this result was clearly statistically significant, the actual prolongation was modest. An alternative dosing schedule was then trialed using the same model to determine if further prolongation of graft survival was possible:

1. IgG: 5 mg/kg on days 0, 2, 4, 6, 8 and 10
2. aNotch-1: 5 mg/kg on days 0, 2, 4, 6, 8 and 10

Using this regimen, mice treated with IgG had an unchanged MST of 7 days, while those treated with aNotch-1 had an MST of 12.5 days ($p<0.0001$; Figure 4.1 B), in the absence of any overt untoward effects. This dosing regimen was then adopted for all further experiments, including mechanistic studies.
**Figure 4.1. Optimisation of dose of aNotch-1 & effect on allograft survival**

Effect of Notch-1 blockade on the survival of fully MHC-mismatched cardiac allografts. Hearts from BALB/c mice were transplanted into WT B6 recipients treated with 5mg/kg of IgG or aNotch-1 on days 0, 3, 7, & 10 post-transplant (A; MST 7 days vs 10 days; p<0.0001) or days 0, 2, 4, 6, 8, & 10 post-transplant (B; MST 7 days vs 12.5 days; p<0.0001). Data shown from pooled experiments; at least 5 mice per group.
4.3.2 Histological & cellular assessment of cardiac allografts following Notch-1 blockade

Histological assessment of grafts harvested on day 7 post-transplant revealed changes consistent with the survival data. In IgG-treated recipients, there was significant destruction of the normal cardiac architecture with severe cellular infiltration; however, in aNotch-1-treated recipients, there was significantly less cellular infiltration and the cardiac architecture was generally preserved.

Figure 4.2. Notch-1 blockade preserves cardiac allograft architecture and reduces graft cellular infiltration

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the grafts isolated. Representative histological slides (H&E stain) from (A) a syngeneic (WT B6 → WT B6) graft, (B) an allograft harvested from an IgG-treated and (C) an aNotch-1-treated recipient are shown.

Grafts isolated from aNotch-1-treated mice weighed significantly less than those isolated from controls (0.096 ± 0.0143 vs 0.1472 ± 0.0081 grammes; p=0.0356; Figure 4.3 A). These grafts were then analysed for their cellular composition: the hearts were first minced and digested with collagenase for 30 minutes at 37C; the cells were isolated using density centrifugation and were obtained by carefully removing theuffy coat; full details are provided in Chapter 2. As shown in Figure 4.3 B, the number of leucocytes isolated from IgG-treated mice was considerably higher than that isolated from mice treated with aNotch-1.
**Figure 4.3. Notch-1 blockade results in considerable reduction in graft-infiltrating lymphocytes**

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the grafts harvested. (A) The weight of the grafts in grammes; ≥3 mice per group. (B) Total leucocytes were isolated from the grafts using density centrifugation. Samples shown are from a single representative mouse from each group. *p<0.05

Following isolation of the graft-infiltrating leucocytes, the cells were mechanically sieved through a 70 μm cell strainer prior to washing and staining for flow cytometry analysis. The lymphocyte population was identified by forward and side scatter characteristics using FlowJo analysis software, and then divided into CD4$^+$ and CD8$^+$ populations. As shown in Figure 4.4, treatment with aNotch-1 effected a decrease in the populations of both total CD4$^+$ and CD8$^+$ graft-infiltrating lymphocytes. The CD4$^+$ population was then further characterised according to the expression of Foxp3 to determine the proportion of graft-infiltrating Tregs, and revealed a significant increase in the Treg population in aNotch-1-treated recipients compared to IgG-treated controls (29.5 ± 3.4 vs 14.73 ± 2.45; p = 0.024; n = 3 per group), as shown in Figure 4.5.
Figure 4.4. Treatment with aNotch-1 is associated with decreased graft-infiltrating lymphocytes

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the grafts harvested. Total leucocytes were isolated from the grafts using density centrifugation and then analysed by flow cytometry using counter-stains for CD4 and CD8. The lymphocyte population was identified by forward and side scatter characteristics using FlowJo analysis software, and then divided into CD4+ and CD8+ populations (upper panel), which revealed a decrease in the populations of both total CD4+ and CD8+ lymphocytes.
Figure 4.5. Inhibition of Notch-1 results in a significant increase in the proportion of graft-infiltrating Tregs

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the grafts harvested. Total leucocytes were isolated from the grafts using density centrifugation and then determined by flow cytometry using counter-stains for CD4 and CD8. The CD4+ fraction was further stained for expression of Foxp3 to determine the proportion of graft-infiltrating Tregs, as shown in flow cytometry plots (A) and graphically (B), and revealed a significant increase in the Treg population. The flow cytometry samples shown are from a single representative mouse from each group; *p<0.05
4.3.3 Characterisation of haematopoietic cells in lymphoid organs upon Notch-1 blockade

To determine the mechanisms by which blockade of Notch-1 confers graft protection, the effect of aNotch-1 was first examined on the spleen and draining lymph nodes (dLN) retrieved on day 7 post-transplantation.

As shown in Figure 4.6, initial macroscopic examination revealed both the spleen and lymph nodes retrieved from aNotch-1-treated mice to be smaller in size than those retrieved from mice treated with IgG.

Figure 4.6. Macroscopic effects of Notch-1 inhibition on lymphoid organs retrieved post-transplant
Macroscopic appearances of lymphoid organs retrieved from either IgG (upper panels) or aNotch-1-treated (lower panels) WT B6 mice 7 days post-transplantation of a BALB/c cardiac allograft. Representative examples of spleens (L) and graft-draining lymph nodes (dLN; R) are shown.
The frequency of overall CD4^+ and CD8^+ effector memory T cells, defined as CD44^{hi}CD62L^{low}, were then investigated using flow cytometry. The lymphocyte gates were determined using the FlowJo analysis program by forward and side scatter characteristics. The populations were then determined according to the CD4 and CD8 counterstains, and thereafter according to their relative expression of CD62L and CD44.

The proportion of splenic CD4^+ effector memory cells was significantly lower in aNotch-1-treated mice compared to IgG-treated controls (17.37 ± 0.9 vs 23.80 ± 0.53%, respectively; p=0.0035; n ≥ 3 per group; Figure 4.7). There was no significant difference in the proportion of CD4^+ effector memory cells isolated from graft-draining lymph nodes in either IgG-treated or aNotch-1-treated mice (7.380 ± 0.834 vs 8.357 ± 0.68%, respectively; p=0.415; n ≥ 3 per group; graph not shown).

Similarly, the splenic CD8^+ effector memory population was significantly reduced in mice treated with aNotch-1 compared to those treated with IgG (17.77 ± 1.1 vs 38.53 ± 0.78%, respectively; p=0.0001; n ≥ 3 per group; Figure 4.8). There was no significant difference in the CD8^+ effector memory population identified within draining lymph nodes in either IgG-treated or aNotch-1-treated mice (7.657 ± 1.88 vs 9.253 ± 2.4%, respectively; p=0.63; graph not shown).
Figure 4.7. Use of aNotch-1 results in significant inhibition of splenic CD4^+ effector memory T cells

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the spleens harvested; a naïve mouse is also shown for comparison. The splenocytes isolated were examined for the frequency of CD4^+ effector memory cells (Teff/mem; defined as CD44^{hi}CD62L^{low}) by flow cytometry. Representative example of at least three repeated experiments shown; n ≥ 3 per group; **p<0.01.
Figure 4.8. Use of aNotch-1 results in significant inhibition of splenic CD8\(^+\) effector memory T cells

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the spleens harvested; a naïve mouse is also shown for comparison. The splenocytes isolated were examined for the frequency of CD8\(^+\) effector memory cells (Teff/mem; defined as CD44\(^{hi}\)CD62L\(^{low}\)) by flow cytometry. Representative example of at least three repeated experiments shown; n ≥ 3 per group; ***p<0.0001
4.3.4 Treatment with aNotch-1 reduces secretion of inflammatory Th1 cytokines in response to allostimulation

Effector memory cells are known to contribute to the secretion of inflammatory cytokines. Given the significant reduction in both the splenic CD4\(^+\) and CD8\(^+\) effector memory populations in mice treated with aNotch-1 post transplantation, as shown above, the effect of Notch-1 blockade on the cytokine milieu was investigated using several different techniques.

Firstly, splenocytes isolated on day 7 post-transplant from WT B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-1, as previously described, were stimulated ex vivo with irradiated donor-type splenocytes and examined for cytokine production using ELISPOT assays for Granzyme B, IFN\(_\gamma\) and IL-4. Full details are provided in Chapter 2, Materials & Methods, section 2.8.

As shown in Figure 4.9, splenocytes isolated from aNotch-1-treated mice secreted significantly lower amounts of Granzyme B (177.3 ± 9.5 vs 313.0 ± 20.2 spots per 0.5 x 10\(^6\) splenocytes in IgG-treated controls; p=0.0037; n ≥ 3 per group; Figure 4.9 A) and IFN\(_\gamma\) (497.7 ± 67.95 vs 1063 ± 29.36 spots per 0.5 x 10\(^6\) splenocytes in IgG-treated controls; p=0.0016; n ≥ 3 per group; Figure 4.9 B), while the secretion of IL-4 was unaffected (117.0 ± 42.55 vs 117.3 ± 16.05 spots per 0.5 x 10\(^6\) splenocytes in IgG-treated controls; p=0.99; n ≥ 3 per group; Figure 4.9 C).
Figure 4.9. Treatment with aNotch-1 reduces secretion of inflammatory Th1 cytokines in response to allostimulation

Splenocytes isolated on day 7 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-1, as previously described, were stimulated ex vivo with irradiated donor-type splenocytes and examined for cytokine production using ELISPOT assays for Granzyme B (A), IFNγ (B) and IL-4 (C). Granzyme B and IFNγ representative examples of three repeated experiments; n ≥ 3 per group; **<0.01.
Secondly, mixed lymphocyte reactions were established, in which $1 \times 10^6$ splenocytes isolated from IgG- and aNotch-1-treated mice on day 7 post-transplantation were incubated with equal numbers of irradiated donor-type splenocytes. Cell-free supernatants were collected after 48 hours of culture, and were then examined for their concentration of individual cytokines using Luminex. Full details are provided in Chapter 2, Materials & Methods, sections 2.10 and 2.17.1.

As shown in Figure 4.10, supernatants collected from aNotch-1-treated mice demonstrated significantly lower levels of pro-inflammatory cytokines compared to IgG-treated controls, including IL-1β ($4.36 \pm 0.71$ vs $12.55 \pm 1.34$ pg/mL; $p=0.0007$), IL-6 ($127.1 \pm 38.68$ vs $383.2 \pm 13.91$ pg/mL; $p=0.0008$), IFNγ ($110.8 \pm 32.09$ vs $2882 \pm 79.13$ pg/mL; $p<0.0001$) and TNFα ($6.66 \pm 0.87$ vs $17.93 \pm 0.44$ pg/mL; $p<0.0001$); the levels of IL-2 were also significantly reduced ($13.58 \pm 0.85$ vs $19.03 \pm 0.99$ pg/mL; $p=0.002$). Neither the levels of IL-17 ($1.37 \pm 0.002$ vs $2.72 \pm 0.83$ pg/mL; $p=0.1$), nor those of IL-5 ($1.75 \pm 0.25$ vs $1.65 \pm 0.12$ pg/mL; $p=0.77$) were significantly different between groups. In keeping with the ELISPOT data, there was no significant difference in the levels of IL-4 between groups ($18.97 \pm 3.16$ vs $14.87 \pm 4.73$ pg/mL; $p=0.49$; data not shown).

There were also significant, although more modest, reductions in the levels of IL-13 ($2.03 \pm 0.59$ vs $6.42 \pm 1.73$ pg/mL; $p=0.03$) and IL-10 ($35.1 \pm 10.95$ vs $98.9 \pm 25.38$; $p=0.04$) in the supernatants collected from aNotch-1-treated mice compared to IgG-treated controls.
Figure 4.10. Inhibition of Notch-1 results in reduced production of inflammatory cytokines following allostimulation in vitro

Cell-free supernatants collected following the incubation of splenocytes isolated from IgG- and aNotch-1-treated mice with irradiated donor-type splenocytes were tested for cytokine production using Luminex. Graphs shown representative of two independent experiments, with the exception of IL-2, which represents one experiment only; n ≥ 3 per group; *p<0.05, **p<0.01, ***p<0.001
4.3.5 Notch-1 blockade results in expansion of the peripheral Treg population

As Notch signaling has been reported to have varying influence on the development and maintenance of Tregs, and given the demonstration of a significantly increased graft-infiltrating Treg population isolated from mice recipient of a cardiac transplant and treated with aNotch-1, as shown in section 4.3.2, the effect of aNotch-1 on peripheral Tregs was next investigated.

Indeed, examination of the splenic subsets in mice recipient of a BALB/c cardiac allograft on day 7 post-transplantation revealed that inhibition of Notch-1 effected a significant increase in the splenic Treg population compared to IgG-treated controls, as determined by the proportion of CD4\(^+\) splenocytes expressing CD25 and Foxp3 (10.01 ± 0.64 vs 6.39 ± 0.39%; \(p=0.0075; n \geq 3\) per group; Figure 4.11 A & B); the proportion of Tregs found in a typical naïve B6 mouse is also shown for comparison (5.02 ± 0.26%; Figure 4.11 A & B). There was no significant difference in the absolute number of Tregs isolated from either group (970470 ± 59392 vs 859014 ± 196232 in IgG-treated controls; \(p=0.616; \) Figure 4.11 C), though the ratio of Tregs/T effector cells was significantly increased in aNotch1-treated recipients (0.59 ± 0.02 vs 0.27 ± 0.01, respectively; \(p=0.0005; \) graph not shown).

The graft-draining lymph nodes retrieved from these mice were also investigated to quantify their resident Treg populations. Mice treated with aNotch-1 were again found to have an increased proportion of CD4\(^+\) cells expressing CD25 and Foxp3 compared to those treated with IgG, although this increase did not reach statistical significance (14.33 ± 0.83 vs 9.573 ± 1.71%, respectively; \(p=0.067; n \geq 3\) per group; graph not shown).
Figure 4.11. Notch-1 blockade results in significant expansion of the splenic Treg population

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the spleens harvested. The splenocytes isolated were examined for the frequency of CD4\(^+\) regulatory T cells (Tregs; defined as CD4\(^+\)CD25\(^+\)FoxP3\(^+\)) by flow cytometry. The proportion of splenic CD4\(^+\) regulatory T cells in aNotch-1-treated mice compared to both naïve B6 mice and IgG-treated controls, as shown in the flow cytometry plots (A) and graphically (B). The absolute number of Tregs isolated from IgG- and aNotch-1-treated mice on day 7 post-transplant (C). Representative example of two repeated experiments shown; **p<0.01
4.3.6 Notch-1 blockade critically affects both the pathological appearance and cellular composition of the thymus in the alloimmune setting

As discussed in Chapter 1, T cells, unlike other blood cell types, complete their differentiation and maturation within the thymus (46), wherein Notch-1 signaling has been shown to play an important role, particularly with regard to T cell maturation (275). Indeed, as shown in Chapter 3, interruption of Notch-1 signaling in naïve mice significantly affected both the number of thymocytes isolated and proportion of cellular subpopulations. The effect of Notch-1 inhibition on thymic architecture and cellular composition in the setting of solid organ transplantation is unknown, and was therefore investigated using the full MHC-mismatch cardiac transplant model.

It was first noticed that treatment with aNotch-1 consistently resulted in a marked reduction in thymic size (Figure 4.12 A; samples retrieved from B6 mice recipient of a BALB/c cardiac allograft on day 7 post-transplant shown). Further histological assessment of the harvested thymi revealed complete loss of the normal thymic cortico-medullary architecture in aNotch-1-treated mice (Figure 4.12 B; 10X view shown for both IgG-treated (left panel) and aNotch-1-treated mice (right panel); images from naïve mice shown; similar appearance from B6 mice recipient of a BALB/c cardiac allograft, image not shown).

As previously discussed, the earliest intrathymic T cell precursors are CD4<sup>+</sup>CD8<sup>−</sup> DN cells; they are further sub-divided into stages of DN development according to their expression of the cell surface markers CD44 and CD25: DN Stage I, CD4<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD25<sup>−</sup>; DN Stage II, CD4<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>; DN Stage III, CD4<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup>CD25<sup>+</sup>. Further stages of T cell development include the development of CD4<sup>+</sup>CD8<sup>+</sup> DP cells and their subsequent differentiation into CD4 and CD8 SP cells that can migrate out of the thymus to the periphery, where they circulate as naïve but immunocompetent T cells. Natural Tregs are known to develop from the CD4<sup>+</sup> SP population.
Figure 4.12. Notch-1 blockade critically affects both the macroscopic and microscopic pathological appearance of the thymus

(A) Macroscopic appearance of thymus harvested from an IgG-treated mouse (left panel) compared to that harvested from an aNotch-1-treated mouse (right panel) on day 7 post-transplantation. Representative example from at least three repeated experiments shown; n ≥ 3 per group. (B) Histological appearance of thymus harvested from naïve B6 mice treated with either IgG (left panel) or aNotch-1 (right panel) as previously described; 10X magnification shown for both samples.
Initial analysis of the cellular composition of the thymus of aNotch-1-treated mice demonstrated complete loss of the CD4⁺CD8⁺ DP thymic population compared to IgG-treated controls (2.295 ± 0.02 vs 87.03 ± 0.62%, respectively; \( p<0.0001 \); Figure 4.13) with a corresponding increase in the CD4⁻CD8⁻ DN (7.54 ± 1.38 vs 2.04 ± 0.21%, respectively; \( p=0.0141 \)) and a marked increase in both the CD4⁺ (65.05 ± 3.15 vs 7.53 ± 0.58%, respectively; \( p=0.0002 \)) and CD8⁺ (25.10 ± 1.8 vs 3.39 ± 0.37%, respectively; \( p=0.0006 \)) SP populations.

There was no significant difference in the absolute counts in any of the subsets between aNotch-1-treated or IgG-treated mice (Table 4.1). It must be noted, however, that the absolute number of DP cells in aNotch-1-treated mice were markedly lower than those in IgG-treated controls, despite not reaching statistical significance.

**Table 4.1. The absolute number of cells in each thymocyte subset isolated from IgG-treated and aNotch-1-treated mice**

<table>
<thead>
<tr>
<th>Subset</th>
<th>IgG control (± SD)</th>
<th>aNotch-1 (± SD)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>25900000 ± 9235000</td>
<td>46590 ± 18090</td>
<td>0.119</td>
</tr>
<tr>
<td>DN</td>
<td>589967 ± 202950</td>
<td>163380 ± 86380</td>
<td>0.212</td>
</tr>
<tr>
<td>CD4⁺ SP</td>
<td>2115000 ± 584028</td>
<td>1293000 ± 440350</td>
<td>0.391</td>
</tr>
<tr>
<td>CD8⁺ SP</td>
<td>1069000 ± 488446</td>
<td>522225 ± 230975</td>
<td>0.464</td>
</tr>
</tbody>
</table>
Figure 4.13. Notch-1 blockade results in complete loss of the double positive CD4+CD8+ thymocyte population

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the thymi harvested; a naive mouse was also sacrificed and the thymus harvested for comparison. Flow cytometry plots (upper panel) depicting the thymocyte populations, initially identified by forward and side scatter characteristics, and thereafter identified by counterstaining for CD4 and CD8. Representative examples of two independent experiments; n ≥ 3 per group; **p<0.01
As was shown in naïve mice in Chapter 3, further analysis of the DN population in transplanted mice treated with aNotch-1 again revealed developmental arrest at DN stage I (Figure 4.14), characterised as CD4⁻CD8⁻CD44⁺CD25⁻, as indicated by a significant increase in the proportion of this population in aNotch-1-treated mice compared to IgG controls (67.03 ± 6.27 vs 28.53 ± 3.49%, respectively; p=0.0058), a marked decrease in the proportion of DN Stage II thymocytes (1.542 ± 0.3 vs 2.46 ± 0.22%, respectively; p=0.068), and complete absence of Stage III DN thymocytes (1.433 ± 0.57 vs 41.5 ± 5.14%, respectively; p=0.0015).

While the development of thymocytes normally progresses from DN to DP, and thence to the single positive CD4⁺ and CD8⁺ populations, the increased proportion of SP cells in this model indicates the presence of a bypass system, whereby DN thymocytes are still able to progress to the SP stages, maintaining the pool of thymocytes ready to exit the thymus and circulate in the periphery. Indeed, there is a trend towards an increase in the proportion of splenic CD4⁺ cells in aNotch-1-treated mice (14.17 ± 0.38 vs 11.87 ± 0.98%, respectively; p=0.095; graph not shown), although no significant difference was noted in the absolute number of splenic CD4⁺ cells between groups (9812000 ± 1052000 vs 13190000 ± 2327000 in IgG-treated controls; p=0.26; graph not shown). There was no significant difference in either the percentage (10.78 ± 0.85 vs 10.87 ± 0.54 in IgG-treated controls; p=0.94) or absolute number (7453000 ± 861340 vs 12170000 ± 2058000 in IgG-treated controls; p=0.1; graphs not shown) of CD8⁺ splenocytes between groups.
Figure 4.14. Notch-1 blockade results in developmental arrest of DN thymocytes at DN Stage 1

Further analysis of the DN population in mice treated with aNotch-1 revealed developmental arrest at DN stage I, (CD4⁻CD8⁻CD44⁺CD25⁻), with complete absence of both Stage II (CD4⁻CD8⁻CD44⁺CD25⁺) and Stage III (CD4⁻CD8⁻CD44⁻CD25⁺) DN thymocytes, as shown in flow cytometry plots (A) and graphically (B). Representative example of at least two independent experiments; n ≥ 3 per group **p<0.01
The significantly increased subset of CD4\(^+\) SP cells was then investigated for expression of Foxp3 to determine whether the aNotch-1-mediated thymocyte disruption had any subsequent influence on the development of thymic Tregs. As shown in Figure 4.15, Notch-1 blockade resulted in a marked increase in the thymic Treg population compared to IgG-treated controls (4.413 ± 1.2 vs 0.258 ± 0.02\%, respectively; p=0.026; n ≥ 3 per group).

**Figure 4.15. Inhibition of Notch-1 results in a significant increase in the proportion of thymic Tregs**

The proportion of Tregs found in SP CD4\(^+\) thymocytes isolated from aNotch-1 treated mice on day 7 post-transplant, as determined by the percentage of CD4\(^+\) SP cells expressing CD25 and Foxp3 (A & B); a naïve mouse is also shown for comparison (B). Representative example of two independent experiments; n ≥ 3 per group; *p<0.05
Peripheral circulating T cells, including Tregs, are known to be capable of trafficking back to the thymus (278). To determine if the Tregs identified within the thymus were all of thymic origin, the CD4+ SP subpopulation was examined for co-expression of Foxp3 and Neuropilin-1, which has been reported as a distinguishing marker of natural thymic Tregs, and has also been shown to be important in maintaining Treg stability and function (279).

BALB/c-sensitised (i.p.) Foxp3-GFP mice were treated with IgG or aNotch-1 on days 0, 2, 4, 6, 8 & 10 and were sacrificed on day 14. Flow-sorted Tregs (splenic/LN) from these mice were isolated and stored for future use. However, thymi harvested from these mice were examined to determine the Treg (CD4+CD25+Foxp3+) populations and the proportion thereof expressing Neuropilin.

As shown in Figure 4.16, a significantly higher percentage of thymic Tregs isolated from aNotch-1-treated mice expressed Neuropilin compared to those isolated from IgG-treated controls: CD4+ CD25+ thymocytes were examined for co-expression of Foxp3 and Neuropilin (74.70 ± 1.76 vs 21.82 ± 0.74%, respectively; p<0.0001; Figure 4.16 A & B).

There was no significant difference between groups in the proportion of CD4+CD25+Foxp3+ splenic Tregs expressing Neuropilin-1 (85.80 ± 0.51 vs 85.73 ± 0.49%, respectively; p=0.93; data not shown). Interestingly, there was a slight, albeit insignificant, increase in the proportion of splenic Tregs expressing Neuropilin-1 in aNotch-1-treated mice compared to IgG-treated mice post-thymectomy (90.23 ± 0.998 vs 87.45 ± 1.29%, respectively; p=0.13; data not shown).
**Figure 4.16. Thymic Tregs isolated from aNotch-1-treated mice are strongly Neuropilin-1⁺**

BALB/c-sensitised (i.p.) Foxp3-GFP mice were treated with IgG or aNotch-1 on days 0, 2, 4, 6 and 8, and were sacrificed on day 10. Thymi harvested from these mice were examined to determine the Treg populations and the expression of Neuropilin-1 therein. (A & B) The proportion of CD4⁺ CD25⁺ thymocytes Foxp3⁺ and Neuropilin-1⁺. ***p<0.0001; n=5 mice per treatment group.
4.3.7 The prolonged graft survival conferred by Notch-1 blockade is critically dependent on the presence of Tregs

As shown in sections 4.3.5 & 4.3.6 above, use of aNotch-1 in transplanted and allo-sensitised mice had a significant effect on Tregs, particularly the thymic nTreg population. To determine whether the expansion of the Treg population was linked to or responsible for the prolonged allograft survival seen in these mice, naïve B6 mice underwent Treg depletion prior to receiving a heterotopic, vascularised, full MHC-mismatched cardiac transplant, as detailed earlier in Section 2.4.1, and were then treated with either IgG or aNotch-1 according to the standard protocols. Treg depletion was achieved by the use of anti-CD25. The dosing regimens are detailed below:

1. aCD25: 250 µg on days -6 & -1; IgG: 5 mg/kg on days 0, 2, 4, 6, 8 & 10
2. aCD25: 250 µg on days -6 & -1; aNotch-1: 5 mg/kg on days 0, 2, 4, 6, 8 & 10

As previously shown, mice treated with IgG alone had an MST of 7 days while those treated with aNotch-1 according to the above schedule had an MST of 12.5 days (p<0.0001). Use of aCD25 had no significant effect on allograft survival in mice treated with IgG compared to those treated with IgG alone (MST 7.5 days vs 7 days; p=0.35; Figure 4.17). However, depletion of Tregs in mice then treated with aNotch-1 near-completely abrogated the survival advantage previously seen with aNotch-1 alone (MST 9 days vs 12.5 days; p=0.0071). Indeed, there was no statistically significant difference between mice treated with IgG + aCD25 and mice treated with aNotch-1 + aCD25 (MST 7.5 days vs 9 days, respectively; p=0.06. This indicates that the graft survival achieved by use of aNotch-1 is critically dependent on the presence of Tregs.
Figure 4.17. The prolonged graft survival conferred by Notch-1 blockade is critically dependent on the presence of Tregs

Treg depletion was performed using aCD25 (250 µg rat anti-mouse CD25 mAb (PC61) at days -6, and -1 before transplantation) in WT B6 mice recipient of a BALB/c cardiac allograft and subsequently treated with either IgG or aNotch-1 on days 0, 2, 4, 6, 8 & 10, according to standard protocols. The survival curves of those mice treated with IgG or aNotch-1 in the absence of Treg depletion are shown for comparison. At least 4 mice per group shown.
4.3.8 Thymectomy does not abrogate the prolongation of graft survival seen following Notch-1 blockade

As detailed above, the expansion of Tregs seen consequent to Notch-1 blockade was shown to be critical to the prolonged allograft survival achieved by treatment with aNotch-1.

To determine whether this Treg effect was due to central (thymic) or peripheral (splenic) Tregs, 8 week old naïve B6 mice underwent a thymectomy two weeks prior to receiving a heterotopic, vascularised, full MHC-mismatched cardiac transplant, as detailed earlier in Section 2.4.1, and were subsequently treated with either IgG or aNotch-1. The thymectomy procedure itself is detailed in Section 2.4.2.

![Diagram of Thymus and Spleen/Lymph Nodes]

**Figure 4.18. Possible mechanisms underlying effect of Notch-1 inhibition on Tregs**
Schematic courtesy of Dr. L.V. Riella.
As expected, prior thymectomy had no effect on allograft survival in mice treated with IgG compared to those in whom thymectomy was not performed (MST 7 days vs 7 days; p=0.65; Figure 4.19). Interestingly, however, thymectomised mice treated with aNotch-1 continued to display a graft survival advantage compared to those treated with IgG (MST 18 days vs 7 days; p=0.0027; Figure 4.19).

Furthermore, performance of thymectomy prior to transplantation failed to abrogate graft survival in mice later treated with aNotch-1 compared to aNotch-1-treated mice in whom thymectomy was not performed (MST 18 days vs 12.5 days; p=0.19; Figure 4.19), indicating that, despite the marked effect of aNotch-1 on thymic Tregs, the prolongation of graft survival seen was due to the effect of Notch-1 blockade on the peripheral Treg population.
Figure 4.19. Pre-transplant thymectomy does not inhibit the graft survival advantage achieved by Notch-1 blockade

The effect of thymectomy two weeks prior to transplantation on the graft survival achieved by treatment with aNotch-1. Hearts from BALB/c mice were transplanted into thymectomised WT B6 recipients treated with 5mg/kg of IgG or anti-Notch-1 on days 0, 2, 4, 6, 8, & 10 post-transplant; survival was compared to WT B6 recipients treated with 5mg/kg of IgG or anti-Notch-1 on days 0, 2, 4, 6, 8, & 10 post-transplant in the absence of thymectomy. At least 4 mice per group shown.
4.3.9 Prior thymectomy does not prevent the peripheral Treg expansion seen following Notch-1 inhibition

Mechanistic experiments were then undertaken to determine what influence prior thymectomy exerted on the relevant peripheral cell compartments.

Firstly, thymectomised aNotch-1-treated mice continued to display decreased frequencies of splenic CD4$^+$ and CD8$^+$ effector memory cell subsets compared to IgG-treated controls (28.05 ± 0.67 vs 36.53 ± 2.99% and 14.23 ± 0.97 vs 31.93 ± 4.66%, respectively; p=0.0324 and p=0.0099; n ≥ 4 per group; graphs not shown).

Interestingly, prior thymectomy did not appear to have any negative effect on the proportion of peripheral Tregs, while administration of aNotch-1 to transplant recipients following thymectomy continued to result in increased frequencies of splenic Tregs compared to IgG-treated controls (11.65 ± 0.218 vs 9.688 ± 0.308%, respectively; p=0.002; Figure 4.20 A & B); the absolute numbers of Tregs were similar between groups (735614 ± 61777 vs 745599 ± 111137 in IgG-treated controls; p=0.94; Figure 4.20 C).

It remained unclear, however, whether the increased peripheral Treg population seen following Notch-1 blockade was due to an expansion in the nTreg population or increased generation of iTregs; experiments to answer this question were then undertaken.
**Figure 4.20. Prior thymectomy does not prevent the peripheral Treg expansion seen following Notch-1 inhibition**

WT B6 recipients underwent thymectomy two weeks prior to transplantation of a BALB/c cardiac allograft and were treated with either 5mg/kg of IgG or aNotch-1 on days 0, 2, 4, 6 prior to sacrifice on day 7. Splenocytes were examined for their proportion of Tregs, as determined by the percentage of CD4⁺ cells expressing CD25 and Foxp3 (A & B); the absolute number of splenic Tregs post thymectomy is also shown (C). At least 4 mice per group shown; **p<0.01
4.3.10 Inhibition of Notch-1 causes an expansion in the nTreg population and does not affect iTreg generation

As discussed previously, in addition to the natural Tregs (nTregs) formed in the thymus, adaptive or induced Tregs (iTregs) may also be generated in the periphery from CD4⁺CD25⁺ cells (58). To determine whether the increased Treg population seen following Notch-1 blockade was due to an increase in the generation of nTregs or iTregs, the effect of aNotch-1 on iTreg generation was investigated both in vitro and in vivo.

Firstly, the influence of aNotch-1 on in vitro Treg generation assay was examined using CD4⁺Foxp3⁻ cells cultured in the presence of APCs, αCD3, IL-2 and TGFβ; a schematic is shown in Figure 4.21 A. The polarising conditions were considered successful, with the population of CD4⁺ cells expressing Foxp3 exceeding 30% of total CD4⁺ cells at the end of the assay.

However, as shown in Figure 4.21 B, neither the addition of 1 nor 10 µg/mL of aNotch-1 in vitro to the assay wells had any significant effect on the proportion of Tregs generated, as determined by the proportion of cells expressing CD4 and Foxp3, compared to the same concentration of IgG control (38.05 ± 2.95 vs 34.30 ± 4%, respectively; p=0.53 and 34.25 ± 1.55 vs 30.20 ± 0.5%, respectively; p=0.13; Figure 4.21 B).
Figure 4.21. Inhibition of Notch-1 does not affect iTreg generation in vitro

(A) Schematic illustrating the in vitro Treg generation assay. (B) The percentage of CD4⁺Foxp3⁺(GFP⁺) cells isolated following incubation of CD4⁺Foxp3⁻(GFP⁻) cells under Treg polarising conditions in vitro, as described in detail in the Methods section, in the presence of varying concentrations of either IgG control or aNotch-1 in vitro.
Secondly, the effect of Notch-1 blockade on Treg generation in vivo was investigated using our ABMtg.Foxp3-GFP mouse model (248). In brief, this is a B6 TCR-tg mouse with CD4+ T cells that express a TCR with defined specificity against the bm12 antigen (ABMtg), and where the expression of Foxp3 is labeled with GFP. The TCR-tg T cells are adoptively transferred into congenic WT B6 mice expressing the surface marker Thy1.1 (CD90.1), such that the adoptively transferred cells can be tracked using the cell surface marker Thy1.2 (CD90.2). 4 x 10⁶ flow-sorted (Thy1.2+) CD4+Foxp3- cells were injected intraperitoneally to WT B6 Thy1.1+ recipients, treated with either IgG or aNotch-1 at 5mg/kg on days 0, 2 & 4; the mice were sacrificed on day 5 and the lymphocyte subsets examined. An illustrative schematic is shown in Figure 4.22 A.

There was no significant difference between either aNotch-1-treated or IgG-treated mice in the number of CD4+ splenocytes (1.425 ± 0.063 vs 0.9825 ± 0.225%, respectively; p=0.11; Figure 4.22 B, left panel) or LN lymphocytes (0.628 ± 0.047 vs 0.4925 ± 0.08%, respectively; p=0.2; Figure 4.22 B, right panel) expressing Thy1.2. In keeping with previous findings, there was a significant increase in the total number of CD4+Foxp3+ cells isolated from lymph nodes in the group treated with aNotch-1 (13.68 ± 0.448 vs 11.95 ± 0.185%, respectively; p=0.012; Figure 4.22 C, left panel). There was also a significant increase in the overall proportion of splenic Tregs (CD4+CD25+Foxp3+) isolated from the spleens of these mice (10.35 ± 0.45 vs 8.713 ± 0.36%, respectively; p=0.03; graph not shown).

However, there were significantly fewer LN Thy1.2+CD4+Foxp3+ cells or iTregs in the aNotch-1 treated group compared to IgG-treated controls (0.3625 ± 0.36 vs 2.180 ± 0.483%, respectively; p=0.024; Figure 4.22 C, right panel). There was no significant difference in the proportion of splenic Thy1.2+CD4+Foxp3+ cells between the groups (0.4625 ± 0.21 vs 0.53 ± 0.27%, respectively; p=0.85; graph not shown). Overall, these data indicate that inhibition of Notch-1 leads to an increase in the nTreg population rather than increased iTreg generation.
Figure 4.22. Inhibition of Notch-1 does not increase the generation of iTregs \textit{in vivo}

(A) Schematic demonstrating the Treg \textit{in vivo} conversion assay using ABMtg.Foxp3-GFP mice. (B & C) Results of the \textit{in vivo} conversion assay using ABMtg.Foxp3-GFP mice treated with either IgG or aNotch-1, according to the usual dosing protocol; mice were sacrificed on day 5 post-adoptive transfer and their lymphoid compartments examined: B (left panel), proportion of CD4$^+$ Thy1.2$^+$ splenocytes isolated; B (right panel), proportion of CD4$^+$ Thy1.2$^+$ (LN) lymphocytes isolated; C (left panel), proportion of total LN Tregs isolated (% CD4$^+$ cells Foxp3$^+$); C (right panel), proportion of induced LN Tregs (iTregs; % CD4$^+$ cells Thy1.2$^+$Foxp3$^+$) isolated. *p<0.05; n ≥ 4 mice per group; representative example of at least two independent experiments shown.
4.3.11 Inhibition of Notch-1 increases the proportion of Tregs by several different mechanisms

To determine the mechanisms underlying the apparently selective preservation of the Treg population, splenic Tregs, identified as described earlier by the markers CD25 and Foxp3, were first evaluated for rates of apoptosis. Using the apoptosis markers Annexin V and 7AAD, we determined that splenic Tregs (CD4+CD25+Foxp3+) isolated from aNotch-1-treated mice on day 7 post-transplant had significantly lower rates of apoptosis compared to IgG-treated controls (11.37 ± 0.52 vs 15.33 ± 0.61%, respectively; p=0.0078; n ≥ 3 per group; Figure 4.23).

Although the presence of a thymus was not shown to be critical to the effect of aNotch-1, rates of apoptosis amongst thymic Tregs were also investigated. Again, thymic Tregs isolated from aNotch-1-treated mice demonstrated lower rates of apoptosis compared to IgG-treated controls (7.19 ± 0.67 vs 17.43 ± 1.27%, respectively; p=0.0021; n ≥ 3 per group; graph not shown).

Furthermore, when examined for expression of Ki67, an intracellular marker expressed by cells in all active stages of the cell cycle, splenic Tregs displayed higher rates of proliferation when compared to those treated with IgG (20.57 ± 0.68 vs 11.50 ± 0.5%, respectively; p=0.0024; Figure 4.24).
Figure 4.23. Treatment with aNotch-1 results in lower rates of apoptosis among the splenic Treg population

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the spleens harvested. The CD4⁺ Treg population was identified using the markers CD25 and Foxp3, and was then analysed for apoptosis using the markers AnnexinV and 7AAD. As shown in both the flow plots (A) and graphically (B), use of aNotch-1 resulted in reduced Treg apoptosis.
Figure 4.24. Treatment with aNotch-1 results in higher rates of proliferation among the splenic Treg population

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and the spleens harvested. The CD4$^+$ Treg population was identified using the markers CD25 and Foxp3, and was then analysed for proliferation using the intracellular marker Ki67. As shown in both the flow plots (A) and graphically (B), use of aNotch-1 resulted in greater Treg proliferation compared to treatment with IgG control. **p<0.01.
4.3.12 Tregs isolated from aNotch-1-treated mice have greater suppressive abilities *in vitro*

To investigate whether inhibition of Notch-1 had any effect on the function of Tregs, in addition to increasing the Treg population, the suppressive abilities of Tregs isolated from mice treated with either IgG or aNotch-1, were examined for their suppressive abilities in a Treg suppression assay.

Purified Tregs, flow-sorted from Foxp3-GFP mice sensitised by intraperitoneal injection of $15 \times 10^6$ BALB/c splenocytes, and thereafter treated with either IgG or aNotch-1 (as detailed in Section 2.11) were added in varying ratios (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) to assays containing conventional T cells (CD4\(^+\)Foxp3\(^-\)) and mitomycin-treated APCs (CD3\(^+\)) isolated from IgG-treated mice for 96 hours. 16 hours before the cells were harvested for analysis, supernatants were collected and 3H-TdR was added to each well. On completion of the incubation period, the plate was analysed using a liquid scintillation counter.

As shown in Table 4.2 and Figure 4.25, Tregs isolated from mice treated with aNotch-1 had greater suppressive properties compared to those isolated from IgG-treated mice at all ratios tested up to the 1:16 dilution.

Table 4.2. Liquid scintillation counts from Treg suppression assay using Tregs isolated from either IgG-treated or aNotch-1-treated mice

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<thead>
<tr>
<th>Treg:Teff/APC</th>
<th>IgG Tregs, cpm</th>
<th>aNotch-1 Tregs, cpm</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>7432 ± 804.9</td>
<td>5075 ± 696.5</td>
<td>0.09</td>
</tr>
<tr>
<td>1:2</td>
<td>17188 ± 1391</td>
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<tr>
<td>1:4</td>
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<td>0.13</td>
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<tr>
<td>1:16</td>
<td>33446 ± 2692</td>
<td>35700 ± 2238</td>
<td>0.55</td>
</tr>
<tr>
<td>1:32</td>
<td>37865 ± 2297</td>
<td>35777 ± 3974</td>
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</table>
Figure 4.25. Tregs isolated from aNotch-1-treated mice have greater suppressive abilities in vitro

Tregs isolated from BALB/c-sensitised Foxp3-GFP mice treated with either IgG or aNotch-1 were examined for their suppressive capacities in a Treg suppression assay. Purified Tregs were added in varying ratios (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) to assays containing conventional T cells (CD4\(^+\)Foxp3\(^-\)) and mitomycin-treated APCs (CD3\(^-\)) isolated from IgG-treated mice for 96 hours. Ratios were tested in triplicate wells.

To determine whether it was possible to confer any additional suppressive capacity upon these Tregs, the experimental plate was divided and aNotch-1 was added in vitro to both IgG and aNotch-1 Tregs plated in the same ratios as those reported above. As shown in Table 4.3, Table 4.4 and Figure 4.26, the addition of aNotch-1 in vitro did not increase the suppressive capacity of Tregs isolated from either IgG-treated or aNotch-1-treated mice. Indeed, where Tregs had been isolated from mice treated with aNotch-1 in vivo, the addition of aNotch-1 in vitro led to less suppression, potentially indicating some cumulative toxicity.
Table 4.3. Liquid scintillation counts obtained from a Treg suppression assay using Tregs isolated from IgG-treated mice with or without the addition of aNotch-1 \textit{in vitro}

<table>
<thead>
<tr>
<th>Treg:Teff/APC</th>
<th>IgG Tregs, cpm</th>
<th>IgG Tregs + aNotch-1, cpm</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>7432 ± 804.9</td>
<td>7779 ± 412.1</td>
<td>0.7207</td>
</tr>
<tr>
<td>1:2</td>
<td>17188 ± 1391</td>
<td>19277 ± 1893</td>
<td>0.424</td>
</tr>
<tr>
<td>1:4</td>
<td>30439 ± 2895</td>
<td>30061 ± 1788</td>
<td>0.917</td>
</tr>
<tr>
<td>1:8</td>
<td>30946 ± 2314</td>
<td>36725 ± 584.7</td>
<td>0.0726</td>
</tr>
<tr>
<td>1:16</td>
<td>33446 ± 2692</td>
<td>35465 ± 1078</td>
<td>0.5246</td>
</tr>
<tr>
<td>1:32</td>
<td>37865 ± 2297</td>
<td>42175 ± 2640</td>
<td>0.2855</td>
</tr>
</tbody>
</table>

Table 4.4. Liquid scintillation counts obtained from a Treg suppression assay using Tregs isolated from aNotch-1-treated mice with or without the addition of aNotch-1 \textit{in vitro}

<table>
<thead>
<tr>
<th>Treg:Teff/APC</th>
<th>aNotch-1 Tregs, cpm</th>
<th>aNotch-1 Tregs + aNotch-1, cpm</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>5075 ± 696.5</td>
<td>6394 ± 264.2</td>
<td>0.1512</td>
</tr>
<tr>
<td>1:2</td>
<td>11720 ± 792.2</td>
<td>12636 ± 558.1</td>
<td>0.3981</td>
</tr>
<tr>
<td>1:4</td>
<td>22519 ± 729.2</td>
<td>26465 ± 837.5</td>
<td>0.0237</td>
</tr>
<tr>
<td>1:8</td>
<td>24723 ± 784.5</td>
<td>39370 ± 1321</td>
<td>0.0039</td>
</tr>
<tr>
<td>1:16</td>
<td>35700 ± 2238</td>
<td>45612 ± 3122</td>
<td>0.0613</td>
</tr>
<tr>
<td>1:32</td>
<td>35777 ± 3974</td>
<td>46461 ± 1820</td>
<td>0.0709</td>
</tr>
</tbody>
</table>
Figure 4.26. The addition of aNotch-1 *in vitro* does not augment the suppressive capacity of Tregs harvested from either IgG- or aNotch-1-treated mice

Tregs isolated from BALB/c-sensitised Foxp3-GFP mice treated with either IgG or aNotch-1 were examined for their suppressive capacities in a Treg suppression assay. Purified Tregs were added in varying ratios (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) to assays containing conventional T cells (CD4⁺Foxp3⁻) and mitomycin-treated APCs (CD3⁺) isolated from IgG-treated mice for 96 hours; aNotch-1 was added at a concentration of 10 µg/ml to all wells. Ratios were tested in triplicate wells.
Allospecificity has been reported to be an important influence on the suppressive or tolerogenic potential of Tregs (280, 281). To investigate whether inhibition of Notch-1 had any differential effect on the function of allospecific Tregs, this suppression assay was repeated using our ABMtg.Foxp3-GFP mouse model (248), as described in Section 2.3.3. In brief, this is a B6 TCR-tg mouse with CD4⁺ T cells that express a TCR with defined specificity against the bm12 antigen (ABMtg), and where the expression of Foxp3 is labeled with GFP (Foxp3-GFP).

Purified Tregs, flow-sorted from ABMtg.Foxp3-GFP mice sensitised by intraperitoneal injection of 15 x 10⁶ bm12 splenocytes, and thereafter treated with either IgG or aNotch-1 (as detailed in Section 2.12) were added in varying ratios (1:4, 1:8, 1:16, 1:32 and 1:64) to assays containing conventional T cells (CD4⁺Foxp3⁻) and mitomycin-treated APCs (CD3⁻) isolated from IgG-treated mice for 96 hours. 16 hours before the cells were harvested for analysis, supernatants were collected and 3H-TdR was added to each well. On completion of the incubation period, the plate was analysed using a liquid scintillation counter.

As shown in Table 4.5 and Figure 4.27, inhibition of Notch-1 did not confer greater suppressive ability upon allospecific Tregs, as indicated by this assay. However, it must be noted that this is representative of one experiment only, wherein the measured scintillation counts were extremely low, such that the assay was not considered successful; indeed, both positive controls (Teff + APCs and Teff alone) registered very low counts: 5714 ± 709.8 and 2838 ± 156.5 cpm (data not shown).

It was unfortunately not possible to repeat this experiment due to a shortage in the number of ABMtg.Foxp3-GFP mice available, and this question therefore remains unanswered.
Table 4.5. Liquid scintillation counts obtained from an allospecific Treg suppression assay using Tregs isolated from IgG-treated or aNotch-1-treated mice

<table>
<thead>
<tr>
<th>Treg:Teff/APC ratio</th>
<th>IgG Tregs, cpm</th>
<th>aNotch-1 Tregs, cpm</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>4817 ± 452.5</td>
<td>5420 ± 61.43</td>
<td>0.2569</td>
</tr>
<tr>
<td>1:8</td>
<td>5078 ± 247.4</td>
<td>5196 ± 477.6</td>
<td>0.8371</td>
</tr>
<tr>
<td>1:16</td>
<td>6078 ± 298.1</td>
<td>6954 ± 834.7</td>
<td>0.3788</td>
</tr>
<tr>
<td>1:32</td>
<td>7995 ± 829.0</td>
<td>7886 ± 862.0</td>
<td>0.9318</td>
</tr>
<tr>
<td>1:64</td>
<td>7753 ± 973.0</td>
<td>5098 ± 375.2</td>
<td>0.0635</td>
</tr>
</tbody>
</table>

Figure 4.27. Allospecific Treg suppression assay

Tregs isolated from bm12-sensitised ABMtg.Foxp3-GFP mice treated with either IgG or aNotch-1 were examined for their suppressive capacities in a Treg suppression assay. Purified Tregs were added in varying ratios (1:4, 1:8, 1:16, 1:32 & 1:64) to assays containing conventional T cells (CD4$^+$Foxp3$^-$) and mitomycin-treated APCs (CD3$^-$) isolated from IgG-treated mice for 96 hours. Ratios were tested in triplicate wells.
4.3.13 Determination of the mechanisms underlying the increased suppressive capacity of aNotch-1-treated Tregs

4.3.13.1 TGFβ

Increased secretion of TGFβ was the first mechanism considered a potential underlying cause of the enhanced suppression seen following Notch-1 inhibition; the effect of aNotch-1 on TGFβ concentrations was therefore investigated in several different settings.

Firstly, supernatants collected from 48 hour allospecific MLRs established using cells isolated from mice recipient of a BALB/c cardiac allograft, treated with IgG or aNotch-1 on days 0, 2, 4 and 6, and sacrificed on day 7, were analysed for their concentration of secreted TGFβ using the Luminex assay. As shown in Figure 4.28 B, while there was no significant difference in the levels of TGFβ between mice treated with aNotch-1 compared to those treated with IgG in the first experiment (321.6 ± 101.9 vs 380.3 ± 32.25 pg/ml, respectively; p=0.69), there was a trend toward increased levels in the second; this did not, however, reach statistical significance (737.2 ± 18.1 vs 671.0 ± 9 pg/ml, respectively; p=0.072). Samples isolated from a further experiment, in which mice underwent thymectomy prior to receiving a full MHC-mismatch cardiac allograft, were also analysed for levels of secreted TGFβ. Again, there was no significant difference in the levels measured between aNotch-1-treated and IgG-treated mice (769.8 ± 52.73 vs 743.0 ± 35.54 pg/ml, respectively; p=0.69).

Secondly, the supernatants collected from the non-allospecific Treg suppression assay (shown in Figure 4.25) just prior to the addition of 3H-TdR were then analysed for levels of TGFβ, again using the Luminex assay. As shown in Figure 4.28 C, there was no significant difference in the levels of TGFβ secreted from cultures containing aNotch-1-treated Tregs compared to IgG-treated Tregs (632±188 vs 392±134 pg/mL; p=0.41; 1:1 ratio shown, similar results at other ratios tested).
Figure 4.28. Treatment with aNotch-1 does not significantly alter the levels of secreted TGFβ1

(A) 5-parameter log standard curve generated from TGFβ1 Luminex assay. (B) Levels of TGFβ1 in supernatants harvested from MLRs from three different experiments – experiment 1 and 2, standard post-transplant mechanisms on day 7; experiment 3, mechanistic experiment on day 7 post-transplant in mice previously subject to thymectomy. All wells tested in duplicate. (C) Levels of TGFβ in supernatants harvested from non-allospecific Treg suppression assay.
4.3.13.2 CTLA4 and Foxp3

Tregs were then examined to determine their expression of Foxp3 and CTLA4, as indicated by mean fluorescence intensity (MFI). The transcription factor Foxp3 has a major role in the development and programming of Tregs, while its level of expression has been shown to correlate with the degree of suppression achieved by Tregs in transplantation (282). The costimulatory molecule CTLA4 (CD152) is constitutively expressed by Tregs, and is central to their normal homeostasis (42, 283), although its role in Treg function is less clear (284).

Purified Tregs, isolated by flow-sorting as described earlier, were stained and analysed by flow cytometry. As shown in Figure 4.29, the MFI of Foxp3 was significantly higher on aNotch-1-treated Tregs compared to IgG-treated controls (3134 ± 22 vs 2821 ± 9, respectively; p=0.0057; n ≥ 2 per group; Figure 4.29 A). However, no significant difference in the MFI of CTLA4 was seen compared to IgG-treated controls (20.90 ± 2.8 vs 24.45 ± 10.55, respectively; p=0.78; n ≥ 2 per group; Figure 4.29 B).

**Figure 4.29. Tregs isolated from aNotch-1 treated mice display increased expression of Foxp3 but not CTLA4**

Flow-sorted Tregs, isolated from BALB/c-sensitised Foxp3-GFP B6 mice treated with either IgG or aNotch-1, were analysed using flow cytometry for their relative expression of Foxp3 (A) and CTLA4 (B), as determined by their measured MFI. **p<0.01
4.3.13.3 TNFR25

TNF receptor superfamily member 25 (TNFR25; also known as death receptor ligand 3 or DR3) is constitutively and highly expressed by Tregs (285, 286). Use of an agonistic TNFR25 antibody led to significant and selective expansion of existing Tregs in vivo but not in vitro, and did not affect the proportion of iTregs; these Tregs were, furthermore, highly suppressive and capable of reducing allergic lung inflammation (286). These data contrasted with previously reported pro-inflammatory effects of TNFR25 signaling, but it was thought that this might have been due to temporal differences in antigen exposure and onset of TNFR25 signaling (286). Although the TNFR25-mediated increase was shown to be dependent on intact Akt signaling, and Notch-1 is known to positively regulate the Akt pathway, due to the similarities in the Treg data, the expression of TNFR25 was investigated in the setting of Notch-1 inhibition.

BALB/c-sensitised (i.p.) Foxp3-GFP mice were treated with IgG or aNotch-1 on days 0, 2, 4, 6, 8 & 10 and were sacrificed on day 14. Flow-sorted Tregs (spleenic/LN) from these mice were isolated and stored for future use. However, thymi harvested from these mice were examined to determine the Treg (CD4+CD25+Foxp3+) populations and the proportion thereof expressing TNFR25. As shown in Figure 4.30, a significantly higher percentage of thymic Tregs isolated from aNotch-1-treated mice expressed TNFR25 compared to those isolated from IgG-treated controls (69.08 ± 5.77 vs 18.30 ± 3.62, respectively; p<0.0001; n=5 mice per group).

However, the expression of TNFR25 was also examined in splenic Tregs isolated from WT B6 mice recipient of a BALB/c cardiac allograft, treated thereafter with IgG or aNotch-1 and sacrificed on day 7: in contrast to the thymic Treg data, there was no increase in the proportion of splenic Tregs expressing TNFR25 (data not shown).
Figure 4.30. Thymic Tregs isolated from aNotch-1-treated mice showed significantly higher expression of TNFR25

BALB/c-sensitised (i.p.) Foxp3-GFP mice were treated with IgG or aNotch-1 on days 0, 2, 4, 6 and 8, and were sacrificed on day 10. Thymi harvested from these mice were examined to determine the Treg (CD4⁺CD25⁺Foxp3⁺) populations and the proportion thereof expressing TNFR25. ***p<0.0001; n=5 mice per treatment group.
Selective deletion of Notch-1 on Tregs enhances immune regulation

The experiments determining the effect of Notch-1 inhibition on Tregs were then replicated utilising Notch-1 conditional knock-out mice (N1cKO mice; Notch1<sup>fl/fl</sup>Foxp3<sup>Cre</sup>), in which Notch-1 is deleted in cells upon their expression of Foxp3, leading to selective absence of Notch-1 in Tregs. This deletion was confirmed using flow cytometry and qPCR, as shown in Figure 4.31. Foxp3<sup>Cre</sup> mice were used as the controls for all experiments utilising Notch1<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice. This set of experiments, with the exception of the Treg suppression assay, was performed by my colleagues Dr. L.V. Riella and Dr. Kassem Safa.

**Figure 4.31. Flow cytometry and QPCR confirmation of Notch1 deletion on Tregs**

Leucocytes were isolated from secondary lymphoid organs of control mice (Foxp3<sup>EGFP-Cre</sup>) or Notch1 conditional knockout mice (Notch1<sup>fl/fl</sup>Foxp3<sup>EGFP-Cre</sup>) and flow sorted for CD4<sup>+</sup>GFP.Foxp3<sup>+</sup> cells. (A) Mean fluorescence intensity (MFI) of Notch1 on Tconv (CD4<sup>+</sup>Foxp3<sup>+</sup>) and Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>). (B) Notch1 determination by QPCR of flow-sorted Tconv or Tregs. ***p<0.0001
As shown in Figure 4.32 A & B, Notch1^{ff}Foxp3^{EGFP-Cre} mice showed an increase in the proportion of CD4^+ cells expressing Foxp3 when compared to Foxp3^{EGFP-Cre} controls (14.9 ± 0.6 vs 10.32 ± 0.93%; p=0.0376) upon their sacrifice on day 7 post transplantation. When examined for Ki67 staining, the N1cKO Tregs again demonstrated a significant increase in proliferation (51.8 ± 5.01 vs 36 ± 1.63%; p=0.04; Figure 4.32 C & D).

Figure 4.32. Selective deletion of Notch-1 on Tregs increases the proportion of peripheral Tregs and the proliferation thereof

Splenocytes isolated from either Foxp3^{EGFP-Cre} mice or Notch1^{ff}Foxp3^{EGFP-Cre} mice recipient of a BALB/c cardiac allograft sacrificed on day 7 post-transplant were examined for the proportion of peripheral Tregs (A & B). These Tregs were then further examined for proliferation using the intracellular marker Ki67 (C & D). *p<0.05; n ≥ 3 mice per group.
These Tregs were then evaluated for rates of apoptosis, again using the cellular markers Annexin V and 7AAD, as described earlier. In contrast to the antibody data, however, there was no significant difference in the measured rate of apoptosis between Tregs isolated from Notch1<sup>f/f</sup>Foxp3<sup>EGFP-Cre</sup> mice compared to those isolated from Foxp3<sup>EGFP-Cre</sup> controls (6.13 ± 1.9 vs 5.77 ± 0.47%; p=0.8819; Figure 4.33 A & B).

Figure 4.33. Selective deletion of Notch-1 on Tregs does not influence the rate of Treg apoptosis

Tregs isolated from either Foxp3<sup>EGFP-Cre</sup> mice or Notch1<sup>f/f</sup>Foxp3<sup>EGFP-Cre</sup> mice recipient of a BALB/c cardiac allograft sacrificed on day 7 post-transplant, as described above, were examined for their rates of apoptosis using the markers Annexin V and 7AAD (A & B).
The Treg suppression assay was also repeated using N1cKO Tregs: as shown in Figure 4.3, the N1cKO Tregs again demonstrated greater suppressive properties compared to control Tregs across all ratios tested.

**Figure 4.3.** Selective genetic deletion of Notch-1 on Tregs renders them more suppressive *ex vivo*

Tregs isolated from either Foxp3\(^{EGFP-Cre}\) mice or Notch1\(^{f/f}\)Foxp3\(^{EGFP-Cre}\) mice were flow-sorted as described previously, and were examined for their suppressive capacities in a Treg suppression assay. Purified Tregs were added in varying ratios (1:1, 1:2, 1:4, and 1:8) to assays containing conventional T cells (CD4\(^{+}\)Foxp3\(^{-}\)) and mitomycin-treated APCs (CD3\(^{-}\)) isolated from Foxp3\(^{EGFP-Cre}\) for 96 hours. Ratios were tested in triplicate wells.

Finally, to assess the function of Notch-1 on Tregs *in vivo*, an adoptive transfer experiment was performed, utilising a cardiac transplant model in which RAG\(^{-/-}\) mice received a BALB/c cardiac allograft; a schematic is shown in Figure 4.35 A. On day 1 post-transplant, the mice were injected with 1 x 10\(^6\) CD4\(^{+}\)GFP.Foxp3\(^{-}\) cells and either 650,000 control (Foxp3\(^{GFP-Cre}\)) Tregs or N1cKO (Notch1\(^{f/f}\)Foxp3\(^{EGFP-Cre}\)) Tregs; graft survival was monitored in the usual manner.
As shown in Figure 4.35 B, graft survival in mice recipient of N1cKO Tregs far exceeded that in recipients of control Tregs (MST >100 vs 47 days; \( p=0.0002; \) \( n=8 & 7, \) respectively).

**Figure 4.35. Selective deletion of Notch-1 on Tregs significantly enhances graft survival in an adoptive transfer model**

(A) Schematic depicting a cardiac transplant model in which RAG\(^{-/-}\) mice on a B6 background received a BALB/c cardiac allograft; on day 1 post-transplant, the mice were injected with \( 1 \times 10^6 \) CD4\(^+\)GFP.Foxp3\(^-\) cells and either 650,000 control (Foxp3\(^{GFP-Cre}\)) Tregs or N1cKO (Notch1\(^{ff}\)Foxp3\(^{EGFP-Cre}\)) Tregs. (B) Graft survival in mice recipient of N1cKO Tregs compared to that in recipients of control Tregs.
4.3.15 Treatment with aNotch-1 results in reduction in levels of donor-specific antibodies

While Notch-2 signaling is known to be critical to normal B cell development, there is evidence that Notch-1 may be important in later stages of B cell activity (232). As discussed earlier, the interaction of Notch-1 and DLL1 was separately shown to both enhance the FO B cell proliferation response following stimulation with anti-CD40 mAb and to promote the differentiation of B cells into ASCs (232). Levels of donor-specific antibodies in both IgG- and aNotch-1-treated mice were therefore evaluated. As per the previous Notch-1 mechanistic studies, WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7. Serum was isolated by centrifuging whole blood at room temperature for 20 minutes at 14000 rpm; detailed methods for serum preparation are described in Section 2.6.5. The serum was then serially diluted (1:4, 1:8, 1:16, 1:32, 1:64) and incubated with naïve donor-type (BALB/c) splenocytes; full details are described in Section 2.9. Negative control serum obtained from naïve BALB/c and WT B6 mice was similarly prepared, diluted and incubated with BALB/c splenocytes. The splenocytes were stained for surface expression of B220, and were counterstained for expression of IgG1 and IgG2a. Lymphocyte gates were identified using the FlowJo analysis program by forward and side scatter characteristics.

Levels of DSA, as determined by the percentage of cells expressing IgG1 and IgG2a, are shown in Figure 4.36. Mice treated with aNotch-1 showed reduction in levels of DSA, as shown by the percentage of cells expressing IgG1 or IgG2a, while the IgG-treated mice showed levels approaching 100% at the 1:4 dilution, declining thereafter. The percentages are also listed separately in Table 4.6.
Figure 4.36. Treatment with aNotch-1 results in reduction in levels of donor-specific antibodies

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7 and serum isolated. Levels of DSA, as determined by the percentage of cells expressing IgG1 and IgG2a, are shown above: IgG-treated mice (blue circles) are compared to aNotch-2-treated mice (red squares); levels from negative control mice (naïve BALB/c and naïve WT B6; black circles and black squares, respectively) are also shown for comparison.
Table 4.6. Percentages of B220 cells positive for IgG1 (A) and IgG2a (B) in diluted serum samples from IgG- and aNotch-1-treated mice

<table>
<thead>
<tr>
<th></th>
<th>IgG Control</th>
<th>aNotch-1</th>
<th>BALB/c Naïve</th>
<th>B6 Naïve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>98.3</td>
<td>92.3</td>
<td>5.42</td>
<td>6.42</td>
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<td>98.7</td>
<td>90.9</td>
<td>4.78</td>
<td>4.91</td>
</tr>
<tr>
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<td>86.6</td>
<td>4.66</td>
<td>3.86</td>
</tr>
<tr>
<td>1:32</td>
<td>98.9</td>
<td>74.4</td>
<td>4.26</td>
<td>3.92</td>
</tr>
<tr>
<td>1:64</td>
<td>98.1</td>
<td>52.8</td>
<td>4.01</td>
<td>3.59</td>
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</tbody>
</table>

<table>
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<tr>
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<th>IgG Control</th>
<th>aNotch-1</th>
<th>BALB/c Naïve</th>
<th>B6 Naïve</th>
</tr>
</thead>
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<td>6.64</td>
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<td>2.81</td>
</tr>
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<td>54.3</td>
<td>4.23</td>
<td>2.19</td>
<td>2.58</td>
</tr>
</tbody>
</table>
4.3.16 Inhibition of Notch-1 leads to a reduction in the proportion of T follicular helper cells

T follicular helper cells are a relatively recently identified subset of T cells whose primary function is to provide help to B cells, regulating the development of effector and memory B cells, thereby controlling antigen-specific B cell immunity and influencing the development of antibodies (287).

The effect of Notch-1 inhibition on the Tfh subset was therefore examined to determine if the reduction in the measured levels of DSA could be attributable to aNotch-1-mediated changes therein. Tfh cells express the chemokine receptor CXCR5 and have been variably shown to express the costimulatory molecules ICOS, CD40L and PD-1 (288, 289). Due to limitations in staining potential, the Tfh subsets were identified using separate staining combinations (CD4⁺B220⁻CXCR5⁺PD1⁺ and CD4⁺B220⁻CXCR5⁺ICOS⁺).

As shown in Figure 4.37, there was a significant reduction in this subset, as identified by either combination of markers, in mice treated with aNotch-1 compared to IgG-treated controls: CD4⁺B220⁻CXCR5⁺PD1⁺ cells, 3.857 ± 0.31 vs 6.587 ± 0.93%, respectively, p=0.0492; CD4⁺B220⁻CXCR5⁺ICOS⁺ cells, 0.7677 ± 0.11 vs 2.247 ± 0.21%, respectively, p=0.0032.
Figure 4.37. Inhibition of Notch-1 leads to a reduction in the proportion of T follicular helper cells

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-1 on days 0, 2, 4, & 6 post-transplant were sacrificed on day 7. The splenocytes isolated were examined for their proportion of T follicular helper cells as determined by the percentage of CD4+ cells shown to be CXCR5+PD1+ (A) and CXCR5+ICOS+ (B). *p<0.05, **p<0.01; n ≥ 3 per group.
4.3.17 Notch-1 blockade acts synergistically with sCTLA4-Ig to prolong allograft survival

Previous work in our laboratory examined the effect of CTLA4-Ig on graft survival and demonstrated that a single dose (sCTLA4-Ig) effected significant prolongation of graft survival. Indeed, CTLA4-Ig has been shown to be highly effective in prolonging allograft survival in multiple rodent models (290, 291); there are, however, concerns about its effects on the Treg population: as CTLA-4-Ig functions by competing with CD28 for B7-1/2 binding, it also blocks the CTLA-4/B7 interaction, and, consequently, inhibitory cell-intrinsic signaling. Indeed, further studies by Dr. L.V. Riella have shown that CTLA4-Ig inhibits the Treg population and is capable of accelerating graft failure in a Treg-dependent model (79). Use of Belatacept, a mutant form of CTLA4-Ig, in human transplant recipients has not been without complication, with higher rates of severe acute rejection reported in the initial trials (78), thought to be partially related to adverse effects on the Treg population (79, 292).

To determine whether use of aNotch-1 could act synergistically with CTLA4-Ig, the two antibodies were administered in combination to WT B6 mice recipient of a heterotopic, vascularised, full MHC-mismatched cardiac transplant, as detailed earlier in Section 2.4.1; allograft survival was compared to that of recipients treated with sCTLA4-Ig at the same dose and on the same schedule:

1. sCTLA4-Ig: 250 µg on day 2 post-transplant
2. aNotch-1: 5 mg/kg on days 0, 2, 4, 6, 8 and 10

Consistent with previously published data, a single injection of CTLA4-Ig (sCTLA4-Ig) significantly prolonged allograft survival in WT B6 recipients (MST 42 days versus 7 days in IgG-treated controls; \( p=0.0046 \)). However, the administration of sCTLA4-Ig in conjunction with a short course of aNotch-1 effected allograft survival exceeding 100 days in all mice treated, compared to a
median survival of 42 days in mice treated with sCTLA4-Ig alone (Figure 4.38; \( p=0.0062 \)) and a median survival of 12.5 days in mice treated with aNotch-1 alone (Figure; \( p=0.0044 \)).

Figure 4.38. Notch-1 blockade acts synergistically with sCTLA4-Ig to prolong allograft survival
Hearts from BALB/c mice were transplanted into WT B6 recipients treated either with 5mg/kg of IgG or aNotch-1 on days 0, 2, 4, 6, 8, & 10 post-transplant and compared to the graft survival of those mice treated with a single dose of CTLA4-Ig (sCTLA4-Ig; 250 μg on day 2) administered on day 2 post-transplant, either alone or in combination with a course of aNotch-1. At least 4 mice per group shown.
To test the level of alloimmune hyporesponsiveness clinically present in these mice, a group of mice (n=3) treated with aNotch-1 and sCTLA4-Ig with initial graft survival exceeding 100 days were subsequently challenged with a second cardiac graft from the same donor strain (BALB/c) in the absence of any further treatment. Survival of the second BALB/c grafts also exceeded 100 days, indicating sustained donor hyporesponsiveness.

Finally, these mice were challenged with a third party (CBA strain) skin transplant; all skin grafts were rejected by day 13, indicating intact third party immunity.
4.3.18 Timelines

Finally, a set of experiments was performed to examine the timing of onset of action of aNotch-1. As the effect of aNotch-1 was most definitively seen on thymocytes, a decision was made to focus on the proportion and absolute number of thymocyte subsets.

In these experiments, mice were given a single dose of aNotch-1 (5 mg/kg) and were sacrificed at different timepoints thereafter. The initial experiment involved testing at 12, 24 and 48 hours following a single dose. Mice recipient of aNotch-1 were compared both to a naïve mouse, and to mice recipient of a single dose of IgG at the same timepoints.

For ease of comparison, only the 12 hour IgG control population is shown graphically in Figure 5.23 B; however, the statistical comparisons detailed below in Table 4.7 are made with their appropriately timed controls. A 1-way ANOVA was also performed to compare differences between all groups, and failed to detect any significant differences (p=0.129)

Table 4.7. Absolute number of thymocytes isolated at 12, 24 and 48 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG Control</th>
<th>aNotch-1</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hours</td>
<td>85000000 ± 38180000</td>
<td>43500000 ± 9192000</td>
<td>0.274</td>
</tr>
<tr>
<td>24 hours</td>
<td>90000000 ± 8485000</td>
<td>42500000 ± 7071000</td>
<td>0.026</td>
</tr>
<tr>
<td>48 hours</td>
<td>72810000 ± 14580000</td>
<td>56000000 ± 4243000</td>
<td>0.258</td>
</tr>
</tbody>
</table>
Figure 4.39. Treatment with aNotch-1 fails to significantly reduce the absolute number of thymocytes by 48 hours following a single dose

As the initial experiment had not shown any significant difference in thymocyte numbers by 48 hours, the experiment was repeated, this time taking its first timepoint at 72 hours. In this experiment, the numbers of thymocytes isolated from aNotch-1-treated mice at varying timepoints were compared only to the number isolated from IgG-treated controls at 72 hours, as the initial experiment had failed to detect any significant difference in thymocyte number in IgG-treated controls across any timepoint.

As shown in Table 4.8 and Figure 4.40, the absolute number of thymocytes had decreased significantly at 72 hours following a single dose of aNotch-1 when compared to IgG-treated controls, and decreased further thereafter to a nadir at 120 hours (or 5 days).

As data already existed on absolute counts and percentages of thymocyte subpopulations on day 7, further testing to ascertain changes on day 6 was not undertaken.
Table 4.8. Absolute number of thymocytes isolated at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean n cells</td>
<td>56500000</td>
<td>23600000</td>
<td>6500000</td>
<td>3400000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>12020000</td>
<td>6223000</td>
<td>2687000</td>
<td>565685</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0048</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.40. Treatment with aNotch-1 significantly reduces the absolute number of thymocytes by 72 hours following a single dose
Figure 4.41. The percentage and absolute number of DN thymocytes and Ki67^hi^ DN thymocytes at 72, 96 and 120 hours following a single dose of control IgG or aNotch-1.

(A & B) The percentage and absolute number, respectively, of DN thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.

(C & D) The percentage and absolute number, respectively, of DN thymocytes that were shown to be Ki67^hi^ isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
Table 4.9. The percentage and absolute number of DN thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>19.40</td>
<td>23.45</td>
<td>22.90</td>
<td>39.45</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>5.091</td>
<td>8.980</td>
<td>5.233</td>
<td>5.303</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.1087</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>10660000</td>
<td>5255000</td>
<td>14180000</td>
<td>13560000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>544472</td>
<td>660155</td>
<td>275206</td>
<td>403475</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.10. The percentage and absolute number of Ki67<sup>hi</sup> DN thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>48.55</td>
<td>20.1</td>
<td>18.3</td>
<td>15.45</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>3.182</td>
<td>0.5657</td>
<td>3.111</td>
<td>0.7778</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>5164000</td>
<td>1058000</td>
<td>255249</td>
<td>207979</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>74699</td>
<td>162417</td>
<td>6239</td>
<td>51787</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.42. The percentage and absolute number of DN thymocytes (Stage I & II) at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

(A & B) The percentage and absolute number, respectively, of DN Stage I thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1. (C & D) The percentage and absolute number, respectively, of DN Stage II thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
Table 4.11. The percentage and absolute number of DN I thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>2.145</td>
<td>2.68</td>
<td>5.575</td>
<td>4.68</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.3323</td>
<td>0.396</td>
<td>1.209</td>
<td>2.291</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.156</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>229455</td>
<td>142136</td>
<td>77401</td>
<td>68097</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>47090</td>
<td>38500</td>
<td>1805</td>
<td>49956</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.0437</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.12. The percentage and absolute number of DN II thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>1.015</td>
<td>0.32</td>
<td>0.505</td>
<td>0.54</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.8415</td>
<td>0.04243</td>
<td>0.02121</td>
<td>0.3677</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.543</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>105858</td>
<td>16955</td>
<td>7133</td>
<td>6582</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>84131</td>
<td>4342</td>
<td>1089</td>
<td>2808</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.189</td>
<td></td>
<td></td>
<td></td>
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</table>
Figure 4.43. The percentage and absolute number of DN thymocytes (Stage III & IV) at 72, 96 and 120 hours following a single dose of control IgG or aNotch-1

(A & B) The percentage and absolute number, respectively, of DN Stage III thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1. (C & D) The percentage and absolute number, respectively, of DN Stage IV thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
Table 4.13. The percentage and absolute number of DN III thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>5.91</td>
<td>1.265</td>
<td>2.25</td>
<td>2.195</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.966</td>
<td>0.1909</td>
<td>0.07071</td>
<td>0.4455</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.0348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>624359</td>
<td>65843</td>
<td>31812</td>
<td>28872</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>177273</td>
<td>1681</td>
<td>5189</td>
<td>2814</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.0062</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.14. The percentage and absolute number of DN IV thymocytes at 72, 96 and 120 hours following a single dose of IgG control or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>90.95</td>
<td>95.4</td>
<td>91.65</td>
<td>92.55</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2.475</td>
<td>0.2828</td>
<td>1.344</td>
<td>1.485</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.1586</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>9697000</td>
<td>5014000</td>
<td>1302000</td>
<td>1252000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>758895</td>
<td>644651</td>
<td>271280</td>
<td>353276</td>
</tr>
<tr>
<td>ANOVA p</td>
<td>0.0003</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 4.44. The percentage and absolute number of DP thymocytes and Ki67\textsuperscript{hi} DP thymocytes at 72, 96 and 120 hours following a single dose of control IgG or aNotch-1.

(A & B) The percentage and absolute number, respectively, of DP thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.

(C & D) The percentage and absolute number, respectively, of DP thymocytes shown to be Ki67\textsuperscript{hi} isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
### Table 4.15. The percentage and absolute number of DP thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>48.4</td>
<td>39.7</td>
<td>36.8</td>
<td>18.95</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>2.97</td>
<td>14</td>
<td>14.42</td>
<td>11.38</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.224</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean n cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27520000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>7496000</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.016</td>
</tr>
</tbody>
</table>

### Table 4.16. The percentage and absolute number of Ki67\(^{hi}\) DP thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>0.925</td>
<td>0.18</td>
<td>0.88</td>
<td>0.3</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.4455</td>
<td>0.01414</td>
<td>0.9617</td>
<td>0.2263</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.4738</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean n cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>237905</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>53277</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0026</td>
</tr>
</tbody>
</table>
Figure 4.45. The percentage and absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> SP thymocytes at 72, 96 and 120 hours following a single dose of control IgG or aNotch-1.

(A & B) The percentage and absolute number, respectively, of CD4<sup>+</sup> SP thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1. (C & D) The percentage and absolute number, respectively, of CD8<sup>+</sup> SP thymocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
Table 4.17. The percentage and absolute number of CD4$^+$ SP thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>4.41</td>
<td>7.965</td>
<td>13.85</td>
<td>11.96</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.3253</td>
<td>3.302</td>
<td>2.899</td>
<td>4.872</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>2472000</td>
<td>1777000</td>
<td>861300</td>
<td>420250</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>346341</td>
<td>283691</td>
<td>183706</td>
<td>233275</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0053</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.18. The percentage and absolute number of CD8$^+$ SP thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>0.585</td>
<td>0.575</td>
<td>1.84</td>
<td>2.235</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.1909</td>
<td>0.3182</td>
<td>0.8061</td>
<td>1.648</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.308</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>319050</td>
<td>125800</td>
<td>108770</td>
<td>80650</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>37547</td>
<td>39315</td>
<td>2956</td>
<td>68660</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.0174</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.46. The percentage and absolute number of CD4$^{+}$Foxp3$^{+}$ thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

(A & B) The percentage and absolute number, respectively, of CD4$^{+}$ Foxp3$^{+}$ thymocytes.

Table 4.19. The percentage and absolute number of CD4$^{+}$Foxp3$^{+}$ thymocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>0.51</td>
<td>3.18</td>
<td>3.31</td>
<td>4.125</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>0.3394</td>
<td>1.909</td>
<td>1.386</td>
<td>1.549</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.204</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>264846</td>
<td>670568</td>
<td>151733</td>
<td>76771</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>258348</td>
<td>560249</td>
<td>28715</td>
<td>17222</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.47. The percentage and absolute number of CD4$^+$Foxp3$^+$ splenocytes and CD4$^+$Foxp3$^+$ splenocytes Ki67$^+$ at 72, 96 and 120 hours following a single dose of control IgG or aNotch-1

(A & B) The percentage and absolute number, respectively, of CD4$^+$Foxp3$^+$ splenocytes isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1. (C & D) The percentage and absolute number, respectively, of CD4$^+$Foxp3$^+$ splenocytes Ki67$^+$ isolated 72, 96 and 120 hours following a single dose of IgG or aNotch-1.
Table 4.20. The percentage and absolute number of CD4^{+}Foxp3^{+} splenocytes at 72, 96 and 120 hours following a single dose of IgG or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>8.345</td>
<td>11.1</td>
<td>13.5</td>
<td>19.4</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>1.492</td>
<td>1.414</td>
<td>2.97</td>
<td>0.7071</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean n cells</td>
<td>175694</td>
<td>493318</td>
<td>643500</td>
<td>1200000</td>
</tr>
<tr>
<td>Std. deviation</td>
<td>96918</td>
<td>133646</td>
<td>532791</td>
<td>293759</td>
</tr>
<tr>
<td>ANOVA</td>
<td>p=0.1196</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.21. The percentage and absolute number of CD4^{+}Foxp3^{+}Ki67^{hi} splenocytes at 72, 96 and 120 hours following a single dose of IgG control or aNotch-1

<table>
<thead>
<tr>
<th></th>
<th>IgG 72°</th>
<th>aNotch-1 72°</th>
<th>aNotch-1 96°</th>
<th>aNotch-1 120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>6.62</td>
<td>8.82</td>
<td>19.45</td>
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<td>300961</td>
</tr>
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<td>4044</td>
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<tr>
<td>ANOVA</td>
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4.4 Discussion

To my knowledge, this is the first report of the effect of selective Notch-1 blockade using an antibody-mediated approach in the setting of solid organ transplantation, and shows that Notch-1 plays a crucial role in alloimmunity.

The alloimmune response is a complex interplay between pathogenic/inflammatory and regulatory/anti-inflammatory immune mechanisms; the supremacy of either process determines whether the ultimate fate of the allograft is rejection or tolerance, respectively (36-38). In transplantation, both CD4\(^+\) and CD8\(^+\) T cells play a dominant role in cell-mediated alloimmunity and have been shown to be of key importance in allograft rejection (254-256), while Tregs, critical to the development and maintenance of self-tolerance (72), are also indispensable to the induction and maintenance of allograft tolerance.

There is accumulating data to support the involvement of Notch signaling in the T cell activation and differentiation pathway, where it is thought to act as a Signal 3 mediator, fine-tuning the immune response (44, 45). These studies have shown increased expression of Notch ligands on APCs following exposure to TLR ligands (69) and increased Notch signaling following TCR ligation (70), while the Notch receptors have been shown to co-localise with CD4 in CD4\(^+\) T cells (70) and the E3 ligase Numb at the T cell – APC interface (71).

Early work indicated that Notch-1 receptor signaling was critical for Th1 cell differentiation in EAE through direct binding of RBPJ to the \(Tbx21\) gene promoter (214). Subsequent data revealed that Notch-1 signaling could directly regulate IFN-\(\gamma\) expression via \(Tbx21\) (293), and, furthermore, the expression of effector CD8\(^+\) T cell molecules such as EOMES, perforin and granzyme B (214, 294).
The potential role of Notch signaling in Tregs was first demonstrated by overexpression of Notch-3 on T cells, which led to accumulation of Tregs in the thymus and periphery (295). Several subsequent studies have investigated the role of Notch ligands in the development and maintenance of Tregs in atopy and autoimmunity, with some conflicting results. Presentation of the house dust mite antigen Der p1 to naïve CD4+ cells by DCs modified to overexpress Jagged1 led to the development of antigen-specific Tregs that were capable of conferring tolerance (218). In an EAE model, inhibition of DLL4 resulted in expansion of the peripheral Treg population and reduction in the severity of disease (221). However, in another study by the same group, inhibition of Jagged1 or DLL1 failed to have an effect on Treg generation either \textit{in vitro} or \textit{in vivo}. Overexpression of Jagged2 on hematopoietic progenitor cells led to enhanced Treg expansion and protection against autoimmune diabetes (296). Use of \(\gamma\)-secretase inhibitors \textit{in vivo} reduced the development and maintenance of peripheral Tregs with consequent development of autoimmune disease (222). In contrast to this, however, inhibition of global Notch signaling in a mouse model of GVHD has been shown to result in accumulation of pre-existing nTregs (224), although the mechanisms underlying this were not explored.

Due to the limitations of individual approaches such as non-physiological overexpression of Notch receptors/ligands, pan-Notch inhibition and artificial polarising conditions \textit{in vitro}, it has so far been difficult to understand the role of individual Notch receptors in Treg homeostasis \textit{in vivo}.

In this study, selective inhibition of Notch-1, achieved by use of the novel, antagonistic antibody aNotch-1, was first shown to effect a significant prolongation of graft survival in a stringent, full-MHC mismatch murine cardiac transplantation model. Commensurate with their enhanced survival, allografts harvested from aNotch-1-treated mice displayed significantly less cellular infiltration by both total CD4+ and CD8+ cells, although, interestingly, a
significantly greater population of graft-infiltrating CD4+ Tregs. Analysis of the splenocyte populations revealed the proportion of splenic CD4+ and CD8+ Teff to be significantly lower in aNotch-1-treated mice compared to IgG-treated controls. In agreement with data showing a critical role for Notch-1 signaling in Th1 differentiation and the secretion of effector cytokines (214, 293, 294, 297), and in support of both previous data from our lab investigating the effect of DLL-1 inhibition (233) and a study that investigated the effect of pan-Notch inhibition of donor alloreactive T cells (224), Notch-1 blockade led to significant reduction in Th1 cells and granzyme B-producing cells. It must be noted that other investigators have also identified Notch-2 signaling as a direct regulator of cytotoxic T lymphocyte differentiation (298, 299); these findings are not necessarily mutually exclusive, and although differences may be related to the experimental context, the effect of Notch-2 inhibition on effector T cell subsets will be investigated in Chapter Five.

The ability to selectively target the Notch-1 receptor in this transplant model using either an antibody or genetic approach has revealed that inhibition of Notch-1 leads to both expansion of Tregs and enhancement of Treg function, while suppressing effector T cells. The replication of these Treg findings in the Notch-1 conditional knock-out model suggests that the increased frequency of Tregs is not simply due to the inhibition of effector T cells, but instead indicates that the effect of aNotch-1 is primarily directed towards and mediated by Tregs. Indeed, Tregs were critical to the enhanced graft survival achieved with Notch-1 blockade, as depletion of Tregs pre-transplant in mice subsequently treated with aNotch-1 near-completely abrogated the survival advantage previously seen with aNotch-1 alone. In agreement with the previous GVHD data (224), the Treg increase seen was demonstrated to be due to expansion of the nTreg population, as no increase in the generation of iTregs, either in vitro or in vivo, was seen upon Notch-1 inhibition. Though preliminary work suggests that adoptive transfer of ex vivo-expanded natural Tregs is effective in controlling rejection (300) and preventing GVHD (301), the ability to expand the Treg
population *in vivo* via blockade of Notch-1 negates the requirement for *ex vivo* Treg expansion and also avoids potential issues of Treg instability (302).

Natural Tregs are known to originate in the thymus and there is convincing evidence to support an indispensable role for Notch-1 in normal thymocyte development (190, 207); indeed, inhibition of Notch-1 was shown to result in complete disruption of normal thymic architecture with associated aberration in normal thymocyte development, characterised by developmental arrest at DN Stage I and subsequent failure to progress to the DP Stage, but yet with maintained progression to the SP subsets. Furthermore, the proportion of Tregs within the thymus was greatly increased, and was shown to consist of Tregs that were of thymic origin, rather than repopulating peripheral iTregs.

Nevertheless, despite the dramatic effect of Notch-1 blockade on the thymus, this was shown not to be central to its overall effect, instead indicating a crucial effect on the periphery. Indeed, the performance of thymectomy two weeks prior to subsequent transplantation and Notch-1 inhibition failed to abrogate either the enhanced graft survival or the expansion and reduced apoptosis seen in the peripheral Treg population. It must be noted, however, that thymectomy was performed in adult mice, in which a full complement of circulating peripheral Tregs would be expected. It is entirely possible that performance of thymectomy in the neonatal period would have produced a different outcome; this may be a question for future study.

The expansion in peripheral Tregs was shown to be due both to a decrease in peripheral Treg apoptosis and increased Treg proliferation. This Treg apoptosis data contrasts with a study by Perumalsamy *et al* that showed Notch-1 signaling protected Tregs from *in vitro* apoptosis induced by the absence of IL-2 (303); however, the susceptibility of Notch1−/− Tregs to apoptosis was inhibited by the addition of exogenous IL-2 *in vitro*, and although Notch1−/− Tregs were also shown to have impaired *in vivo* survival in congenic hosts (303), these
Tregs were activated \textit{in vitro} and were not subjected to an antigenic challenge \textit{in vivo}, which may account for the differences seen.

In this study, Tregs isolated from aNotch-1-treated mice were demonstrated to be more suppressive than those isolated from IgG-treated controls, and, although did not appear to have greater expression of CTLA4 or secrete higher amounts of TGF\(\beta\), were shown to have higher expression of Foxp3 than controls. The transcription factor Foxp3 has a major role in the development and programming of Treg cells. The level of Foxp3 expression on Tregs was previously shown to correlate with the degree of suppressive function of Tregs in transplantation, suggesting that the beneficial effect of Notch-1 blockade could be in part attributed to a higher Foxp3 expression (282).

Use of an agonistic TNFR25 antibody was previously shown to effect significant and selective expansion of existing Tregs \textit{in vivo} but not \textit{in vitro}, and did not affect the proportion of iTregs; these Tregs were, furthermore, highly suppressive and capable of reducing allergic lung inflammation (286). However, the effect of TNFR25 signaling was shown to be dependent on intact Akt signaling, and, as discussed earlier in Section 1.5.1, Notch-1 signaling has been shown to positively influence the Akt pathway. Nonetheless, given the similarities in the effects of aNotch-1 and TNFR25 signaling, the expression of TNFR25 in the setting of Notch-1 blockade was also examined. Interestingly, a significantly higher proportion of thymic Tregs isolated from aNotch-1-treated mice demonstrated expression of TNFR25, but this did not extend to peripheral Tregs, and the significance of this is unknown.

In agreement with previous data indicating that Notch-1 signaling was important for later stages of B cell activity, particularly the differentiation of B cells into ASCs (232), blockade of Notch-1 in this stringent transplant model was shown to inhibit the formation of donor-specific antibodies. This may be partly attributable to a reduction in the proportion of T follicular helper cells, known to
play a crucial role in normal antibody responses (287). Given the increasingly recognised contribution of antibodies to the alloimmune response, the ability of Notch-1 blockade to affect both the T and B cell responses may be of particular benefit in potential future use as an immunosuppressive strategy.

Finally, transient Notch-1 inhibition was revealed to act synergistically with a single dose of CTLA4-Ig to effect long-term graft survival and sustained donor hyporesponsiveness. While CTLA4-Ig has been shown to be highly effective in prolonging allograft survival in multiple rodent models (290, 291), there are concerns about its effects on the Treg population: as CTLA-4-Ig functions by competing with CD28 for B7-1/2 binding, it also blocks the CTLA-4/B7 interaction, and, consequently, inhibitory cell-intrinsic signaling. Indeed, use of Belatacept, a mutant form of CTLA4-Ig, in human transplant recipients has not been without complication, with higher rates of severe acute rejection reported in the initial trials (78), thought to be partially related to adverse effects on the Treg population (79, 292). However, given the significant survival advantage seen in the combination-treated mice, it is possible that the beneficial effects of Notch-1 blockade on the Treg population have superseded the possible disadvantage conferred by sCTLA4-Ig alone, and suggest that this may be a promising therapeutic combination.

In summary, Notch signaling is critical for the fine-tuning of the immune response. Most immunosuppressive agents in clinical use induce global inhibition of the immune system, including both effector and regulatory components. One of the striking findings upon Notch-1 blockade in transplantation was the simultaneous suppression of effector T cells and function with expansion and enhanced function of Tregs. This immunomodulatory component could be vitally important for the improvement of long-term graft survival and minimisation of maintenance immunosuppression. These data demonstrate a critical role of Notch-1 in alloimmunity and identify a promising target for manipulation of the T cell response and promotion of
immune regulation. Furthermore, the availability of a selective, human Notch-1 antibody, which requires only transient use to achieve long-lasting results, suggests that this approach could be efficiently translated into novel strategies of immunosuppression in solid organ transplantation in humans.
Chapter Five: The effect of selective inhibition of the Notch-2 receptor on the alloimmune response

5.1 Introduction
Following the seminal studies by Terasaki and Patel (304) in the early period of transplant immunology, research efforts were predominantly focused upon histoincompatibility and the humoral component of the alloimmune response. In the subsequent decades, however, the attention of the transplant community was largely directed at the T cell contribution to the alloimmune response, and the development of potential therapeutic techniques to modify this, particularly following reports of the existence of different T cell lineages (305).

Latterly, the role of B cells in alloimmunity has gained increasing prominence, both in terms of antigen presentation and alloantibody production. Indeed, a recent large series from the Mayo Clinic revealed that the majority of cases of late renal allograft loss were due to chronic antibody-mediated injury as a manifestation of the alloimmune response (5). It is also clear from studies using mice with B cells capable of antigen presentation, but not alloantibody production, that B cells contribute to the development of CAV, a hallmark of chronic rejection, independent of antibody production, by supporting T cell responses through antigen presentation and maintenance of lymphoid architecture (306). These findings were confirmed by the use of chimeric mice, where B cells were present but could not present antigen, and in which both T cell responses and CAV were markedly reduced (306).

Notch-2 signaling has been shown to play a critical, non-redundant role in the development of marginal zone B cells (104), a subset of mature peripheral B cells that have been shown to be both very efficient antibody producers and activators of naïve T cells. Mice conditionally deficient in Notch-2 displayed a severely diminished MZB population (104), and, furthermore, a significant reduction in the T2 population, providing evidence that the MZB precursors lie
within this population (104). Deletion or absence of other elements of the Notch signaling pathway, including RBP-J (226), MAML1 (227, 228) and ADAM10 (229), yielded similar results. Later stages of B cell activity, including B cell activation and terminal differentiation into antibody-secreting cells (ASCs), have also been shown to be regulated by Notch signaling (111).

Figure 5.1. B cell schematic with stages of B cell development associated with Notch-2 signaling highlighted

The involvement of Notch-2 signaling in these particular stages of B cell development renders it of great interest as we seek to manipulate the B cell response in alloimmunity. The aim of this chapter was to investigate the role of Notch-2 within the alloimmune response, particularly that pertaining to the B cell component, by selectively inhibiting the Notch-2 receptor.
5.2 Methods
All experimental methods are described in detail in Chapter 2, so for the purposes of this chapter will be only briefly described.

5.2.1 Mice
6 – 8 week old C57BL/6 (B6; Thy1.1+) and BALB/c (H-2d) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in our facility. Naïve B6 mice were used as recipients of a BALB/c cardiac allograft in the transplantation experiments.

5.2.2 Transplant Model
Vascularised intra-abdominal heterotopic cardiac allograft transplants were performed using sterile microsurgical techniques (258) by Tetsunosuke Shimizu (T.S.) or Shunsuke Ohori (S.O.), surgical fellows in our laboratory, as described in detail in Chapter 2.4.1. General anaesthesia was achieved either by intraperitoneal injection of a combination of ketamine and xylazine or by inhaled isoflurane. Graft survival was assessed by daily palpation; rejection was defined as complete cessation of cardiac contractility, and was confirmed by direct visualisation at laparotomy.

5.2.3 Antibodies

5.2.3.2 Notch receptor antibody
The anti-Notch-2 (aNotch-2) antibody was generously provided by Dr. Christian Siebel of Genentech, Inc. (South San Francisco, CA, USA). As described earlier, it is an antagonistic, fully human IgG1 antibody which potently inhibits Notch-2 but not other Notch receptors, and which binds with similarly high affinity to both the mouse and human orthologues (243). Control IgG, also obtained from Genentech, Inc., was administered as a control antibody at the same dose and on the same schedule as aNotch-2, according to the respective experiments.
5.2.3.3 Anti-CD20

The aCD20 clone 5D2 (murine IgG2a) was purchased from Genentech, Inc. (South San Francisco, CA, USA). It was initially administered at a dose of 200 µg on day -1 before transplantation; the protocol was subsequently amended to dose 200 µg on days -4 and -1.
5.3 Results

5.3.1 Use of anti-Notch-2 significantly prolongs cardiac allograft survival

Following on from the trial of aNotch-2 in naïve mice, it was next administered, according to the dosing schedule below, to B6 mice recipient of a heterotopic, vascularised, full MHC-mismatched cardiac transplant, as detailed earlier in Section 2.4.1. Allograft survival was compared to that of recipients treated with control IgG at the same dose and on the same schedule:

1. IgG: 5 mg/kg on day 0, 2.5 mg/kg on days 3, 7 and 10
2. aNotch-2: 5 mg/kg on day 0, 2.5 mg/kg on days 3, 7 and 10

Mice treated with IgG had a median survival time (MST) of 7 days, while those treated with aNotch-2 had an MST of 8.5 days (p=0.0193; see Figure 5.2 A). An alternative dosing schedule was then trialed using the same model to determine if further prolongation of graft survival was possible:

1. IgG: 5 mg/kg on day 0, 2.5 mg/kg on days 3, 5, 7, 9 and 11
2. aNotch-2: 5 mg/kg on day 0, 2.5 mg/kg on days 3, 5, 7, 9 and 11

Using this regimen, mice treated with IgG had an unchanged median survival time of 7 days, while those treated with aNotch-2 had an MST of 9 days (p=0.064; see Figure 5.2 B), in the absence of any overt untoward effects.

A further modification was then made to the dosing protocol:

1. IgG: 5 mg/kg on days 0, 3, 5, 7, 9 and 11
2. aNotch-2: 5 mg/kg on days 0, 3, 5, 7, 9 and 11
Using this regimen, mice treated with IgG again had an MST of 7 days; however, in those mice treated with the increased dose of aNotch-2, the MST was significantly prolonged at 12 days (p=0.0003; see Figure 5.2 C), again in the absence of any overt untoward effects. This dosing protocol was then adopted for all further experiments, including mechanistic studies.

Figure 5.2. Optimisation of dose of aNotch-2 & effect on the survival of fully MHC-mismatched cardiac allografts

Hearts from BALB/c mice were transplanted into WT B6 recipients treated with either 5mg/kg of IgG or aNotch-2 on day 0, 2.5 mg/kg on days 3, 7, & 10 post-transplant (A; MST 7 vs 8.5 days), 5mg/kg of IgG or aNotch-2 on day 0, 2.5 mg/kg on days 3, 5, 7, 9 & 11 post-transplant (B; MST 7 vs 9 days) or 5mg/kg of IgG or aNotch-2 on days 0, 3, 5, 7, 9 & 11 post-transplant (C; MST 7 vs 12 days). Data shown from pooled experiments; n ≥ 3 mice per group.
5.3.2 Histological assessment of cardiac allografts following inhibition of Notch-2

Histological assessment of grafts harvested on day 8 post-transplant revealed changes consistent with the survival data. In IgG-treated recipients, there was significant destruction of the normal cardiac architecture with severe cellular infiltration (Figure 5.3 B); however, in aNotch-2-treated recipients, there was significantly less cellular infiltration and the cardiac architecture was generally preserved (Figure 5.3 C).

Figure 5.3. Notch-2 blockade preserves cardiac allograft architecture and reduces graft cellular infiltration

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and the grafts isolated. Representative histological slides (H&E stain) from a syngeneic (WT B6 → WT B6) graft (A), an allograft harvested from an IgG-treated (B) and aNotch-2-treated (C) recipient are shown.

As previously described, these grafts were further analysed to determine the proportion of graft-infiltrating leucocytes. The hearts were first minced and digested with collagenase for 30 minutes at 37C; the cells were then isolated using density centrifugation and were obtained by carefully removing the buffy
coat; full details are provided in Chapter 2. As shown in Figure 5.4, the number of leucocytes isolated from the grafts of IgG-treated mice was considerably higher than that isolated from mice treated with aNotch-2.

Figure 5.4. Inhibition of Notch-2 results in marked reduction in number of graft-infiltrating lymphocytes
WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and the grafts harvested. Total lymphocytes were isolated from the grafts using density centrifugation. The samples shown are from a single representative mouse from each group.
5.3.3 Characterisation of haematopoietic cells in lymphoid organs following inhibition of Notch-2

To determine the mechanisms by which inhibition of Notch-2 improves graft survival, the effect of aNotch-2 was first examined on the lymphoid organs retrieved on day 8 post-transplantation. As shown in Figure 5.5, initial macroscopic examination revealed a modest reduction in the size of the spleens retrieved from aNotch-2-treated mice compared to those treated with IgG; in contrast to the earlier data with aNotch-1 shown in Chapter 4, however, there was no significant difference in the size of either the draining lymph nodes or thymi harvested from IgG- or aNotch-2-treated mice.

![Image of thymi (A), draining lymph nodes (B), and spleens (C) isolated from IgG- (left panel) or aNotch-2-treated (right panel) WT B6 mice on day 8 post-transplant of a BALB/c cardiac allograft. Images from single experiment shown, representative of multiple repeat experiments.](image)

Figure 5.5. Macroscopic effects of Notch-2 inhibition on lymphoid organs retrieved post-transplant
Images of thymi (A), draining lymph nodes (B), and spleens (C) isolated from IgG- (left panel) or aNotch-2-treated (right panel) WT B6 mice on day 8 post-transplant of a BALB/c cardiac allograft. Images from single experiment shown, representative of multiple repeat experiments.
Notch-2 is primarily involved in B cell development and fate; independent of their direct involvement in the alloimmune response, however, B cells are also known to contribute to T cell responses by activating naïve T cells. Therefore, taking the same approach as that used to investigate the effect of Notch-1 inhibition, the frequencies of splenic and dLN CD4$^+$ and CD8$^+$ effector memory T cells, defined as CD44$^{hi}$CD62L$^{lo}$, were examined using flow cytometry. As described previously, the lymphocyte gates were determined using the FlowJo analysis program by forward and side scatter characteristics. The populations were then determined according to the CD4 and CD8 counterstains, and thereafter according to their relative expression of CD44 and CD62L.

As shown in Figure 5.6, the proportion of splenic CD4$^+$ effector memory cells was significantly, albeit modestly, reduced in aNotch-2-treated mice compared to IgG-treated controls (21.65 ± 0.7 vs 25.87 ± 0.81%, respectively; p=0.011; n ≥ 3 per group; Figure 5.6). There was no significant difference in the percentage of CD4$^+$ effector memory cells identified within draining lymph nodes in either IgG-treated or aNotch-2-treated mice (19.50 ± 3.7 vs 13.39 ± 2.65%, respectively; p=0.22; n ≥ 3 per group; graph not shown).

There was, however, a highly significant reduction in the splenic CD8$^+$ effector memory population in mice treated with aNotch-2 compared to those treated with IgG (13.30 ± 1.05 vs 35.13 ± 2.71%, respectively; p=0.0004; n ≥ 3 per group; Figure 5.7). Again, there was no significant difference in the CD8$^+$ effector memory population isolated from graft-draining lymph nodes in either IgG-treated or aNotch-2-treated mice (12.32 ± 2.86 vs 7.945 ± 2.15%, respectively; p=0.27; n ≥ 3 per group; graph not shown).
**Figure 5.6. Use of aNotch-2 results in significant inhibition of splenic CD4$^+$ effector memory T cells**

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and the spleens harvested; a naïve mouse is also shown for comparison. The splenocytes isolated were examined for the frequency of CD4$^+$ effector memory cells (Teff/mem; defined as CD44$^{hi}$CD62L$^{low}$) by flow cytometry. Representative example of at least three repeated experiments shown; n ≥ 3 per group; *p<0.05.
Figure 5.7. Use of aNotch-2 results in significant inhibition of splenic CD8\(^+\) effector memory T cells

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and the spleens harvested; a naïve mouse is also shown for comparison. The splenocytes isolated were examined for the frequency of CD8\(^+\) effector memory cells (Teff/mem; defined as CD44\(^{hi}\)CD62L\(^{low}\)) by flow cytometry. Representative example of at least three repeated experiments shown; n \(\geq\) 3 per group; ***p<0.0001
5.3.4 The effect of Notch-2 blockade on T follicular helper cells

As discussed previously in section 4.3.16, T follicular cells are a subpopulation of Th cells involved in T and B cell cross-talk, providing help to B cells and thereby influencing antigen-specific B cell immunity and the development of antibodies (287).

As described in section 4.3.16, Tfh cells were identified according to the phenotype CD4⁺B220⁻CXCR5⁺PD1⁺. As shown in Figure 5.8 A & B, the use of aNotch-2 led to a modest but significant reduction in the Tfh population in initial experiments (4.66 ± 0.29 vs 5.83 ± 0.26%, respectively; p=0.022); however, this was not borne out in repeated experiments, with one showing an increase in Tfh cells in aNotch-2-treated mice (6.2 ± 0.74 vs 4.08 ± 0.18%, respectively; p=0.02; graph not shown), while the third showed no difference between either group (4.98 ± 0.24 vs 3.85 ± 1.8%, respectively; p=0.66; graph not shown).

The Tfh population was also identified according to the phenotype CD4⁺B220⁻CXCR5⁺ICOS⁺: no significant difference was detected between groups (5.1 ± 1.9 vs 4.03 ± 1.18, respectively; p=0.65; graph not shown), although it must be acknowledged that this staining combination was performed on one experiment only.
Figure 5.8. The effect of Notch-2 blockade on the splenic Tfh population

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and the spleens harvested; a naïve mouse is also shown for comparison. The splenocytes isolated were examined for the frequency of CD4+ T follicular helper cells (Tfh; defined as CD4+B220−CXCR5+PD1+) by flow cytometry; *p<0.05; n ≥ 3 per group
5.3.5 Effect of Notch-2 blockade on secretion of pro-inflammatory cytokines

Firstly, splenocytes isolated on day 8 post-transplant from WT B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were stimulated ex vivo with irradiated donor-type splenocytes and examined for cytokine production using ELISPOT assays for Granzyme B, IFNγ, IL-4 and IL-6. Full details are provided in Chapter 2, Materials & Methods, section 2.8.

As shown in Figure 5.9, treatment with aNotch-2 resulted in a modest, but significant, reduction in the secretion of Granzyme B (297.0 ± 9.34 vs 384.3 ± 36.84 spots per 0.5 x 10^6 splenocytes in IgG-treated controls; p=0.0445; n ≥ 3 per group; Figure 5.9 A), although this was not replicated in a duplicate experiment (308.0 ± 58.00 vs 321.0 ± 38.68 spots per 0.5 x 10^6 splenocytes in IgG-treated controls; p=0.857; graph not shown). Levels of secreted IFNγ, however, were consistently significantly lower in splenocytes isolated from aNotch-2-treated mice (634.0 ± 76.64 vs 1301 ± 0.58 spots per 0.5 x 10^6 splenocytes in IgG-treated controls; p=0.0007; n ≥ 3 per group; Figure 5.9 B).

There was no significant difference in the secretion of either IL-4 (87.00 ± 9.772 vs 136.0 ± 28.92 spots per 0.5 x 10^6 splenocytes in IgG-treated controls; p=0.1272; n ≥ 3 per group; Figure 5.9 C) or IL-6 (176.5 ± 22.50 vs 255.0 ± 43.71 spots per 0.5 x 10^6 splenocytes in IgG-treated controls; p=0.2746; n ≥ 3 per group; Figure 5.9 D).
Figure 5.9. Treatment with aNotch-2 reduces secretion of inflammatory cytokines in response to allostimulation

Splenocytes isolated on day 8 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were stimulated ex vivo with irradiated donor-type splenocytes and examined for cytokine production using ELISPOT assays for Granzyme B (A), IFNγ (B), IL-4 (C) and IL-6 (D); *p<0.05, ***<0.001; n ≥ 3 per group.
Secondly, mixed lymphocyte reactions were established, in which $1 \times 10^6$ splenocytes isolated from IgG- and aNotch-2-treated mice on day 8 post-transplantation were incubated with equal numbers of irradiated donor-type (BALB/c) splenocytes. Cell-free supernatants were collected after 48 hours of culture, and were then examined for their concentration of individual cytokines using Luminex. Samples were tested in duplicate wells, and the average value used. Full details are provided in Chapter 2, Materials & Methods, sections 2.10 and 2.17.1.

As shown in Figure 5.10, supernatants collected from aNotch-2-treated mice demonstrated significantly lower levels of pro-inflammatory cytokines compared to IgG-treated controls, including TNF$\alpha$ (21.44 ± 0.73 vs 34.30 ± 4.856 pg/mL; p=0.0397), IFN$\gamma$ (1720 ± 283.3 vs 9041 ± 2813 pg/mL; p=0.0412), IL-1$\beta$ (12.34 ± 0.86 vs 22.78 ± 1.71 pg/mL; p=0.0016) and IL-17 (7.379 ± 5.66 vs 49.85 ± 14.58 pg/mL; p=0.02); the secretion of IL-6 was also reduced, although this did not reach significance (184.4 ± 39.50 vs 369.7 ± 112.3 pg/mL; p=0.17).

There was no difference in the levels of secreted IL-2 (52.11 ± 3.15 vs 45.26 ± 4.21 pg/mL; p=0.241), IL-13 (14.36 ± 2.19 vs 17.49 ± 3.16 pg/mL; p=0.35) or IL-4 (34.17 ± 8.61 vs 37.22 ± 8.55 pg/mL; p=0.81). The levels of IL-10, however, were significantly reduced in aNotch-2-treated mice compared to controls (137.5 ± 16.22 vs 225.6 ± 26.72 pg/mL; p=0.03).
Figure 5.10. Levels of pro-inflammatory cytokines are reduced upon Notch-2 inhibition

Cell-free supernatants collected following the incubation of splenocytes isolated from IgG- and aNotch-2-treated mice with irradiated donor-type splenocytes were tested for the levels of various secreted cytokines using Luminex. Samples were tested in duplicate wells, and values averaged; *p<0.05 **p<0.01
5.3.6 Notch-2 blockade inhibits T2 B cells but increases follicular B cells

As discussed earlier, following the emergence of immature B cells from the bone marrow, they home to the spleen, where they progress through two transitional B cell stages, type 1 (T1) and type 2 (T2), before developing into the main peripheral mature B cell subsets, marginal zone B cells and follicular B cells, and, from there, to plasma cells.

The T1, T2 and FO subsets were identified by the expression of the surface marker B220, and thereafter according to their relative expression of CD21 and CD24 (260): T1 cells were classified as B220\(^+\)CD24\(^{hi}\)CD21\(^{lo/int}\), T2 cells as B220\(^+\)CD24\(^{hi}\)CD21\(^{int/hi}\), and mature FO B cells as B220\(^+\)CD24\(^{int}\)CD21\(^{int}\).

As shown in Figure 5.11, there was no significant difference in the proportion of T1 B cells in aNotch-2-treated mice compared to IgG-treated controls (11.04 ± 0.76 vs 11.56 ± 1.07%, respectively; p=0.7). The T2 population, however, was significantly reduced in aNotch-2-treated mice (6.813 ± 0.61 vs 17.70 ± 2.91%, respectively; p=0.0079), while there was a significant increase in the proportion of mature FO B cells (78.73 ± 1.36 vs 63.83 ± 1.3%, respectively; p=0.0006).

The absolute numbers of these cell subsets were also calculated and revealed changes mirroring the percentages of these populations: a similar number of T1 B cells was found in aNotch-2-treated mice compared to IgG-treated controls (3829000 ± 516935 vs 3221000 ± 541018, respectively; p=0.447; graph not shown); there was a reduction in the number of T2 B cells (2369000 ± 363431 vs 5476000 ± 1163000, respectively; p=0.0435; graph not shown); and finally, the absolute number of FO B cells was increased in aNotch-2-treated mice (26840000 ± 1369000 vs 19050000 ± 2450000, respectively; p=0.0322; graph not shown).
Figure 5.11. Effect of Notch-2 inhibition on B cell development and differentiation

Splenocytes isolated on day 8 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were examined for their proportions of the T1, T2 and FO subsets, according to the expression of B220, and thereafter, the relative expression of both CD21 and CD24. Representative example of three repeated experiments shown; n ≥ 3 per group; *p<0.05, ***p<0.001.
5.3.7 The proliferation of FO, T1 and T2 B cells is significantly reduced upon Notch-2 blockade

Having determined the effect of aNotch-2 on the proportion of the T1, T2 and FO B cell subsets, the rates of proliferation therein were next investigated; as previously described in Section 4.3.11, the individual populations were examined for their expression of Ki67.

As shown in Figure 5.12, there was reduced proliferation across all three subsets in mice treated with aNotch-2. Within the T1 cell population, the proliferation was reduced by approximately 60%, although this did not reach statistical significance (13.45 ± 0.05 vs 33.87 ± 5.75%, respectively; p=0.07).

Interestingly, despite the diametrically opposite effects of Notch-2 inhibition on the number and proportion of T2 and FO B cells, there was a significant reduction in cellular proliferation within both subsets in mice treated with aNotch-2 compared to IgG-treated controls (10.80 ± 0.7 vs 19.67 ± 1.19% and 0.75 ± 0.17 vs 3.42 ± 0.32%, respectively; p=0.0119 and p=0.0087).
Figure 5.12. The proliferation of FO, T1 and T2 B cells is significantly reduced upon Notch-2 blockade

Splenocytes isolated on day 8 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were examined for their proportions of the T1, T2 and FO subsets, as previously described, and thereafter for their expression of Ki67 (A & B); *p<0.05, **p<0.01.
5.3.8 Inhibition of Notch-2 results in loss of the marginal zone B cell subset

MZ B cells mediate rapid antibody responses to blood-borne antigens in both a T cell-dependent (105) and -independent manner (106). Compared to FO B cells, they have been shown to be partially activated, express higher basal levels of costimulatory molecules and develop into plasma cells more rapidly following activation (107, 108); furthermore, they are far superior to FO B cells in inducing naïve CD4^+ T cell expansion (109).

The marginal zone B cell population was identified by first gating on B220^+ splenocytes, and thereafter according to their expression of CD21 and CD1d (B220^+CD21^{hi}CD1d^{hi}).

As shown in Figure 5.13, use of aNotch-2 effected a marked reduction in the marginal zone population, essentially resulting in complete absence of the cell subset (0.898 ± 0.076 vs 11.27 ± 0.26% in IgG-treated controls; p<0.0001; n ≥ 3 per group; Figure 5.13 A & B).

The absolute number of marginal zone cells was also calculated, and revealed a corresponding, significant reduction in the number of these cells in aNotch-2-treated mice (311926 ± 47232 vs 3389000 ± 459451, respectively; p=0.006; Figure 5.13 C).
Figure 5.13. The splenic marginal zone B cell population is near-completely eliminated upon Notch-2 blockade

Splenocytes isolated on day 8 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were examined for the marginal zone B cell population, according to the expression of B220, and thereafter, the relative expression of both CD21 and CD1d; both the proportion (A & B) and absolute number (C) of this population are shown. Representative example of three repeated experiments shown; n ≥ 3 per group; ***p<0.001.
5.3.9 The proportion of plasma cells in both spleen and bone marrow is reduced upon inhibition of Notch-2

As discussed previously, plasma cells, a subset containing both short- and long-lived B cells (96, 97) that develop in the periphery following the activation of mature marginal zone or follicular B cells, are also known to return to populate the bone marrow (98, 99). They are highly effective antibody-producing cells: indeed, plasma cells have been shown to secrete antibodies at rates of up to 10,000 molecules per second per cell (307). Given the alteration in both the MZ and FO B cell populations, the downstream effect of this on the plasma cell populations was investigated. Plasma cells do not express the surface marker B220 but highly express the surface marker CD138, also known as Syndecan-1.

As shown in Figure 5.14, the proportion of splenic plasma cells was significantly, albeit modestly, reduced in aNotch-2-treated mice compared to IgG-treated controls (0.4630 ± 0.033 vs 1.717 ± 0.298%, respectively; p=0.0472; Figure 5.14 A & B). There was also a significant reduction in the percentage of plasma cells identified within the bone marrow in aNotch-2-treated mice (0.1985 ± 0.004 vs 0.2990 ± 0.013%, respectively; p=0.0103; Figure 5.14 C & D). However, this latter reduction was not seen in a duplicate experiment (0.709 ± 0.04 vs 0.598 ± 0.0312%, respectively; p=0.11; n ≥ 3 per group; graph not shown).
Figure 5.14. The proportion of plasma cells in both spleen and bone marrow is reduced upon inhibition of Notch-2

WT B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were sacrificed on day 8 post-transplant, and the splenocytes and bone marrow isolated. The proportion of plasma cells identified by flow cytometry using the cell surface markers B220\(^*\)CD138\(^+\) in both the spleen (A & B) and bone marrow (C & D) are shown; \(*p<0.05\)
5.3.10 Notch-2 blockade inhibits the regulatory B cell subset

Recognition of the regulatory B cell (Breg) population remains a relatively recent discovery, while the combination of phenotypic markers used to identify Bregs is somewhat variable. In this study, Bregs were initially identified as B cells expressing CD5⁺CD1d⁺, a population thought to identify most IL-10-producing Bregs, including many previously attributed to the MZ or T2-MZ subsets (80, 308). As regulatory B cells have more recently been identified by the expression of TIM-1 (309), later experiments were expanded to include this population.

As shown in Figure 5.15 A & B, use of aNotch-2 resulted in a significant reduction in the CD5⁺CD1d⁺ Breg population compared to IgG-treated controls (2.045 ± 0.155 vs 5.567 ± 0.558, respectively; p=0.017)

The population of B220⁺ TIM-1⁺ cells was also examined. Again, there was a significant reduction in the size of this subset in mice treated with aNotch-2 (7.550 ± 1.61 vs 15.10 ± 0.72%, respectively; p=0.0156). Although it must be noted that this staining combination was performed on only one set of these mechanistic experiments, it serves to reinforce the data provided by the earlier experiments examining the CD5⁺CD1d⁺ population.
Figure 5.15. Notch-2 blockade inhibits the CD5⁺CD1d<sup>+</sup> Breg population

Splenocytes isolated on day 8 post-transplant from B6 mice recipient of a BALB/c cardiac allograft and treated with either IgG or aNotch-2, as previously described, were examined for their expression of B220, and thereafter, the relative expression of both CD5 and CD1d (A & B). Representative example of three repeated experiments shown; n ≥ 3 per group; *p<0.05.
5.3.11 Treatment with aNotch-2 results in near-total inhibition of donor-specific antibodies

To determine potential down-stream sequelae of the altered B cell profile seen in aNotch-2 treated mice, particularly in the MZ B and plasma cell populations, levels of donor-specific antibodies in both IgG- and aNotch-2-treated mice were evaluated. As per the previous Notch-2 mechanistic studies, WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8. Serum was isolated by centrifuging whole blood at room temperature for 20 minutes at 14000 rpm; detailed methods for serum preparation are as described in Section 2.6.5. The serum was then serially diluted (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024) and incubated with naïve donor-type (BALB/c) splenocytes; the methods are described in detail in Section 2.9. Negative control serum obtained from naïve BALB/c and WT B6 mice was similarly prepared, diluted and incubated with BALB/c splenocytes. The splenocytes were stained for surface expression of IgG1 and IgG2a, and counter-stained with CD4 and B220. Lymphocyte gates were determined using the FlowJo analysis program by forward and side scatter characteristics, and were then further identified according to the CD4 and B220 counterstain.

Levels of DSA, as determined by the percentage of B220\(^+\) or CD4\(^+\) cells expressing IgG1 and IgG2a, are shown in Figure 5.16. Mice treated with aNotch-2 showed near-total inhibition of DSA, as shown by both the percentage of B220 and CD4 cells expressing IgG1 or IgG2a, while the IgG-treated mice showed levels approaching 100% at the 1:4 dilution, declining thereafter. The percentages are also listed separately in Table 5.1.
Figure 5.16. Treatment with aNotch-2 results in near-total inhibition of donor-specific antibodies

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and serum isolated. Levels of DSA, as determined by the percentage of B220^+ or CD4^+ cells expressing IgG1 and IgG2a, are shown above: IgG-treated mice (blue circles) are compared to aNotch-2-treated mice (red squares); levels from negative control mice (naïve BALB/c and naïve WT B6; black circles and black squares, respectively) are also shown for comparison. Percentages of B220 cells positive for IgG1 (A) and IgG2a (B) and CD4 cells positive for IgG1 (C) and IgG2a (D). A representative example from two separate experiments is shown.
Table 5.1. Percentages of B220 cells positive for IgG1 (A) and IgG2a (B) and CD4 cells positive for IgG1 (C) and IgG2a (D) in diluted serum samples from IgG- and aNotch-2-treated mice

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Table 5.1. Percentages of B220 cells positive for IgG1 (A) and IgG2a (B) and CD4 cells positive for IgG1 (C) and IgG2a (D) in diluted serum samples from IgG- and aNotch-2-treated mice

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5.3.12 Notch-2 blockade selectively inhibits the secretion of IgG and IgM

To determine if there was any selective inhibition in the subtype of antibody resulting in the reduced levels of DSA seen in aNotch-2 treated mice, levels of IgG subclasses, IgM and IgA were evaluated in both IgG- and aNotch-2-treated mice. As per the previous Notch-2 mechanistic studies, WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8. Serum was isolated by centrifuging whole blood at room temperature for 20 minutes at 14000 rpm; detailed methods for serum preparation are described in Section 2.6.5.

The serum was then assayed for levels of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA using individual ELISA kits, purchased from Life Diagnostics, Inc. The standards, sample dilutions and enzyme conjugate reagent required for each kit varied and were prepared as described in Section 2.16.2. The optical density of each well was read within 5 minutes at 450 nm using a microplate reader. A standard curve was generated and the concentration multiplied by the dilution factor of each sample (all reported as ng/ml). The duplicate readings for each standard and sample were averaged.

Compared to treatment with IgG, use of aNotch-2 leads to a significant decrease in the serum levels of IgG1 (647903 ± 110486 vs 308621 ± 34345 ng/ml, respectively; p=0.0216), IgG2a (85100 ± 22949 vs 12293 ± 3091 ng/ml, respectively; p=0.0127), IgG2b (147354 ± 24176 vs 62069 ± 8365 ng/ml, respectively; p=0.008), IgG3 (325596 ± 38698 vs 121869 ± 10924 ng/ml, respectively; p=0.0003) and IgM (212818 ± 24949 vs 78045 ± 17544 ng/ml, respectively; p=0.0007); the levels of IgA (36629 ± 6133 vs 26877 ± 6145 ng/ml, respectively; p=0.28) were unaffected.
Figure 5.17. Notch-2 blockade selectively inhibits IgG subclasses and IgM
WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and serum isolated. Serum levels of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA were determined using a quantikine ELISA, performed as detailed in Section 2.16.2. The combined results of three experiments are shown; n ≥ 3 per group per experiment.
5.3.13 Notch-2 inhibition only partially prevents the development of DSA when administered following intra-peritoneal allo-sensitisation

Following the DSA experiments in the solid organ (cardiac) transplant model, I attempted to determine whether delayed administration of aNotch-2 could prevent or inhibit the development of previously formed or existing DSA. As the cardiac transplant model is so stringent, delayed administration was not thought to be feasible as rejection in this model occurs at approximately days 6 – 7. Therefore, a decision was made to use an alternative allo-sensitisation model, in which 15 x 10⁶ BALB/c splenocytes were injected intraperitoneally into naïve WT B6 mice. Mice were treated with either IgG or aNotch-2 on days 0, 3, 5, 7 & 10 post-sensitisation and were sacrificed on day 14. A third group of mice was sensitised at the same time as the initial two groups of mice but received no treatment until day 14, at which point they were treated with aNotch-2 on days 14, 17, 19, 21 & 24; they were sacrificed on day 28. Confirmation of the effectiveness of intraperitoneal allo-sensitisation was made using an IFNγ ELISPOT (data not shown).

The serum was prepared, serially diluted (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512) and incubated with naïve donor-type (BALB/c) splenocytes as previously described; the methods are listed in detail in Section 2.9. Negative control serum obtained from naïve BALB/c and WT B6 mice was similarly prepared, diluted and incubated with BALB/c splenocytes. The splenocytes were again stained for surface expression of IgG1 and IgG2a, and counterstained with B220. Lymphocyte gates were determined using the FlowJo analysis program by forward and side scatter characteristics, and were then further identified according to the B220 counterstain.

As shown in Figure 5.18, the levels of DSA measured in the IgG-treated mice were similar to those seen in the cardiac transplant model, again confirming the intra-peritoneal injection of donor splenocytes as a true sensitising event.
However, the levels of DSA measured in both the early and late aNotch-2 treatment groups were significantly higher than those measured in aNotch-2-treated recipients of a cardiac transplant: levels of IgG1\(^+\) B220 lymphocytes were similar to those measured in IgG-treated recipients until the 1:64 dilution, and were thereafter less; levels of IgG2a\(^+\) B220 lymphocytes were significantly lower than IgG-treated controls in the early aNotch-2 treatment group at all dilutions, while the late aNotch-2 treatment group demonstrated lower levels from the 1:64 dilution onwards.
Figure 5.18. Administration of Notch-2 on either an early or delayed dosing schedule only partially inhibits the development of DSA following intra-peritoneal sensitisation

(A) Percentage of B220⁺ lymphocytes expressing IgG1; (B) percentage of B220⁺ lymphocytes expressing IgG2a.
Table 5.2. Percentages of B220 cells positive for IgG1 (A) and IgG2a (B) in diluted serum samples from IgG- and aNotch-2-treated mice, administered according to either a standard (early) or delayed dosing schedule

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5.3.14 Notch-2 blockade leads to increased levels of soluble BAFF

Given the documented role of BAFF in B cell development, as discussed in Section 1.3.2, the effect of the altered B cell profile consequent to Notch-2 blockade on BAFF levels was investigated.

Using serum isolated from IgG- or aNotch-2-treated mice on day 8 post transplantation, levels of BAFF were measured using a quantikine ELISA, performed as detailed in Section 2.16.1.

As shown in Figure 5.19, use of aNotch-2 leads to a significant increase in the serum levels of BAFF (20881 ± 456.2 pg/ml) compared to both mice treated with IgG (11222 ± 850.7 pg/ml; p<0.0001) and a naïve mouse (1944.2 pg/ml).

**Figure 5.19. Notch-2 blockade leads to increased levels of soluble BAFF**

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8 and serum isolated. Serum levels of BAFF were determined using a quantikine ELISA. A representative example from three separate experiments is shown. ***p<0.001
5.3.15 Inhibition of Notch-2 increases the proportion of peripheral Tregs

As BAFF has also been shown to be important in Treg survival, it was thought possible that Notch-2 inhibition, with its demonstrated attendant increase in BAFF levels, would lead to downstream effects on the Treg population; the proportion of peripheral Tregs in both IgG- and aNotch-2-treated mice was therefore examined on day 8 post-transplantation.

As previously, peripheral Tregs were identified as the population of CD4$^+$ splenocytes expressing CD25 and Foxp3. Indeed, as shown in Figure 5.20, there was a significant increase in the splenic Treg population in mice treated with aNotch-2 compared to those treated with IgG (11.4 ± 0.1 vs 8.46 ± 0.46%, respectively; p=0.0165).

![Figure 5.20. Inhibition of Notch-2 increases the proportion of peripheral Tregs](image)

WT B6 mice recipient of an allogeneic (BALB/c) cardiac graft and treated with either IgG or aNotch-2 on days 0, 3, 5, & 7 post-transplant were sacrificed on day 8. The splenocytes isolated were examined for the frequency of CD4$^+$ regulatory T cells (Treg; defined as CD4$^+$CD25$^+$FoxP3$^+$) by flow cytometry. Representative example of two repeated experiments shown; *p<0.05
5.3.16 Secreted levels of IL-21 were unaffected by Notch-2 blockade

IL-21 is known to be important in the regulation of both innate and adaptive immune responses; from a B cell perspective, it has a major role in driving terminal B cell differentiation to plasma cells and in the production of immunoglobulins (310). Efforts were therefore undertaken to investigate if inhibition of Notch-2 was associated with dysregulation of IL-21 secretion.

MLRs composed of splenocytes isolated from IgG- or aNotch-2-treated recipients on day 8 post transplant and incubated with irradiated donor-type (BALB/c) splenocytes were established; cell free supernatants were collected after 48 hours and were examined for levels of IL-21 using Luminex. As shown in Figure 5.21 B, there was no significant difference between levels found in aNotch-2-treated mice compared to controls (3.67 ± 0.76 vs 2.77 ± 0.89 pg/ml, respectively, p=0.47), although the levels in both groups were very low.

A further experiment was performed to investigate the levels of secreted IL-21 in serum: the levels therein were essentially undetectable (data/graph not shown). However, it must be noted that this is representative of one experiment only, and it was thought likely that this was attributable to a technical problem with the assay.
Figure 5.21. Notch-2 inhibition does not influence the secretion of IL-21
(A) 5-parameter log standard curve generated from the IL-21 Luminex assay.
(B) Levels of IL-21 in cell-free supernatants harvested from MLRs composed of
splenocytes from IgG- or aNotch-2-treated recipients on day 8 post transplant
incubated with irradiated donor-type (BALB/c) splenocytes for 48 hours. All
wells tested in duplicate; n = 4 per group.
5.3.17 Effect of B cell depletion on allograft survival in recipients subsequently treated with aNotch-2

As previously shown, Notch-2 inhibition significantly affects the development and maintenance of B cell subsets, particularly marginal zone B cells, while also exerting effects on certain T cells subsets. To try and determine which was leading to the prolongation of graft survival, mice underwent B cell depletion prior to transplantation using aCD20 and were subsequently treated with either IgG or aNotch-2, according to the protocol below. Survival was also compared to mice treated with aNotch-2 alone.

1. aCD20: 200 µg on day -1; IgG: 5 mg/kg on days 0, 3, 5, 7, 9 and 11
2. aCD20: 200 µg on day -1; aNotch-2: 5 mg/kg on days 0, 3, 5, 7, 9 and 11

![Graph showing survival rates](image)

**Figure 5.22.** The administration of a single dose of aCD20 prior to transplantation does not significantly improve graft survival in mice subsequently treated with aNotch-2

Graft survival in WT B6 mice recipient of a BALB/c cardiac allograft treated with either a single dose of aCD20 (200 µg on day -1 pre-transplant), aNotch-2 (standard regimen) or a combination of the two regimens.
Although not shown above, there was no significant difference in the survival of mice treated with IgG alone compared to those who were treated with both aCD20 and IgG (MST 7 days vs 7 days; p=0.7; n ≥ 4 per group). While the BALB/c → B6 cardiac transplant model is stringent and rejection known to be predominantly T-cell-mediated in the early post-transplant period, previous work has shown that maximal B cell depletion was achieved 3 days following administration of aCD20 (J.S. Obhrai, Department of Medicine, Oregon Health & Science University; unpublished data). Therefore, to ensure that there had been sufficient B cell depletion, a second trial was undertaken with the addition of a second dose of aCD20 prior to transplantation, administered according to the protocol below:

1. aCD20: 200 µg on days -4, -1; IgG: 5 mg/kg on days 0, 3, 5, 7, 9 and 11
2. aCD20: 200 µg on days -4, -1; aNotch-2: 5 mg/kg on days 0, 3, 5, 7, 9 and 11

Using this protocol, use of aCD20 significantly prolonged graft survival when compared to IgG alone (MST 10 days vs 7 days, respectively; p=0.042; Figure 5.23), indicating efficacy of the B cell depletion regimen and, furthermore, providing evidence of B cell involvement in the early post-transplant alloimmune response.

Interestingly, when aCD20 was combined with aNotch-2, the effect was highly significant, both compared to aNotch-2 alone and to the combination of aCD20 + IgG (MST 19 days vs 12 days and MST 19 days vs 10 days, respectively; p=0.002 and p=0.0029; Figure 5.23).
Figure 5.23. The administration of two doses of aCD20 prior to transplantation significantly improves graft survival in mice subsequently treated with aNotch-2 compared to treatment with aNotch-2 alone.

Graft survival in WT B6 mice recipient of a BALB/c cardiac allograft treated with either aCD20 (200 µg on days -4, -1 pre-transplant), aNotch-2 (standard regimen) or a combination of the two regimens.
5.3.18 Timeline

To determine the onset of effect of Notch-2 inhibition, the effect of aNotch-2 on the marginal zone B cell subset was examined at 12, 24, 48 and 72 hours following administration of a single 5mg/kg dose. Mice recipient of aNotch-2 were compared both to a naïve mouse, and to mice recipient of a single dose of IgG at the same timepoints.

As shown in Table 5.3 and Figure 5.24, the effect of administration of aNotch-2 can be seen as early as 24 hours, and is near-complete at 72 hours. For ease of comparison, only the 12 hour IgG control population is shown graphically in Figure 5.24 B; however, the statistical comparisons detailed below are made with their appropriately timed controls. A 1-way ANOVA was also performed to compare differences between all groups, and revealed a p=0.0266.

Table 5.3. Percentages of MZ B cells isolated at varying timepoints (indicated) following a single dose of IgG Control or aNotch-2

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<td>24 hours</td>
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<tr>
<td>48 hours</td>
<td>4.03 ± 0.04%</td>
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<td>72 hours</td>
<td>5.11 ± 0.49%</td>
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Figure 5.24. Timeline showing effect of aNotch-2 on marginal zone B cells
12, 24, 48, and 72 hours following administration of a single dose
*p<0.05 **p<0.01
5.4 Discussion

To my knowledge, this is the first report of the effect of selective Notch-2 inhibition using an antibody-mediated approach in the setting of solid organ transplantation, and, as for Notch-1, shows that Notch-2 plays a crucial role in alloimmunity, particularly with respect to the B cell response and antibody secretion.

B cells play a critical role in the adaptive immune response, with principal functions of antigen presentation, antibody formation and the generation of memory subsets; recently, a subset of B cells with suppressor functions has also been described (80, 81), which is thought to be important in allograft tolerance. The development of de novo anti-HLA antibodies post-transplant has been shown to be associated with allograft dysfunction, rejection and allograft loss, particularly where those antibodies are directed against donor HLA (311-314), although it is now clear that B cells also contribute to the development of chronic rejection independent of antibody production, by supporting T cell responses through antigen presentation and maintenance of lymphoid architecture (306).

Although the treatment of ABMR is usually multi-faceted (142), involving strategies to remove or neutralise existing antibodies and prevent further antibody secretion using B cell depleting agents (e.g., anti-CD20 or rituximab), until recently, there was no treatment to mitigate the contribution of plasma cells to the B cell response: as plasma cells do not express the surface marker CD20, they fail to be inhibited by rituximab. Recruitment of the proteasome inhibitors (e.g., bortezomib), which can inhibit plasma cells, has expanded our therapeutic arsenal, although at present these agents are predominantly being used in a limited, rescue capacity.
While expression of Notch-2 has been demonstrated on B cells at varying stages of development, Notch-2 signaling has definitively been shown to be critically involved in the development of marginal zone B cells: mice conditionally deficient in Notch-2 were noted to have a markedly diminished MZ B cell population, and, furthermore, a significant reduction in the T2 population (104). Although determination of the expression and involvement of the Notch ligands in normal B cell development has been challenging, the ligand involved in MZ B cell development has been shown to be DLL1: mice conditionally deficient in DLL1 display the same MZ B cell-deficient phenotype as that seen in Notch-2-deficient mice (206), as do mice lacking Mib-1, an E3 ligase known to regulate DLL1 endocytosis (230). Furthermore, activation of FO B cells ex vivo was enhanced in the presence of DLL1, while the production of IgG1+ cells was also increased (231).

The functional characteristics of these B cell subsets make them a very attractive target in attempts to manipulate the B cell contribution to alloimmunity. To date, however, there have been no studies examining the effect of inhibition of Notch-2, and the potential downstream disruption to normal B cell development, on the alloimmune response.

In this study, inhibition of Notch-2, achieved by use of the novel, blocking aNotch-2 antibody, was first shown to effect a significant prolongation in graft survival in the fully MHC-mismatched heterotopic cardiac transplant model.

MZ B cells have been shown to mediate rapid antibody responses in both a T cell-dependent (105) and -independent manner (106), thought to be partially due to their location in the splenic marginal zone, a point at which they are uniquely positioned to interact with blood-borne antigens (101). Compared to FO B cells, they have been shown to be partially activated, express higher basal levels of costimulatory molecules and develop into plasma cells more rapidly following activation (107, 108); furthermore, they are far superior to FO
B cells in inducing naïve CD4$^+$ T cell expansion (109). As discussed above, a lack of Notch-2 signaling, achieved previously by conditional genetic deficiencies, has been shown to critically interfere with the development of MZ B cells. In this study, the achievement of Notch-2 blockade by use of aNotch-2 was equally effective in interfering with MZ B cell development; indeed, as mentioned previously, the effect on this subset was so striking and so consistent that it acted as a useful marker of satisfactory delivery of the antibody.

Downstream of this effect on the marginal zone B cell population was a significant reduction in the splenic plasma cell population with an attendant, although less consistent, reduction in the percentage of bone marrow plasma cells. The only agent currently in clinical use with the ability to inhibit the plasma cell population is the proteasome inhibitor bortezomib. In both mouse and human studies (315, 316), use of bortezomib was shown to significantly reduce the population of alloantibody-secreting plasma cells, in some cases to a degree sufficient to allow transplantation in patients with previously prohibitively positive B cell crossmatches (316); it must be stressed, however, that its use as a desensitising agent pre-transplant has only occurred in isolated, pilot cases. While bortezomib has been shown to be effective in reducing the plasma cell population, it fails to inhibit the MZ B cell population (315), which, as previously discussed, is also a highly efficient source of antibody production.

Indeed, administration of aNotch-2 resulted in a reduction in the development of DSA to an extent commensurate with the reduction in the MZ B cell compartment: mice recipient of a fully MHC-mismatched cardiac allograft and treated simultaneously with aNotch-2 displayed levels of B220$^+$ IgG1$^+$ and IgG2a$^+$ DSA approaching those seen in naïve mice. Interestingly, the use aNotch-2 in an alternative sensitisation model, in which B6 mice were injected i.p. with BALB/c splenocytes, failed to inhibit the development of DSA, irrespective of whether treatment was administered simultaneously or on a
delayed schedule, perhaps suggesting that the either the difference in antigenic composition, antigenic load, or manner of antigen presentation may be important. It therefore remains unclear whether treatment with aNotch-2 can inhibit the secretion of pre-existing antibodies. Clearly, while the achievement of inhibition or reduction in the secretion of de novo anti-HLA antibodies is highly desirable in transplantation, a treatment that inhibits the secretion of pre-existing antibodies may be useful as a desensitising agent but pose additional immunosuppressive risk. Further investigation of the Ig subclasses affected revealed that treatment with aNotch-2 selectively inhibited IgG and IgM, and did not affect the levels of secreted IgA. It is thought that the most active plasma cells, those secreting the highest amounts of immunoglobulin, are the most sensitive to the effects of bortezomib (317); as the acute humoral response to transplantation is likely characterised by the greatest activity in IgM and IgG-secreting cell subsets, it is possible that this renders them similarly most sensitive to the effects of aNotch-2.

Perhaps unsurprisingly given the disruption to the latter stages of B cell development, there was a significant increase in the secreted levels of BAFF following inhibition of Notch-2. BAFF is known to be important for the development and survival of both transitional B cells and mature B cells. Where BAFF is present in excess, autoreactive B cells can mature into follicular B cells, leading to development of B cell-mediated autoimmune disease (115, 116), although, in contrast, an excess of BAFF signaling has also been shown to result in Treg expansion with consequent prolongation of graft survival (122). Crucially, however, there was no evidence of autoimmune disease in mice treated with aNotch-2 despite the increased secretion of BAFF, likely due to the downstream inhibition of B cell development and activity. Interestingly, though, there was an increase in the Treg population, which may be attributable to the higher levels of BAFF in the groups treated with aNotch-2. In addition to their influence on the T effector cell subsets, and effect on T cell-dependent B cell immunoglobulin secretion, Tregs have also been shown to be capable of
directly suppressing B cells (318). The ability of aNotch-2 to disrupt normal B cell development and function while maintaining the Treg population may alleviate some of the concerns in the transplant community associated with the use of anti-BAFF, and presents Notch-2 as a potentially superior target, although it must be acknowledged that the use of aNotch-2 also interfered with the Breg population.

Interestingly, the combination of aNotch-2 and aCD20 effected further prolongation of graft survival than aNotch-2 alone, reinforcing the theory that the effect of Notch-2 inhibition is mediated both by an effect on T cells and B cells. It must be noted, however, that the use of aCD20 alone in this study resulted in modest but significant prolongation of allograft survival, an effect that has not been borne out in other studies; the reasons for this are unclear.

In summary, these data reveal a crucial role for Notch-2 in B cell differentiation and function in transplantation, particularly in the production of alloantibodies, and indicate that selective blockade of Notch-2 may be a useful therapeutic strategy to prevent the alloantibody-mediated injury so resistant to current therapies. Again, the availability of a selective, human Notch-2 antibody suggests that this approach could be efficiently translated into novel strategies of immunosuppression in solid organ transplantation in humans.
Chapter Six: Summary Discussion

6.1 Discussion

In this study, I have investigated the effect of selective, discrete blockade of the Notch-1 and Notch-2 receptors in the setting of solid organ transplantation with particular focus on their roles in T and B cell development and behaviour, respectively. While previous research from our laboratory had provided evidence that the Notch ligands DLL1 and Jagged2 were involved in the alloimmune response (233, 234), this is the first study demonstrating the important, individual roles both Notch-1 and Notch-2 play therein, and reveals them to be promising, novel targets for immune modulation.

The Notch pathway has previously been identified as a target of interest for therapeutic manipulation in a variety of clinical diseases, from haematopoietic and solid organ cancers to structural cardiac and vascular anomalies (257). γ-secretase inhibitors, pan-Notch receptor inhibitors originally developed to block the production of the amyloid-β peptide in efforts to treat Alzheimer's Disease, have been predominantly trialed in the cancer setting, although progress to clinical use has been hindered by adverse on-target effects. Indeed, while use of the GSI DBZ in a bone marrow transplant model achieved some encouraging results in attenuation of the GVHD response, it resulted in rapidly fatal gastrointestinal toxicity (238). As it was thought that the severity of this toxicity may have been due, at least in part, to pre-existing intestinal injury consequent to total body irradiation (238), the first part of this study assessed the potential of pan-Notch inhibition in the solid organ transplant model. As was unequivocally shown, however, the use of DBZ in this model was not a viable therapeutic option. The focus of my project was therefore directed towards selective Notch-1 and Notch-2 blockade. The novel aNotch-1 and aNotch-2 antibodies were well tolerated by naïve mice when administered individually, and yielded some promising results in the T and B cell compartments,
respectively, justifying further investigation of their use in the heterotopic vascularised cardiac transplant model. Interestingly, concurrent use of aNotch-1 and aNotch-2 led to death in all mice treated with evidence of gastrointestinal toxicity similar to that observed in DBZ-treated mice; in keeping with previous data (240), this suggests that the majority of the gastrointestinal side-effects noted following treatment with GSIs are due to combined Notch-1 and Notch-2 inhibition, rather than Notch-3 or Notch-4 blockade.

Use of aNotch-1 alone in the cardiac transplant model engendered significant prolongation of graft survival, an effect that was associated with marked reduction of the T effector cell population and, importantly, expansion of the peripheral Treg subset. The selective, divergent nature of these effects places aNotch-1 in contrast to the majority of clinical immunosuppressive agents which result in global inhibition of the effector and regulatory T cell components of the immune system (319). As it is increasingly clear that it is the balance of these opposing subsets, rather than any absolute reductions thereof, that determines the outcome of a transplant, this immune-modulatory element could be vitally important as we seek therapeutic options that optimise long-term graft survival and permit minimisation of maintenance immunosuppression. The increase in nTregs observed following Notch-1 inhibition was shown to be due to a combination of decreased Treg apoptosis with increased Treg proliferation; furthermore, Tregs isolated from aNotch-1-treated mice were more suppressive ex vivo. These results were confirmed by selective genetic deletion of Notch-1 on Tregs which increased the proportion, proliferation and suppressive function of Tregs both in vitro and in vivo. In agreement with previous data (190, 198, 199), inhibition of Notch-1 revealed it to have a critical, non-redundant role in normal thymocyte development. Despite a clear block in DN thymocyte maturation, however, the single positive CD4+ and CD8+ thymocyte populations were preserved, indicating a bypass system; furthermore, the peripheral CD4+ and CD8+ populations (both percentages and absolute counts) were preserved. A substantial increase was observed in the thymic nTreg population upon
Notch-1 blockade; interestingly, however, the thymus was not central to the mechanism of action of aNotch-1. The thymus naturally involutes with age, with a reported rate of loss of thymic epithelium of 3% per year during adulthood; these changes are associated with perivascular expansion and progressive replacement with adipocytes and peripheral lymphocytes (320-322). Were the effects of aNotch-1 to be dependent on an intact, albeit adult, thymus, this may have had important implications for potential clinical responsiveness, given an increasingly elderly population of transplant recipients.

The final part of this project assessed the impact of Notch-2 blockade on the alloimmune response. Administration of aNotch-2 to mice recipient of a fully MHC-mismatched cardiac transplant also produced a significant prolongation of allograft survival, albeit to a lesser extent than Notch-1 blockade. In keeping with previous data that identified Notch-2 signaling as a direct regulator of cytotoxic T lymphocyte differentiation (298, 299), interruption of Notch-2 signaling in this model was associated with a reduction in T effector cells, and, furthermore, an increased Treg population. Most strikingly, however, use of aNotch-2 resulted in marked changes in the B cell subsets and downstream function thereof. Although the majority of immunosuppressive agents in clinical use target T cells rather than B cells, there is a large body of data to support the contribution of B cells to the alloimmune response: there is incontrovertible evidence that the development of donor-specific anti-HLA antibodies is detrimental to allograft outcomes (311-314), while it now known that B cells contribute to the development of chronic rejection independent of antibody production, by supporting T cell responses through antigen presentation and maintenance of lymphoid architecture (306). In this study, inhibition of Notch-2 effected near-complete loss of the marginal zone B cell subset and, interestingly, a reduction in the plasma cell population, both known to be extremely efficient sources of antibody production. Indeed, administration of aNotch-2 resulted in a highly significant reduction in the levels of donor-specific antibodies following cardiac transplantation. While the proteasome inhibitor
bortezomib, a recent recruit to the immunosuppressive repertoire, inhibits plasma cells, it does not affect the marginal zone population (315). It remains to be seen if the inhibition of Bregs noted upon Notch-2 blockade has any detrimental consequences. Interestingly, although the influence of aNotch-2 was most evident in the B cell populations, combined treatment with aNotch-2 and aCD20 effected further prolongation of graft survival than aNotch-2 alone, indicating that its effects on the T effector cell and Treg compartments were an important early effect on graft survival. These data present aNotch-2 as a possible therapeutic agent capable of interfering with T and B cell cross-talk, in addition to potentially highly clinically relevant effects on downstream cellular function.

In summary, these studies reveal a promising, novel approach for immune modulation in transplantation by selectively targeting Notch-1 and Notch-2. As with any data generated in animal models, there is a clear caveat that these results may not be directly translatable to humans. Importantly, however, the antibodies used herein are fully human IgG1 antibodies, the availability of which suggests that this approach could be efficiently developed.
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**Appendix 1**

**PROTOCOL NUMBER: 02943**

Harvard Medical Area (HMA) Standing Committee on Animals – “Established in 1907”
180 Longwood Avenue, Suite 113, Boston, MA 02115
Tel: 617-432-3192 Fax: 617-432-3169
http://www.hms.harvard.edu/orsp/ iacuc@hms.harvard.edu

**ANIMAL EXPERIMENTATION PROTOCOL**

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<th>Nader Najafian, MD</th>
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<td>Contact Person: Phone &amp; Email</td>
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**Protocol Title**
Mechanisms of allorecognition and allograft rejection

**Funding Source(s) and Grant Title(s)**
1) American Society of Transplantation; 7/1/09 to 6/30/11; Role of TIM-3/Galectin-9 Pathway in Allograft Rejection and Tolerance
2) American Society of Transplantation; 7/1/10 to 6/30/12; Novel Role of the Notch Pathway in Transplantation
3) NIH; 5P01AI041521-13; 7/1/07 to 6/30/12 The Role of Novel T Cell Costimulatory Pathways in Allograft Rejection and Tolerance
4) NIH; R01AI051559-08; 4/1/08 to 3/31/13 Role of Negative T Cell Costimulatory Pathways in Allograft Rejection and Tolerance
5) HMS; 2P01AI056296-06A1; 9/4/09 to 8/31/14 T Cell Costimulatory Pathways; Functions and Interactions (project 1)

**Funding Application Due Date And Grant Number (if known)**
As above

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This proposal has been reviewed and approved by the HMA Standing Committee on Animals

HMA Standing Committee on Animals ____________________________ Date: ____________
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Check one:

X New Protocol
Three Year Review of Existing Protocol

This proposal has been reviewed and approved by the HMA Standing Committee on Animals

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Minireview

The Role of Costimulatory Molecules in Directing the Functional Differentiation of Alloreactive T Helper Cells

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Costimulatory molecules are a heterogenous group of cell surface molecules that act to amplify or counteract the initial activating signals provided to T cells from the T cell receptor following its interaction with an antigen/major histocompatibility complex, thereby influencing T cell differentiation and fate. Although costimulation was previously thought to be indispensable for T cell activation at all stages of development, it is now known that the requirements for costimulation, and the costimulatory molecules involved, vary according to the stage of T cell differentiation. The ability to influence T cell fate is of paramount interest in the field of transplantation as we seek therapeutic options that inhibit detrimental alloimmune responses whilst simultaneously promoting allograft tolerance. As with many immune mechanisms, there is a degree of functional overlap between certain costimulatory molecules, whereas some have diametrically opposite effects on different T cell subsets despite sharing common ligands. This is a critical point when considering these molecules as therapeutic targets in transplantation, as blockade of a costimulatory pathway, although desirable in vitro, precluding an effective T cell response and promoting tolerance (4). Knowledge of the different costimulatory pathways involved in the various stages of the immune response is crucial: it is now apparent that memory T cells resist many of the therapeutic strategies employed to target naive alloimmune responses, while there is increasing interest in the feasibility of manipulating signals involved in T cell exhaustion for therapeutic benefit in chronic viral infections and malignancy. The focus of this minireview will be directed to the role of costimulation in the functional differentiation of effector T helper cells and the alloimmune response.

Introduction

Costimulatory molecules belong to three major families, namely the immunoglobulin (Ig) superfamily, the tumor necrosis factor (TNF)—TNF receptor (TNFR) superfamily and the emerging T cell Ig and mucin (TIM) domain family (Table 1); they may additionally be classified according to the nature of the signal they provide. They cannot activate T cells independently, but are critical to the functional naive T cell response, the nature of which depends upon the outcome of integration of these stimulatory or inhibitory signals (1,2). Upon activation, the naive T cell develops into an effector cell, and may subsequently develop into an effector or central memory cell (3); depending upon the nature and duration of antigenic stimulus, it may later display markers of exhaustion. Costimulation is critical to the naive T cell response: in the absence of CD28 ligation, TCR stimulation of naive T cells induces T cell anergy in vitro, precluding an effective T cell response and promoting tolerance (4). Knowledge of the different costimulatory pathways involved in the various stages of the immune response is crucial: it is now apparent that memory T cells resist many of the therapeutic strategies employed to target naive alloimmune responses, while there is increasing interest in the feasibility of manipulating signals involved in T cell exhaustion for therapeutic benefit in chronic viral infections and malignancy. The focus of this minireview will be directed to the role of costimulation in the functional differentiation of effector T helper cells and the alloimmune response.

Effector T Cells

CD4+ T cells, or T helper cells, play a central role in the determination of the adaptive immune response in the settings of autoimmunity, allergy and alloimmunity. Upon interaction with their cognate antigen via the TCR, naive CD4+ T cells can differentiate into various lineages, including the classical effector T helper type 1 (Th1) and type 2 (Th2) subsets, and the more recently identified Th17, T follicular helper (Tfh), Th9 and induced regulatory T cell populations (iTreg; 5–9; Figure 1). These subsets are functionally distinct and may be identified by the production of characteristic cytokines (Table 2). The differentiation decision is influenced by a variety of factors, including the cytokine
Role of Costimulation in T Cell Differentiation

<table>
<thead>
<tr>
<th>Costimulatory Molecule</th>
<th>Ligands</th>
<th>Family</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28</td>
<td>B7-1 (CD80); also binds CTLA-4 (4), PD-L1 (60)</td>
<td>IgG–CD28/B7</td>
<td>Constitutive–all naive CD4 and CD8 T cell subsets (4)</td>
</tr>
<tr>
<td></td>
<td>B7-2 (CD80); also binds CTLA-4 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>B7-1 (CD80; 4)</td>
<td>IgG–CD28/B7</td>
<td>Constitutive–Tregs (4)</td>
</tr>
<tr>
<td></td>
<td>B7-2 (CD80; 4)</td>
<td></td>
<td>Inducible–activated T cells (4)</td>
</tr>
<tr>
<td>ICOS</td>
<td>ICOS-L (2)</td>
<td>IgG – CD28/B7</td>
<td>Inducible (135)</td>
</tr>
<tr>
<td>PD-1</td>
<td>PD-L1 (136); also binds B7–1 (60)</td>
<td>IgG – CD28/B7</td>
<td>PD-L1: Constitutive and Inducible–APCs, T cells, Tregs, nonhematopoietic cells (4,136)</td>
</tr>
<tr>
<td></td>
<td>PD-L2 (136)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD27</td>
<td>CD70 (2)</td>
<td>TNF/TNFR</td>
<td>Constitutive–naive T, B and NK cells (2)</td>
</tr>
<tr>
<td></td>
<td>CD30L (2)</td>
<td></td>
<td>Inducible–CD70–T and B cells (2)</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 (2)</td>
<td>TNF/TNFR</td>
<td>Constitutive–Tregs (2)</td>
</tr>
<tr>
<td></td>
<td>CD40L (2)</td>
<td></td>
<td>Inducible–activated T effector/memory (2)</td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td>TNF/TNFR</td>
<td>Inducible–activated T cells, NK cells, eosinophils, platelets (2)</td>
</tr>
<tr>
<td></td>
<td>CD40 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L</td>
<td></td>
<td>TNF/TNFR</td>
<td>Inducible–activated T cells, NK cells, eosinophils, platelets (2)</td>
</tr>
<tr>
<td>OX40</td>
<td>OX40L (61)</td>
<td>TNF/TNFR</td>
<td>Constitutive–Tregs (2)</td>
</tr>
<tr>
<td></td>
<td>GITR (2)</td>
<td></td>
<td>Inducible–activated T effector cells, B cells, NK and macrophage (2)</td>
</tr>
<tr>
<td>TIM-1</td>
<td>TIM-1 (48)</td>
<td>TIM</td>
<td>Inducible–activated CD4 and CD8 T cells (31,32)</td>
</tr>
<tr>
<td></td>
<td>TIM-4 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM-2</td>
<td>H-ferritin (48)</td>
<td>TIM</td>
<td>Inducible–activated T cells (Th2), splenic B cells (48)</td>
</tr>
<tr>
<td></td>
<td>Semaphorin 4A (Sem4A; 48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM-3</td>
<td>Galectin-9 (48)</td>
<td>TIM</td>
<td>Constitutive–Tregs (52)</td>
</tr>
</tbody>
</table>

CD80 and CD86, constitutively expressed on B cells, macrophages and dendritic cells (DCs), is itself increased upon activation, promoting CD40–CD154 interactions and intensifying antigen-specific signaling. Interruption of the CD40–CD40L pathway, achieved by use of anti-CD40L monoclonal antibodies or genetic knockout mice, initially showed great promise in transplantation, significantly enhancing allograft survival and preventing acute rejection in rodents (20,21) and primates (22,23), an effect associated with inhibition of Th1 responses and deviation to a Th2 phenotype (24). Furthermore, anti-CD40L mAbs have been shown to act synergistically with other therapies including CTLA4-Ig (20), and, when given in concert with donor-specific transfusion (DST), to generate chimerism in the absence of bone marrow conditioning, leading to donor-specific tolerance (25). Interestingly, the combination of anti-CD40L with the immunosuppressive agents cyclosporin A (CsA) and methylprednisolone, but not rapamycin or mycophenolic acid, inhibited anti-CD40L mAb-induced cardiac allograft survival in a murine transplant model (26), thought to be due to marked inhibition of CD40L expression by CsA and methylprednisolone (26); in another study, use of calcineurin inhibitors or anti-IL2R were also shown to abrogate anti-CD40L-induced graft survival (27). Rapamycin, however, demonstrated marked synergy with anti-CD40L, achieving indefinite graft
survival in all recipients, with significant reduction in the frequency of alloreactive IFN-γ-secreting cells and inhibition of chronic rejection (27); this effect was not diminished by the addition of calcineurin inhibition (27). Although these interactions have clear clinical implications, efforts to develop anti-CD40L antibodies for clinical use were unfortunately hindered by the unexpectedly high incidence of thrombotic complications in both nonhuman primate studies and phase I clinical trials (28), thought to be due to the interruption of CD40L signaling on platelets (29). Efforts have now focused on the development of anti-CD40 monoclonal antibodies; early results in transplantation are promising (30).

Although not found on naive CD4+ T cells, TIM-1 is rapidly expressed upon their activation (31,32), and is capable of orchestrating the differentiation of several Th lineages depending upon the avidity with which it is engaged (33,34). Interestingly, following differentiation, TIM-1 expression is maintained by Th2, but not Th1 or Th17 cells (32,35). In a mouse model of multiple sclerosis, the use of a high-affinity agonistic anti-TIM-1 mAb enhanced both the Th1 and Th17 response (33); conversely, use of an antagonistic anti-TIM-1 antibody in a murine transplant model improved the survival of fully MHC-mismatched cardiac allografts, an effect dependent on the presence of Tregs and associated with inhibition of the alloreactive Th1 response but maintenance of the Th2 response (36). A recent study also demonstrated that use of a recombinant human TIM-1 fusion protein (TIM-1-Fc) inhibited the proliferation of TIM-4+ CD4+ cells, an effect associated with inhibition of Akt and Erk1/2 phosphorylation (37). Furthermore, TIM-1-Fc inhibited the proliferation of alloreactive CD4+ T cells and led to significant prolongation of allograft survival in a murine cardiac transplant model, with inhibition of the Th1 response but preservation of both Th2 and natural regulatory T cell (nTreg) function, effects thought to be achieved by the binding of Tim-1-Fc to a receptor other than TIM-4 (37).

CTLA-4 (CD152) is a receptor structurally related to CD28 that also binds to the ligands B7–1 and B7–2, albeit with much greater affinity (4). CTLA-4 is not constitutively expressed on conventional T cells, but is upregulated upon activation in a CD28-dependent manner (38,39). CTLA-4 directly antagonizes CD28 signaling by inhibiting Akt (40), providing negative costimulatory signals that inhibit T cell proliferation, IL2 production and cell cycle progression. Indeed, signaling through CTLA-4 inhibits both Th1 and Th2 differentiation, and is also thought to enhance the
Role of Costimulation in T Cell Differentiation

Table 2: The functions and differentiation characteristics of T helper cell lineages

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Physiological function</th>
<th>Disease state</th>
<th>Costimulatory molecules that promote lineage differentiation</th>
<th>Costimulatory molecules that inhibit lineage differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Cellular immunity against intracellular pathogens</td>
<td>Hypersensitivity</td>
<td>CD40L (18)</td>
<td>TIM-3 (47,50–52)</td>
</tr>
<tr>
<td>Viral immunity</td>
<td></td>
<td>Autoimmunity</td>
<td>TIM-1 (33)</td>
<td>CTLA-4 (41)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD28 (10)</td>
<td>PDL-1 (59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OX40 (61)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ICOS (76, 77)</td>
<td></td>
</tr>
<tr>
<td>Th2</td>
<td>Humoral immunity</td>
<td>Allergy</td>
<td>CD28 (10, 65, 66)</td>
<td>TIM-1 (33)</td>
</tr>
<tr>
<td>B cell help—regulate B cell activation and antibody class switching</td>
<td>Atopy</td>
<td></td>
<td>OX40 (61, 69, 70)</td>
<td>TIM-4 (81, 82)</td>
</tr>
<tr>
<td>Extracellular parasitic/helminthic infections</td>
<td></td>
<td></td>
<td>ICOS (76, 77, 79)</td>
<td></td>
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<tr>
<td>Th17</td>
<td>Mediate responses to extracellular bacteria and fungi</td>
<td>Autoimmunity</td>
<td>CD28 (18, 78, 87)</td>
<td>TIM-3 (51, 52)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>ICOS (78, 89, 90)</td>
<td>TIM-4 (92)</td>
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<td></td>
<td></td>
<td>TIM-1 (34)</td>
<td>CTLA-4 (87)</td>
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<td></td>
<td>CD40L (18, 93)</td>
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<td></td>
<td>OX40 (95, 96)</td>
<td></td>
</tr>
<tr>
<td>Th9</td>
<td>Helminthic infections</td>
<td>Atopy</td>
<td>CD28 (128)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>ICOS (124–126, 128)</td>
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<td>CD40L (121, 126, 129)</td>
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<td></td>
<td>OX40 (123, 124)</td>
<td></td>
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<tr>
<td>Th</td>
<td>Provide help to B cells</td>
<td>Autoimmunity</td>
<td>PD-1, PDL-1 (130, 132, 133)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CD28 (129)</td>
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<td>ICOS (124–126, 128)</td>
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<td>CD40L (121, 126, 129)</td>
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<td></td>
<td>OX40 (123, 124)</td>
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<td></td>
<td></td>
<td></td>
<td>CD28-nTregs (97–100)</td>
<td></td>
</tr>
<tr>
<td>Treg</td>
<td>Regulation/suppression of the immune response</td>
<td>Critical for maintenance of self-tolerance</td>
<td>CD28-nTregs (103)</td>
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<td></td>
<td></td>
<td></td>
<td>TIM-3 (47, 50)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>PD-1, PDL-1 (53, 113)</td>
<td>TIM-1 (52–54)</td>
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<td></td>
<td></td>
<td></td>
<td>GITR (117)</td>
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</table>

7 = Unknown.

susceptibility of effector T cells to suppression (41). Further insights into its immunoregulatory activity were provided by a recent study which demonstrated that CTLA-4 captures its ligands B7–1 and B7–2 from other cells by trans-endocytosis, leading to their degradation inside CTLA-4 expressing cells, and preventing their ligation with CD28 (42). CTLA-4-Ig, a fusion protein that contains the extracellular CTLA-4 domain and the Fc portion of IgG1, and potently inhibits CD28 signaling, was shown to be highly effective in prolonging allograft survival in multiple rodent models (43,44), although combination therapy with DST was required to induce tolerance in most models (45). Investigation of potential interactions with other immunosuppressive agents failed to demonstrate any abrogation of allograft survival following the addition of calcineurin inhibition, methylprednisolone or rapamycin (27). However, disappointing results seen upon translation of therapy into transplantation. Use of belatacept in human transplant recipients has not been without complication, with higher rates of severe acute rejection reported in the initial trials (46); as this is thought to be primarily related to effects on the Treg population, it will be discussed in more detail later.

TIM-3, originally identified on activated Th1 cells (47), is now known to be expressed by cells of both the innate and adaptive immune systems (35,48). Galectin-9, the identified ligand of TIM-3 (49), is widely expressed in various tissues, but in the nonactivated immune system, is predominantly found on CD4+25+nTregs and naive CD4+ T cells. TIM-3, in contrast, is not expressed by naive CD4+ T cells, but is expressed upon their activation and differentiation to the Th1 phenotype (47,50). The TIM-3–Galectin-9 pathway has been shown to act as a negative T cell costimulatory pathway: Galectin-9 is upregulated by IFN-γ, and upon interaction with TIM-3, preferentially inhibits Th1 cells by promoting cell death, thought to be due to a combination of apoptosis and necrosis (49). TIM-3 blockade accelerates Th1-mediated autoimmune disease (50); conversely, TIM-3 has been shown to negatively regulate the secretion of Th1 and Th17 cytokines in human studies, but, in contrast to murine TIM-3, does not appear to induce cell death (51). Our group has previously shown that TIM-3 blockade, achieved by use of a blocking antibody RMT3–23, accelerates allograft rejection in murine transplant models in the absence of CD80/CD86 or CD28 signaling (52), an effect both dependent on the presence of CD4+ T cells and associated with expansion of the allospecific CD4+ T cell population (52). TIM-3 blockade was also associated with
enhanced Th1 polarization and increased frequencies of alloreactive IFN-γ-, IL-17-, IL-6- and granzyme B-producing cells (52).

There is a significant body of data to demonstrate that the PD-1–PDL1 pathway is centrally involved in the alloimmune response, and acts to promote engraftment (53–55). PD-1, PDL1 and PDL2 are prominently expressed in acutely rejecting cardiac allografts (55), although there is evidence that each plays a distinct role: blockade of either PD-1 or PDL1 accelerated allograft rejection in CD28−/− mice recipient of a fully MHC-mismatched cardiac allograft (56), but only PDL1 blockade precipitated rejection in WT mice recipient of a fully MHC-mismatched (56) or single MHC Class II-mismatched (53) cardiac allograft, indicating relative contributions to the alloimmune response (2,53). Furthermore, donor, but not recipient, expression of PDL1 was critical to allograft survival and the prevention of vasculopathy in a single MHC-mismatched cardiac transplant model (57), while donor endothelial PDL1 expression was shown to be essential for the maintenance of allograft tolerance induced by CTLA4-Ig in a fully MHC-mismatched model (54). PDL1-Ig acts synergistically with both CsA and anti-CD154 to prolong graft survival in models of cardiac (55) and islet (58) transplantation, respectively. Conversely, PDL1 blockade has been shown to enhance alloreactive Th1 differentiation (59), while there is also evidence that PDL1 functions in a PD-1-independent manner to mediate apoptosis of alloreactive T cells (59). Interestingly, B7−1 has been recently identified as an additional ligand for PDL1, with evidence that this interaction functions as a bidirectional inhibitory pathway (60). Furthermore, specific blockade of the PDL1–B7−1 pathway has been demonstrated to precipitate allograft rejection independent of PD-1 signaling, with enhanced Th1 and Th2 alloreactivity (53).

Although thought to have a central role in Th2 differentiation, CD28, OX40 and TIM-1 instead promote Th1 differentiation upon strong TCR signaling (10), the production of IFN-γ or IL-12 (61) or high affinity binding (33), respectively. These molecules will be discussed in more detail below.

**Th2 cells**

The role of Th2 cells in the alloimmune response is unclear; while there is some evidence to suggest that Th2 cells may play a regulatory role (62), there are also data that indicate their involvement in the rejection response (63,64).

CD28 signaling has been shown to play an important role in the differentiation of naive T cells to the Th2 lineage (65), a mechanism dependent on the presence of IL4 and achieved via the induction of GATA-3 (66). It is also dependent on the strength of signaling through the TCR: weak TCR signaling in concert with CD28 indeed promotes IL4 and, consequently, Th2 cells (10); strong TCR signaling, however, promotes the production of IFN-γ and the differentiation of Th1 cells (10). Much of the data regarding the contribution of CD28 signaling in transplantation is provided by studies investigating the use of CTLA4-Ig, which competes with CD28 for binding to CD80/86. However, there are also data in animal models regarding the use of agonistic anti-CD28 antibodies, which have been shown to prolong allograft survival in rodent models of transplantation (67). Recent progress has been made in the development of a chimeric human/primate mAb which selectively blocks CD28 signaling without agonistic or superagonistic activity, and was shown to inhibit both acute and chronic rejection in nonhuman primates, with evidence of synergistic activity when combined with CsA (68).

OX40 plays a central role in the differentiation of naive CD4+ T cells to the Th2 lineage, an effect mediated initially by IL4R-independent induction of nuclear factor of activated T cells c1, and subsequently by an IL4R-dependent effect on GATA-3 (69). OX40 is known to act on naive T cells in concert with CD28, and indeed, the initial effects of OX40 on IL-4 transcription are dependent on intact CD28 signaling (69); moreover, previous data have demonstrated that deficiency of CD28 results in impaired Th2 responses (65). However, production of IFN-α or IL-12 (61) has been shown to divert Th2 differentiation, instead promoting Th1 responses. Interestingly, although OX40 signaling has also been shown to promote the Th2 differentiation of human naive CD4+ T cells in vitro (70), it did not inhibit IL-12-mediated upregulation of IFN-γ production (70). Furthermore, stimulation of OX40 via an agonistic anti-OX40 mAb in a murine cardiac transplant model, wherein the absence of CD40-CD40L signaling had achieved tolerance, precipitated rejection with evidence of both Th1 and Th2 donor-reactive responses, mediated by CD8+ and CD4+ T cells, respectively (71). Isolated blockade of OX40 signaling in rodent models of transplantation has little effect on allograft survival (72). However, the combination of anti-OX40L mAb with rapamycin resulted in significant enhancement of allograft survival compared to rapamycin alone, although in contrast to hCTLA4-Ig, failed to demonstrate any such enhancement when combined with CsA (72). Furthermore, OX40 signaling has been shown to have a critical role in CD28- and CD40L-independent rejection: use of a blocking anti-OX40L mAb in the absence of CD28-CD40L signaling, achieved by use of double deficient mice or blocking antibodies, leads to significant prolongation of skin graft survival (73), although OX40 blockade was shown to significantly prolong both cardiac and skin graft survival when combined with CD28-B7 blockade, inhibiting both alloreactive IFN-γ production and the generation of activated/effector lymphocytes (72).

ICOS, a member of the Ig superfamily, is inductively expressed upon cell activation, and has complex links to both CD28 and CTLA-4. ICOS is upregulated upon CD28 costimulation, although ligation of ICOS-L leads to downregulation of CD86 on APCs (74); conversely, CTLA-4 signaling inhibits ICOS expression. Interestingly, despite the role of CD28 costimulation in ICOS expression, ICOS costimulation is an important mechanism for T cell
activation in the absence of CD28 signaling (79). ICOS has been reported to regulate both Th1 and Th2 (76,77), and, more recently, Th17 differentiation (78); it appears to be more crucial for the Th2 lineage, with evidence that its functions via enhancement of IL-4R-mediated signaling (79), although the requirements for ICOS signaling may depend on the experimental model used and the timing of signaling. Indeed, a recent study of ICOS-deficient patients revealed impaired polarization to Th1, Th2 and Th17 subsets, with further deficiencies in CD4+ effector and central memory subsets (80). In transplantation, the expression of ICOS has been shown to be markedly upregulated in allografts undergoing both acute and chronic rejection (76), while ICOS blockade significantly prolonged allograft survival in a fully MHC-mismatched murine model (75,76). The timing of therapy was shown to be important, with delayed blockade shown to effect the greatest prolongation of graft survival (76); mice defective in either STAT-4 or STAT-6 signaling (defective Th1 and Th2 responses, respectively) displayed a similar tempo of rejection to their WT counterparts, although only STAT-4−/− mice demonstrated prolonged allograft survival upon ICOS blockade, indicating that this effect is dependent upon an intact STAT-6 pathway and, by extension, an intact Th2 response (75). ICOS blockade has further been shown to act in concert with anti-CD40L, preventing the development of chronic rejection seen with anti-CD40L therapy in the absence of DST (76), while the combination of ICOS blockade and a short course of CsA effected permanent engraftment of fully mismatched cardiac allografts with normal histology at day 100 (76).

In addition to its role in Th1 and Th17 differentiation, the TIM-1–TIM-4 pathway is also involved in Th2 differentiation. Interestingly, data from autoimmune and atopic models indicate that, in addition to strength of signal provided to TIM-1 (via different affinity antibodies), the differentiation decision appears to be influenced by the specific anti-TIM antibodies used, indicating that distinct TIM-1 epitopes have widely varying effects. Although administration of a high affinity agonistic antibody in a model of experimental autoimmune encephalomyelitis (EAE) enhanced Th1 and Th17 responses and aggravated disease severity, use of a lower affinity antibody promoted Th2 responses and inhibited disease development (33). In models of allergy, the upregulation of TIM-4 on intestinal (81) and bone marrow-derived DCs (82) in vivo following exposure to Staphylococcus enterotoxin B or cholera toxin and peanut extract, respectively, has been shown to promote Th2 polarization and intestinal allergy. Little data exists regarding its role in transplantation.

TIM-2, a TIM molecule to date identified only in mice, is primarily expressed by activated CD4+ T cells, particularly those of Th2 phenotype. Despite this, however, TIM-2 is believed to be a negative regulator of Th2 cells in models of allergy and autoimmunity (83). There is little data regarding the role of TIM-2 in transplantation.

**Role of Costimulation in T Cell Differentiation**

**Th17 cells**

In the setting of transplantation, there is increasing evidence that Th17 cells contribute to the rejection response (84,85), although many studies have focused on the contribution of IL-17, which may be secreted by cell types other than Th17 (86). Our group has previously shown Th17 cells to be capable of mediating a severe, accelerated form of vasculopathy in a cardiac transplant model which lacks Th1 cells (84); a recent study further demonstrated Th17 cells to be capable of mediating rejection in the absence of other lymphocyte populations and despite neutralization of IL-17A (85). Overall, the exact role of Th17 cells in normal alloimmune conditions, in which the Th1 response dominates, remains to be determined.

In addition to its role in other T helper subsets, CD28 costimulation is reportedly involved in Th17 differentiation, although this may reflect its general role in T helper cell activation (18,78). More recently, the use of anti-CTLA-4, which potentiates CD28 responses, was shown to promote Th17 differentiation in vitro and in vivo, whereas the use of CTLA-4-Ig, and consequent CD28 blockade, inhibited Th17 differentiation (87). However, in another mouse model, CD28 ligation with an anti-CD28 mAb was itself demonstrated to inhibit Th17 differentiation of naïve cells, but appeared to enhance IL-17 production from previously differentiated Th17 cells (88), while CTLA4-Ig facilitated both murine and human Th17 differentiation (88).

The role of ICOS signaling in Th17 differentiation has yet to be fully determined. Along with CD28, ICOS has been shown to be involved in Th17 cell development both in vitro and in vivo (78), with ICOS signaling capable of inducing optimal IL-17 secretion from Th17 cells (78). More recent data has demonstrated that ICOS is also important in the maintenance of the Th17 phenotype and expansion of previous differentiated Th17 cells, the mechanism of which involves the induction of c-maf and upregulation of the stimulatory cytokine IL-21 (89). Its role in the differentiation decision, however, is controversial. Although shown to be crucial for Th17 maintenance, as discussed above, ICOS was not a prerequisite for Th17 differentiation in a murine model (89), a recent study, however, reported ICOS to play a vital role in both the differentiation and expansion of human Th17 cells (90), while, conversely, evaluation of T cell subsets in ICOS-deficient patients revealed impaired polarization to Th1, Th2 and Th17 subsets, with further deficiencies in CD4+ effector and central memory subsets (90).

TIM-1 was initially thought to be primarily involved in Th1/Th2 differentiation. However, more recent data has shown that TIM-1 also plays a role in the differentiation of Th17 cells. Use of an agonist anti-TIM-1 mAb in an islet transplantation model prevented the development of tolerance, and in vitro, was shown to convert existing Tregs into IL-17-producing cells (34). Furthermore, inhibition of TIM-1 signaling by anti-TIM-1 was shown to overcome the...
resistance to tolerance mediated by IL-17-producing CD8 T cells in the absence of CD28 and CD40L signaling (91). Interestingly, a recent study has also provided evidence that TIM-3 inhibits both the differentiation of naïve CD4+ T cells to the Th17 lineage and the production of IL-17 in Th17-polarized cultures in a TIM-1-independent manner (92).

As discussed earlier, our group has previously shown that TIM-3 blockade, achieved by use of a blocking antibody RMT3–23, accelerates allograft rejection in murine transplant models in the absence of CD80/CD86 or CD28 signaling (52), an effect associated with both enhanced Th1 and Th17 polarization and increased frequencies of alloreactive IFN-γ-, IL-17-, IL-6- and granzyme B-producing cells (52). Inhibition of TIM-3 in vitro also increased production of IL-17 from effector T cells (52). TIM-3 also inhibits the secretion of Th17 cytokines in human studies, but, in contrast to murine TIM-3, does not appear to induce cell death (51).

In addition to its role in Th1 differentiation, recent autoimmune data have demonstrated a role for the CD40L–CD40 pathway in Th17 differentiation (118). In the setting of strong antigenic stimulation, CD40 ligation was shown to promote the secretion of IL6 by DCs via pattern recognition receptors in vitro, which, when combined with TGFβ, provided the optimal milieu for Th17 differentiation (93). Furthermore, CD40+ mice were shown to be incapable of mounting a normal Th17 response despite high antigen stimulation and were completely protected from the development of EAE (93).

The effect of OX40 on Th17 differentiation in transplantation is unclear. There are in vitro data indicating that OX40 inhibits Th17 cell induction, an effect mediated by IFN-γ (94); however, this data does not concur with in vivo studies demonstrating that OX40 activity is necessary for the Th17-mediated diseases EAE (95) and rheumatoid arthritis (96).

Tregs
Natural Tregs (nTregs) are formed in the thymus and are characterized by the expression of the transcription factor FoxP3. Induced Tregs may also be generated in the periphery from CD4+CD25− cells in the presence of TGFβ(17). Tregs are critical to the development and maintenance of self-tolerance, and, in transplantation, are indispensable to the induction and maintenance of allograft tolerance.

CD28 is critical to the development of nTregs in the thymus (97), and mice deficient in CD28 or either of its ligands have been shown to have significantly reduced numbers of nTregs (97–100). CD28 costimulation is also involved in the generation of Tregs from CD4+CD25− T cells through the production of IL2 (101), although this requirement is not absolute (102). However, high levels of CD28 costimulation have been shown to both inhibit the generation of iTregs and promote the development of T effector cells through a mechanism involving Lck signaling, independent of IL2 (103). In vivo, mice deficient in CD28 show prolonged acceptance of fully MHC-mismatched cardiac allografts (100,104), but paradoxical accelerated rejection of single MHC Class II-mismatched cardiac allografts, accompanied by both an increased ratio of T effector cells to Tregs and increased production of Th1 and Th2 cytokines (100). Use of a superagonistic anti-CD28 mAb in rodent models was shown to preferentially activate and expand functional Tregs both in vitro and in vivo (105,106); unfortunately, attempts to translate this therapy into clinical use were disastrous: in Phase I clinical trials, six healthy volunteers developed a massive cytokine storm upon administration of a superagonistic anti-CD28 mAb (107).
emergence of Tregs (110). Evaluation of a small cohort of renal transplant patients treated with anti-IL2R and randomly assigned to belatacept or calcineurin inhibitor maintenance therapy, revealed a significant, albeit transient, reduction in the frequency of circulating CD4+CD25+FoxP3+ Tregs in both groups which was attributed to treatment with anti-IL2R (111); however, no reduction in Treg frequency or potency was seen in either group long term (111,112).

In addition to its inhibitory effects on alloreactive effector cells, the PD-1–PD-L1 pathway is centrally involved in the differentiation and maintenance of FoxP3+ Tregs, resulting in increased generation of Tregs, sustained FoxP3+ expression and enhanced suppressor function, effects achieved by blockade of the Akt/mTor pathway and enhancement of the Treg phenotypic (119). Furthermore, OX40 ligation leads to decreased gene expression of FoxP3, whereas OX40 stimulator cells (119) has been shown to promote allograft survival in models of skin transplantation (116), cardiac (116) and renal (117). Its precise role in the induction of FoxP3+ Tregs (119,120). Notably, there is no difference in Treg number or suppressive capacity between OX40 KO and WT mice, demonstrating that OX40 is not involved in the homeostatic regulation of Tregs (119).

The use of different agonistic anti-TIM-1 antibodies in murine models of allergy, allo- and autoimmune disease has revealed TIM-1 to be variously involved in the differentiation of Th1, Th2 and Th17 cells, while inhibiting both the commitment to and suppressor function of the Treg phenotype (32–34); accordingly, use of an agonist antibody prevented the induction of allograft tolerance in an islet transplantation model (34).

**Role of Costimulation in T Cell Differentiation**

**Tfh cells**

Tfh cells are a subpopulation of Th cells primarily involved in providing help to B cells, regulating the development of effector and memory B cells, thereby controlling antigen-specific B cell immunity (121); their exact role in the alloimmune response has yet to be determined. Tfh cells express the chemokine receptor CXCR5 and the costimulatory molecules ICOS, CD40L and PD-1 (121,122); the expression of OX40 has also been identified among some mouse strains (123,124).

ICOS signaling has been shown to be centrally involved in the induction of Tfh cells via upregulation of the transcriptional repressor Bcl-6 (125) and the maintenance of germinal centers (GC; 124). ICOS deficiency is associated with decreased Tfh cell development (126) and profound defects in antibody production, isotype switching and antibody production in both murine models and humans (124,127). Notwithstanding this, the presence of Tfh cells, albeit limited, and Bcl-6 upregulation in ICOS−/− mice suggests that the requirement for ICOS signaling is not absolute, although clearly their function is grossly impaired. Recent data suggests that there is some functional overlap between ICOS and CD28, as ICOS deficiency is capable of substituting for CD28, reversing the Tfh and B cell defects seen in CD28-deficient mice (128).

The CD40–CD40L pathway is known to play a critical part in humoral immunity and in the provision of T cell help to B cells. Interruption of the CD40–CD40L pathway in mice or humans has been shown to result in impaired humoral immune responses, including loss of GC formation, impaired isotype switching and failure of memory B cell responses (121). Specifically, however, deficiency of circulating Tfh cells has been demonstrated in humans with CD40L mutations (126).

The role of OX40 costimulation in the function and maintenance of Tfh cells is controversial. Interruption of the OX40–OX40L interaction was only demonstrated to negatively impact Tfh development in isolated mouse models (124); however, other studies have shown such an interruption to prevent the accumulation of Tfh cells in the follicular B-cell regions.
regions of lymph nodes, indicating a role in Thf migration, while the maintenance of OX40 expression on effector Thf cells is thought to suggest a role in the determination of cell fate upon effector Thf–B cell interactions (123, 129).

Thf cells also express PD-1, which is upregulated because of Bcl-6 expression (130), although its role in the differentiation or maintenance of the lineage is incompletely understood. The highest levels of PD-1 are found on GC Thf cells (131), whereas GC B cells express PDL1. Interruption of the PD-1–PDL1 interaction via PD-1 blockade was shown to enhance Thf cell number, although with reportedly variable effect on GC formation (132, 133).

**Th9 cells**

Th9 cells, a recently discovered T helper subset, are characterized by the production of IL9 and IL10. Shown to promote tissue inflammation (6, 9), they have been implicated in asthma and autoimmune disease (134). Their role in transplantation is, as yet, unknown. Interestingly, the combination of TGFβ and IL-4 was shown to direct Th9 differentiation (6, 9), although the mechanism by which this is achieved requires further elucidation: one hypothesis suggests that the Th9 phenotype is generated by TGFβ-induced “reprogramming” of Th2-differentiated cells (9), another that IL-4 inhibits the induction of FoxP3 by TGFβ, instead generating Th9 cells (6). The transcription factors IRF4 (134) and STAT6 (6) are required for their differentiation, but it is unclear whether a Th9-specific transcription factor exists, or indeed which costimulatory molecules play a role therein. We await the outcome of further investigation into these matters, and indeed their role in transplantation, with interest.

**Conclusions**

Costimulatory molecules continue to hold great promise as therapeutic targets in transplantation. However, a thorough understanding of how these pathways cooperatively function to direct T cell differentiation is critical, as, given their complex relationships and shared ligands, blockade of a costimulatory pathway, although desirable in itself, may prevent the ligation of an essential regulatory costimulatory molecule.

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