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Antibody-mediated chronic rejection in CD52 transgenic mice after T cell depletion with alemtuzumab

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

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Abstract

Alemtuzumab, with its ability to provide profound T-cell depletion without major side effects, looks promising as it can potentially reduce the amount of calcineurin inhibitors used in transplant patients. Unfortunately, the lack of understanding about the mechanisms of alloreactive T-cell repopulation and antibody-mediated rejection remains an unsolved issue in the clinical care of patients after alemtuzumab treatment. In this study, using human CD52 transgenic mice treated with alemtuzumab, we performed detailed examinations of graft survival, level of serum alloantibody, and the effects of lymphocyte depletion on the development of antibody-mediated chronic rejection. Fully MHC-class mismatched donor cardiac allografts were transplanted into alemtuzumab-treated CD52Tg mice and showed no acute rejection for 200 days after transplantation while untreated recipients acutely rejected their grafts within 10 days after transplantation. However, approximately half of long-term alemtuzumab-treated mice showed increased degrees of vasculopathy and C3d deposition at day 100 post-transplant. The histological development of chronic rejection correlated with donor-specific alloantibody production and C3d deposition in the graft. Furthermore, alloreactive B cells were shown to increase in accordance with Donor Specific Antibody detection (DSA). The potential significance of this work is not only to elucidate the mechanisms that govern a humoral component of the immune response but also to create a discriminating model in which in vivo data of antibody-mediated chronic rejection can be studied.

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I. Introduction

1.1 Transplantation in Medicine Today

Solid organ and bone marrow transplants are currently used to treat a variety of diseases, ranging from leukemia to diabetes-triggered kidney failure to liver cirrhosis caused by hepatitis, among others. Organ transplantation represents a triumph for the field of medicine. Over the past fifty years, the field has matured from being considered an unattainable dream to being a reality with advancements in immunosuppressant, tissue typing, and surgery techniques. The number of solid organ transplants performed in the U.S. steadily increased in the past two decades, from 12,623 performed in 1988 to 26,245 transplants in 2011 (2a). Despite the increasing use of transplantation in medicine, however, certain factors have limited its success. One limiting factor worthy of mention, but beyond the main focus of this study, is the shortage of available organs: of the nearly 50,000 people removed from solid organ transplant waiting lists in 2011, 6505 of them were taken off because they died while waiting for a donor organ (2b). The main obstacle in transplantation is the immune system's capacity to respond to "non-self" cells and tissue. Following a successful transplantation, the immune system recognizes and attacks the transplanted organ as a foreign entity despite its usefulness to the recipient.

1.2 Types of transplants (mainly adapted from Kindt et al., 2007)

Organs transplanted from a donor to a unique recipient can be categorized into three types.

Xenografts refer to organs transplanted from one species to another. A ubiquitous example in modern medicine is porcine heart valve and islet cells transplanted into human recipients. Another example is attempted piscine-primate (fish to non-human primate) transplant of islet (i.e. pancreatic or insular tissue) tissue. However, xenotransplantation is often an extremely dangerous type of transplant because of the increased risk of genetic non-compatibility.

Allografts refer to organs transplanted between two genetically distinct organisms of the same species. Most of transplants in human, such as the general liver, kidney and heart transplantation, fall under the category of allografts.

Isografts or syngeneic grafts refer to organs transplanted between genetically identical animals or people. An example of this is skin transplantation from one member of an inbred mouse strain to another mouse from the same strain. In humans, a syngeneic graft refers to a transplant in which the donor-recipient pair consists of identical twins.

Isografts are differentiated from other types of transplants because they do not trigger an immune response. Isografts do not elicit an immune response because there are minimal genetic differences and the recipient's immune system will be recognized as self-antigens. Allografts and xenografts, on the other hand, introduce genetically different proteins into the recipients and will elicit an adaptive immune response.

1.3 Types of rejection

There are three types of immune-system-mediated rejection of transplants: hyperacute, acute, and chronic. Hyperacute rejection occurs in a very short time span, within minutes to hours after transplantation. It results from preformed anti-donor antibodies that circulate in the recipient's body prior to the transplant. These pre-circulating antibodies cause pro-coagulatory changes which result in extensive hemostasis, eventually resulting in destruction of the graft (5). Such pre-circulating antibodies can be the result of pregnancy, a previous blood transfusion, or a blood type mismatch, among other factors. Numerous approaches have been suggested to circumvent this problem, such as pre-testing of recipients for reactivity against donors, or by matching blood and HLA types (38).

Acute rejection occurs within two to sixty days following organ transplantation. This type of rejection results from the mounting of an adaptive immune response against the transplanted organ. The recipient's lymphocytes recognize the organ as foreign and begin to proliferate and attack it. A variety of immunosuppressive drugs can prevent and manage acute rejection. Examples of such drugs include cyclosporine A and tacrolimus, which inhibit calcineurin signaling to prevent T cell activation (3). T cell-mediated injury generally has been considered to be the main cause of acute rejection, and most immunosuppressive therapies have been developed to target T cells. Improvements in T cell-directed immunosuppressants have consequently decreased the incidence of acute rejection (4,5). From1988 until 1996, both acute rejection rates and graft survival rates improved, thanks to cyclosporine A and tacrolimus (2). One-year survival of kidney transplants, for example, has increased to around 90% over the past few years from a 40% survival rate in 1975 (6).

On the other hand, new therapeutic regimens, while reducing the incidence of acute rejection, have often times failed to show significant beneficial effects on long-term graft survival. Such long-term rejection, which usually takes place years after transplantation, is termed chronic rejection. Long-term graft survival has not improved during the past two decades and currently, most organs rejected in human organ transplantations are lost to chronic rejection (7). The 10-year graft survival rate of transplanted kidneys has not improved from the 1975 figure of 50% (6). Chronic rejection of organ transplants remains an unsolved problem in the field of transplantation and is the major reason for late graft failure (8). Pathologically, common features of chronic rejection include vasculopathy leading to ischemic injury, and fibrosis associated with replacement of normal tissue architecture by fibrous elements. Each transplanted solid organ type develops manifestations of chronic rejection unique to that organ, but fibrosis and vasculopathy are common to all.

1.4 Chronic rejection – biggest impediment in long-term survival

Recent studies have identified B cell-mediated alloantibody rejection as the leading cause of chronic rejection in kidney, lung, and heart transplant recipients (11-14). The ability to correctly diagnose antibody-mediated rejection is important to deliver appropriate therapy and to reduce allograft loss. Among many techniques to detect antibody-mediated chronic rejection are: the presence of allograft injury by histology, donor-specific antibody in blood (DSA), and evidence of C3d (complement component) by histology (19). In human renal transplantation, DSA infiltration to donor tissue can be identified by peritubular capillary staining of C3d, which correlates with the presence of donor-reactive serum alloantibodies (15). About 50% of patients with chronic rejection and transplant glomerulopathy or arteriopathy have C3d deposition in the

organ (16). Thus, the deposition of the complement split fragment C3d in allografts is considered a reliable histologic marker of antibody-mediated chronic rejection.

The emergence of chronic antibody-mediated rejection has resulted in new challenges for scientists and clinicians to face in managing troubled transplant recipients. Unfortunately, the mechanisms that govern chronic humoral responses are not well defined. In antibody-mediated responses, the antigen is a class I or II MHC or ABO blood group antigen. When encountering appropriate antigens, the alloreactive B cells form a structure called a germinal center (GC), which allows the quick proliferation of B cells and T helper (T_h) cells (22). The activated B cells inside the GC have two different developmental pathways: some B cells will proliferate and differentiate into short-lived plasma cells, initiating first-antibody production, and others will differentiate into memory B cells and higher affinity antibodies that maintain continuous humoral immunity (23). Interestingly, B and T responses are not independent from each other, but closely linked and coupled. They share a common arrangement of ligand-binding receptors and signal transduction molecules that utilizes analogous signaling pathways needed to respond to foreign antigens.

The finding that B cell activation will not lead to an effective antibody response unless help is provided by T cells was a great step forward in terms of understanding the mechanisms that produce humoral immunity (24). A subset of $CD4^+$ T cells, named T follicular helper cells (T_{fh}), is the subset that is functionally responsible for aiding B cell activation and the formation of the humoral antibody response. The chemokine receptor CXCR5, which is one of the factors that promotes the localization of this subset of T cells into the GC, serves as the identifying marker for humoral antibody response (36, 37). Recent experiments have shown that T_{fh} cells are

required for the formation of the GC and provide developmental signaling required for the production of memory B cells as well as the high affinity plasma cells (25, 26).

1.5 Alemtuzumab as induction therapy

Because of its ability to deplete both T and B lymphocytes, natural killer cells, monocytes, and macrophages, alemtuzumab can be used as an induction therapy for transplant recipients while reducing the amount of current immunosuppressant drugs (17). The success of current immunosuppressive therapies is limited by the nephrotoxicity of the drugs. Minimizing the detrimental effects while suppressing the immune system is the crux of current transplant immunology research. Knechtle et al. studied 10 renal transplant recipients who received alemtuzumab and reduced tacrolimus treatment. All patients are alive with a functioning kidney graft at 27-39 months of follow-up, suggesting alemtuzumab's great potential for suppressing the immune system effectively with minimal maintenance of toxic drugs (18). In spite of the potential of alemtuzmab to solve one of the biggest dilemmas in the field of transplantation, there have been concerns regarding the usage of alemtuzumab. Recent recognition of the increased incidence of rejection with a humoral component in patients receiving alemtuzumab induction has garnered interest in understanding the mechanisms by which DSA mediates graft injuries (21).

To understand the exact mechanism by which alemtuzumab mediates its biological effect in vivo, a transgenic mouse expressing human CD52 was created (28). Alemtuzumab is approved as a first-line treatment against B cell chronic lymphocytic leukemia and results in the depletion of CD52 tumor cells. The CD52 antigen is expressed on B and T lymphocytes, natural killer cells, macrophages, monocytes, and neutrophils. In a study conducted by Hu et al, the transgenic mice treated with the monoclonal antibody displayed the same phenomena observed

in humans: the transient increase in serum cytokines and depletion of peripheral blood lymphocytes (20). The CD52 transgenic mouse is a useful model and can provide mechanistic explanations of antibody-mediated chronic rejection in the activity of alemtuzumab.

1.6 Goals and objectives of this study

The ultimate goal of my project is to understand the immune mechanisms that govern the humoral chronic rejection observed after heart transplant patients have been treated with alemtuzumab. Recent recognition of the high incidence of humoral rejection following alemtuzumab treatment has garnered much interest in the field of transplant immunology. Many studies are being devised to understand B cells' effector role in graft rejection, alloantibody production after T lymphocyte depletion, and repopulating T cells' role in chronic rejection. However, the signals and pathways that mediate chronic rejection are still the least understood, with very limited in vivo data available. The proposed project will use our murine transplant model system, which utilizes CD52 transgenic mice, advanced flow cytometric techniques, and the detailed observation of donor-specific B cells and antibodies. The specific objectives of this project are:

1. To test alemtuzumab's efficacy in both repressing T-cell mediated responses to allografts and T-cell depletion in major immune organs.
2. To simulate heart-transplanted patients using a CD52 transgenic mouse model, and perform examinations of graft survival and levels of serum alloantibody after alemtuzumab treatment.
3. To determine if T lymphocyte depletion and repopulation correlate with the development of antibody-mediated rejection.

II. Methods

2.1 Mice

Homozygous huCD52Tg (H-2^k) mice were kindly donated by Herman Wladman. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbour, ME). Mice were housed in a specific pathogen-free barrier facility and used at 6-12 weeks of age. To induce T cell depletion in vivo, 10µg of alemtuzumab (in 200µL phosphate buffered saline) were intra-peritoneally administered on days -2,-1, +2 and +4 of transplantation. The Emory University Animal care and Use Committee approved all studies.

2.2 Heart transplantation

Fully MHC-mismatched C57BL/6 (H-2^b) donor hearts were transplanted into CD52Tg (H-2^k) recipients. The recipient aorta and vena cava were prepared and the donor heart was transplanted into the abdominal cavity of the recipients. Donor aorta and pulmonary artery were anastomosed in an end-side fashion. The grafts were monitored by daily palpation and graded from 4+ (strong beat) to 0 (no beat), which was confirmed by laparotomy at the time of sacrifice. Animals were sacrificed on the day of rejection (cessation of beating) or a designated time point (day 100).

2.3 Flow Cytometry

Cell processing: Mice were sacrificed on the day of rejection and 100 days after transplantation. Spleens were extracted at the time of sacrifice and peripheral blood was drawn bi-weekly. Cells were spun in a centrifuge for 5 minutes at 15,000 rpm. The supernatants were then aspirated from the samples leaving just a cellular pellet. Cells were suspended in 5mL of

High Yield Lysing Buffer (Invitrogen, San Diego, CA) in order to lyse red blood cells. After 5 minutes, 5mL of R2 buffer were added to the samples to suspend the lysing process. Cells were then centrifuged for 5 minutes at 15,000 rpm. Supernatants were aspirated and cells were suspended in 10mL of R2 buffer and counted using a light microscope. Cells were re-suspended in PBS(phosphate buffered saline) solution containing 2% FBS (fetal bovine serum) Cells were then surface stained for the appropriate cell surface markers and incubated in the dark and on ice for 20 minutes. The cell markers used in staining were: CD4, CD8, Cxcr3, CCR5, CD25, FoxP3, NK1.1, CD44, CD11a, CD62L for T cells and GL7, IgG, IgM, IgD, CD38, CD94, CD4/CD8/F4/80 for B cells(BD Pharmagen, San Diego, CA). Cells were then washed twice with 200µL of FACS. Cells were stained with allogeneic (H-2K^{k}, and H-2D^{k}) and syngeneic MHC monomers (H-2K^{b}, H-2D^{b}) were generated from NIH tetramer facility. Cells were then incubated on ice and in the dark for 40 minutes. Cells were then washed 3 times in 1x Wash/Perm and resuspended in 300µl of FACs. Flow cytometry data were collected on a BD FACS Caliber and BD FACS LSRII bench-top analyzer (BD Bioscience, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR)

2.4 Detection of donor-specific antibody (DSA)

Flow cross match was performed using naïve CD52Tg, pre-transplant recipient, and post-transplant recipient serum. C57BL/6 splenocytes were isolated from the spleen. Mononuclear target cells (1x10^{6}) were re-suspended and incubated with equal volume of Fetal Bovine Serum (FBS) then washed with PBS containing 2% FBS. Recipient serum (1:32 dilution) was incubated with donor splenocytes for 20 min at 4°C in the dark. 5µL of FITC-conjugated anti-mouse Ig was added to the samples and incubated for 20 minutes after washing. The T and B cells were stained with APC-conjugated anti-CD3 and PE-conjuated anti B220, respectively. Samples were

analyzed on a FACS caliber (Beckman Coulter, Brea, CA) and LSR II (Beckman Coulter). The alloantibody production of each serum sample was calculated as fold increase compared to the negative control (naïve CD52Tg mice serum) in the same test run.

2.5 Immunohistochemistry

The grafts from alemtuzumab-treated recipients were harvested 100 days after transplantation. The grafts were bisected and fixed in 10% formalin. The grafts were stained with mouse T-cell marker CD3 (BD Pharmingen, San Diego, CA) and were incubated overnight at 4°C. Endogenous peroxidase quenching was performed post-primary antibody incubation with 1% hydrogen peroxide in TBS for 10 minutes. A donkey anti-rat IgG-Horseradish peroxidase (HRP) conjugated secondary antibody from Jackson Laboratories was used for detection and incubated for 45 minutes at room temperature. The signal was visualized using diaminobenzidine (DAB) chromogen (DakoCytomation, Carpinteria, CA) followed by counterstaining with hematoxylin. A histotechnologist with expertise in transplant immunohistochemistry prepared the grafts.

2.6 Morphometric Analysis

Whole slide of grafts from CD52 Tg recipients were stained with elastic trichrome and then were scanned with an Aperio ScanScope XT (Aperio Technologies, Inc., Vista, CA) and analyzed using the Image Scope (Aperio Techonologies) for morphometric analysis. Scanned images of all vessels in a plane were analyzed and measured with computer-based software (Aperio Techonologies). Neointimal hyperplasia was calculated ([intima area/(lumen+intima area)] x 100) from captured vessels for each graft. Vessels with a neointimal hyperplasia over 20% were considered diseased vessels. For T cell infiltration, trichrome stained and anti-CD3

mAb stained sections were scanned with an Aperio ScanScope XT and analyzed using the Image Scope Positive Pixel Count algorithm. A pathologist with expertise in transplant pathology viewed C3d, H&E, and trichrome stained slides to evaluate C3d deposition and vasculopathy semi-quantitatively.

III. Results

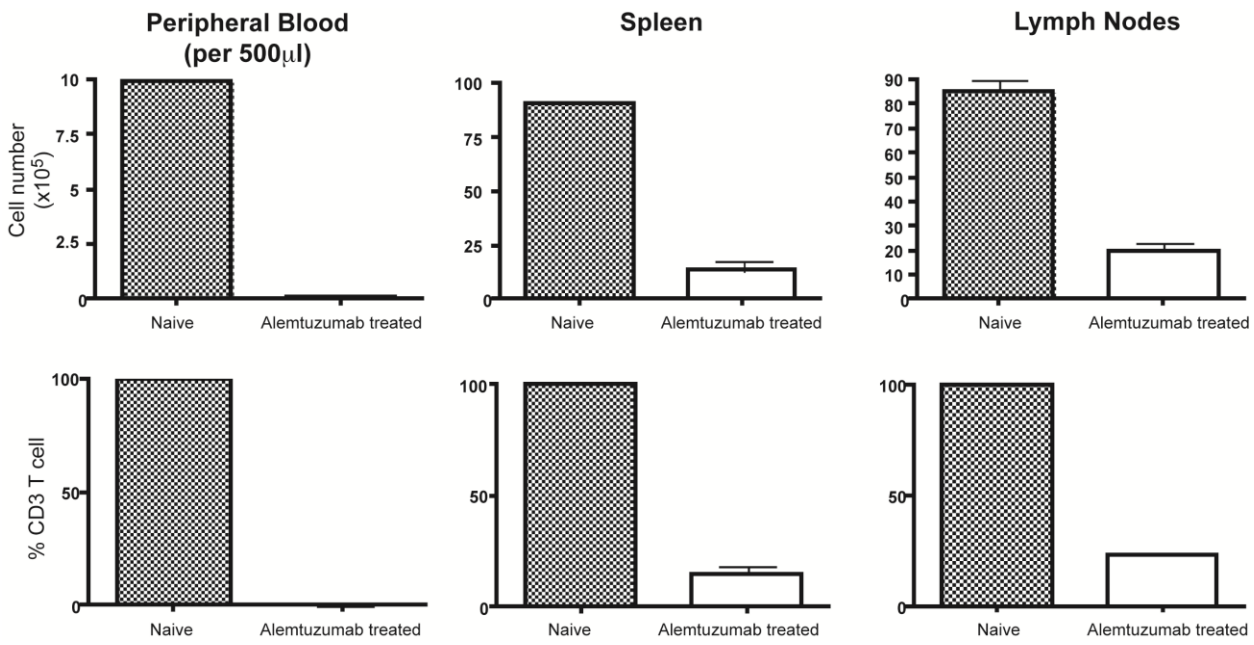

Figure 1. T cell depletion after alemtuzumab treatment in human CD52 transgenic mice
T cell depletion was observed in the lymph node, spleen, and peripheral blood

The role of alemtuzumab in CD52 transgenic mice with respect to its ability to deplete lymphocytes was investigated in completely MHC-mismatched C57BL/6 (H-2^b) heart transplants in CD52 Tg mice (H-2^k). Our blood analysis showed that after four injections of alemtuzumab, there was a >99.5% depletion of peripheral T cells at 24 hours post-treatment. The number of CD3+ T lymphocytes in 500 µL of blood decreased from 993,600 in naïve mice to 6398. In the spleen, the same phenomenon was observed. Alemtuzumab was able to deplete 85.61 ± 6.37 % of T lymphocytes when compared to naïve spleen. The number of CD3+ T lymphocytes in the spleen of the treated mice decreased from 9,100,000 to 1,350,900. Although the general trend of T cell depletion was observed in both lymph node and spleen, lymph node was the most resilient against alemtuzumab's depletion effect (76.51 ± 4.94%).

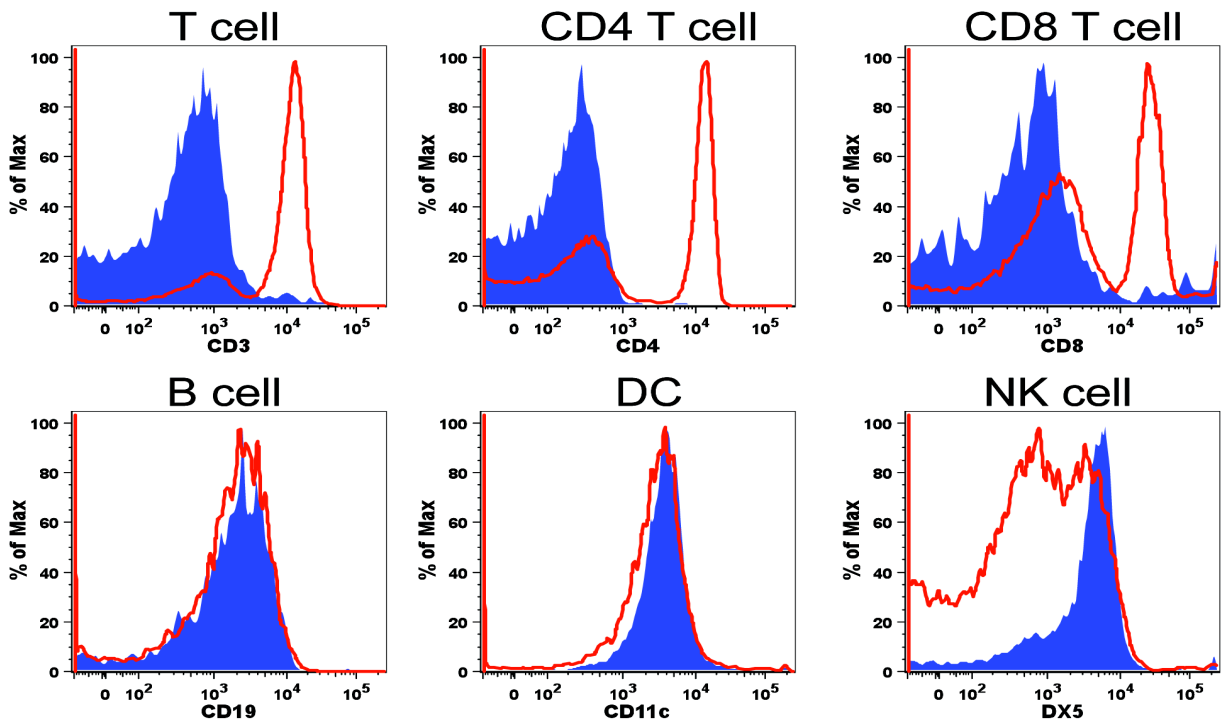

Figure 2. Characterization of exclusive cross-reactivity of alemtuzumab against T cells in human CD52 transgenic mice

No cross-reactivity of alemtuzumab with murine CD52 on B cells(CD19+), dendritic cells (CD19+) and NK cells (DX5+) in human CD52 transgenic mice. Cell populations that express human CD52 are depleted, such as CD4+T cells and CD8+T cells. Untreated control staining is shown by red line and alemtuzumab-treated staining is shown by blue solid area.

After confirming alemtuzumab's exhaustive ability to wipe out T cells in blood, spleen, and lymph nodes, we next analyzed alemtuzumab's effect on sub T cell populations (CD4+ and CD8+) as well as other immune-responsible cells such as B cells, dendritic cells, and natural killer cells (NK cells). The red line shown on the graph represents untreated mice while the blue solid area shows alemtuzumab-treated mice. Flow cytometry analysis showed positive cross-reactivity of alemtuzumab to T helper lymphocytes, as shown by the blue solid area shift to the left side on the forward scatter graph. The peripheral blood was also stained with fluorochrome CD3 and CD8, glycoprotein markers present on the surface of T effector cells or T cytotoxic

cells. In agreement with the general decrease of T cell population, T effector cells showed significant decrease after the treatment.

B cells, dendritic cells, and NK cells were also stained with CD19, CD11c, and DX5, respectively. Interestingly, our data showed that there was no significant cross-reactivity to alemtuzumab as shown by the red line coinciding with blue solid area, suggesting there was minimal change of the immune cell population after the treatment. In contrast to the effect of alemtuzumab's depletion ability in the human body as reported by Hwang et al, (28) the effect of the monoclonal antibody in CD52 transgenic mice was exclusively selective against T lymphocytes.

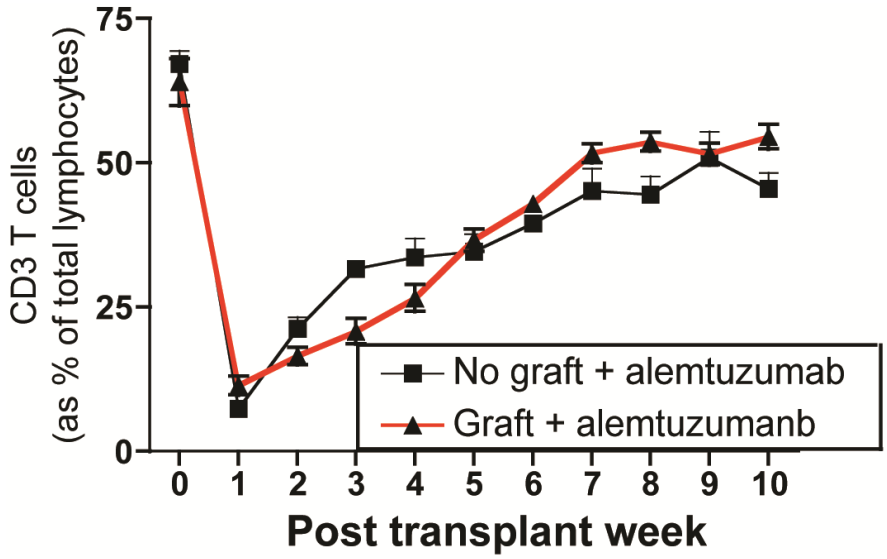

Figure 3. T cell repopulation kinetics after alemtuzumab treatment with or without cardiac allograft.
Serial frequencies of repopulating T cells were expressed as percent of T cells (CD3+) and of total lymphocytes (CD45+).

After confirming the T cell depletion after administration of alemtuzumab, we next analyzed T cell repopulation kinetics in mice with and without cardiac allograft to determine the

rate of the T cell repopulation as well as to find out whether the cardiac graft would expedite the repopulation of T cell. Out of the total number of lymphocytes, which was confirmed by staining with a CD45 fluorochrome marker, the $CD3^+$ T lymphocytes accounted for 64.167 ± 13.262% in pre-transplant mice periphery blood (n=10). At 1 week after transplantation, there was not a significant difference between the number of T cellsin mice that underwent transplantation and the control group (7.4540± 0.7647% vs. 8.581±1.400%). At 3 weeks after transplantation, CD3 T cells repopulated slightly faster in mice with no graft compared to mice with grafts (25.967 ± 6.555% vs. 20.871 ± 5.705%). However, the trend reversed at 8 weeks and transplanted mice showed more rapid repopulation (53.223 ± 4.353% vs. 48.343% ± 3.821). Overall, the difference in T cell population in both groups did not exceed more than 4.880% and heart allografts did not necessarily expedite the repopulation.

According to an in vivo study conducted in human patients by Huang et al, gradual repopulation of T lymphocytes began one month after transplantation while our data suggest that repopulation began in mice as early as the second week after transplantation. In addition, by week 9, the T lymphocyte population came back to 85.039±5.197 % of the pre-transplantation level. Not only did the repopulation begin much earlier in mice but also the T-cell repopulation kinetics were much more rapid compared to those of humans.

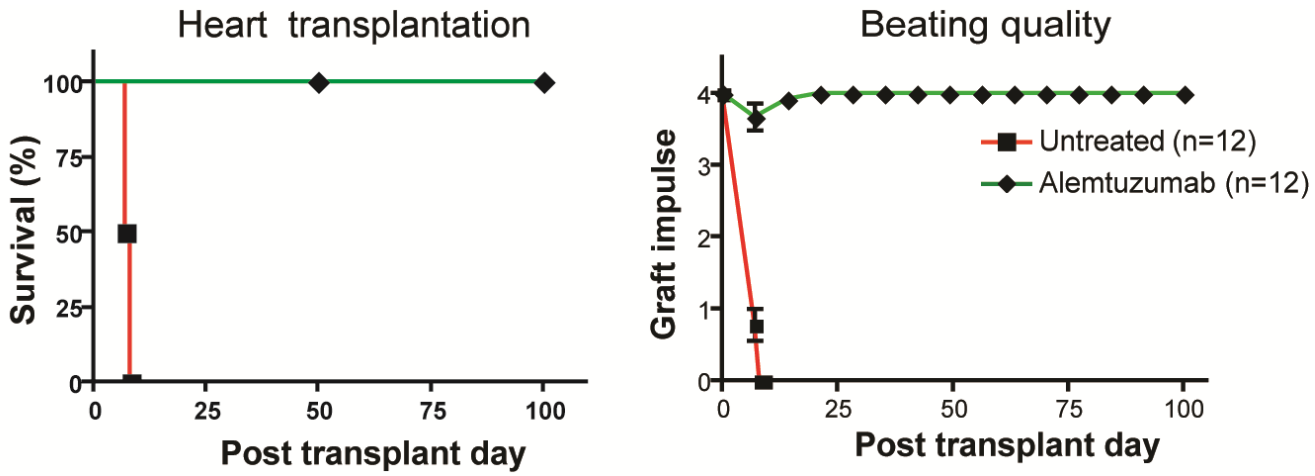

Figure 4. Cardiac allograft survival and beating quality
Heart transplant recipients showed significantly prolonged survival and improved beating quality with alemtuzmab treatment.

Knowing the repopulating kinetics of T cells after alemtuzumab treatment, we then performed heart transplantations to measure the efficacy of alemtuzumab in suppressing allograft rejections. The status for the allografts was measured by daily palpation and graded from 4+ (strong beat) to 0 (no beat). In the case when the beating score was 0, rejection was confirmed by laparotomy and visual inspection. The mice that were treated with the monoclonal antibodies showed neither significant decrease in beating quality nor any rejection by day 100 after transplant. However, the untreated mice showed an accelerated decrease in beating quality compared to the treated mice and showed acute rejection on 8.31 ± 0.712 days.

Human patients who are treated with alemtuzumab showed a state of donor-specific immune hypo-responsivness or tolerance despite the gradual post-transplant T-cell repopulation (29). Strikingly, we were able to observe the same T cell hypo-responsiveness against allografts in our transgenic mice: despite returning to 85.039±5.197 % (Figure 3) of its pre-transplant T lymphocyte levels by week 9, there were no signs of rejection as confirmed by palpations of the allograft. Alemtuzumab successfully prevented early acute rejection beyond post-transplant day 100 and induced hypo-responsiveness of T cells against the allograft.

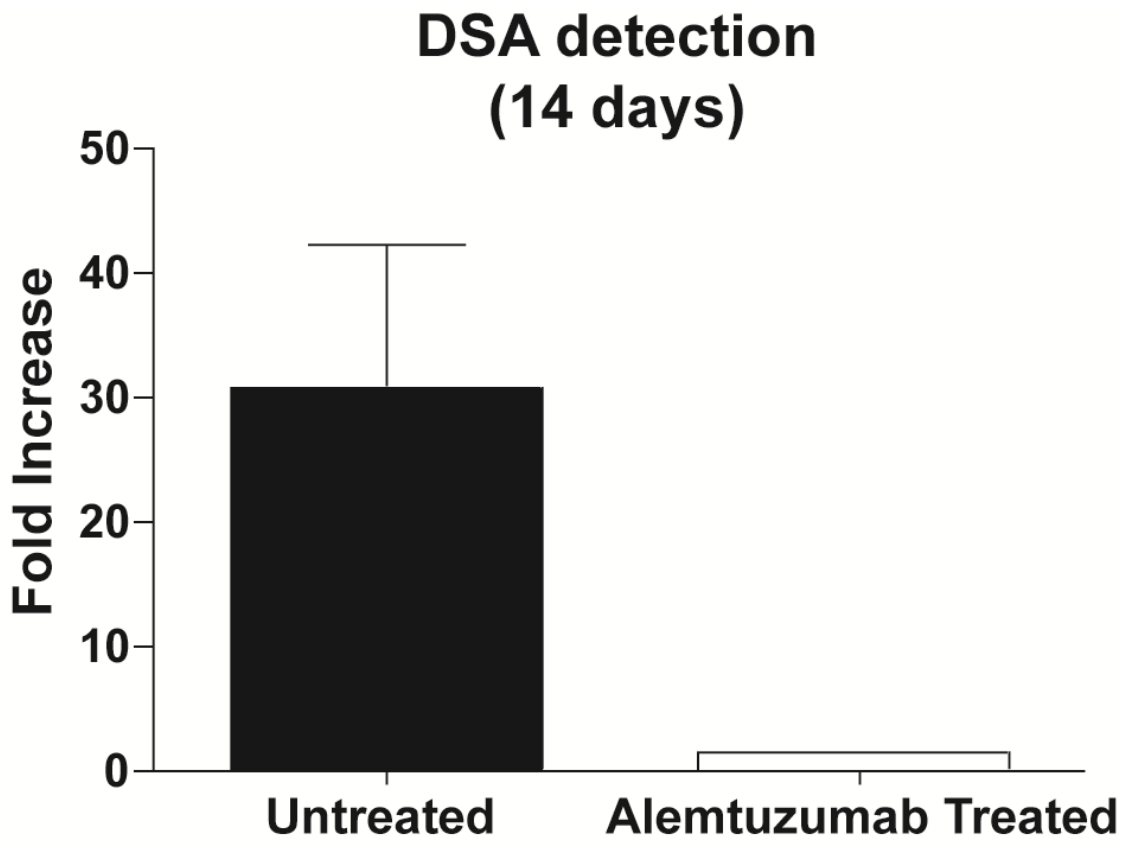

Figure 5. Donor-specific antibody (DSA) detection of untreated mice and alemtuzumab treated mice
Our results confirmed that alemtuzumab treatment successfully suppressed alloantibody responses by 14 days after transplantation. On the other hand, untreated recipients showed significantly elevated DSA levels in serum.

Knowing that alemtuzumab can induce hypo-responsiveness of T cells against the allograft, we then decided to see if the monoclonal antibody would have any effects on antibody-mediated responses against the allograft. We used the flow cross-match test to measure circulating serum levels of donor specific alloantibodies (DSA) and to verify that alemtuzumab-mediated T cell depletion can effectively manage B cell responses in the early phases of transplantation. Gauging DSA levels is crucial in understanding the progression of the humoral immune response as antibodies are the ultimate effectors of humoral response. Donor C57BL/6 mice splenocytes were sorted and mixed with serum samples from the heart-transplant recipients

(hCD52). The mixed samples were diluted with phosphate buffered solution (PBS) in a 1:32 dilution, and co-cultured with donor splenocytes.

Our results revealed that the untreated mice showed a significantly higher number of DSA compared to the mice treated with alemtuzumab. The response of pre-transplanted C57BL/6 naïve mice with no heart transplant to hCD52 mouse splenocytes was used as the base level for measuring the fold increase of treated and untreated groups. The untreated group showed numbers of DSA 30.66± 11.38 times higher than that of the control group. On the other hand, the treated group showed no significant increase of DSA (1.29 ± 0.26). Alemtuzumab was able to successfully suppress antibody-mediated response against the allograft14 days after transplantation.

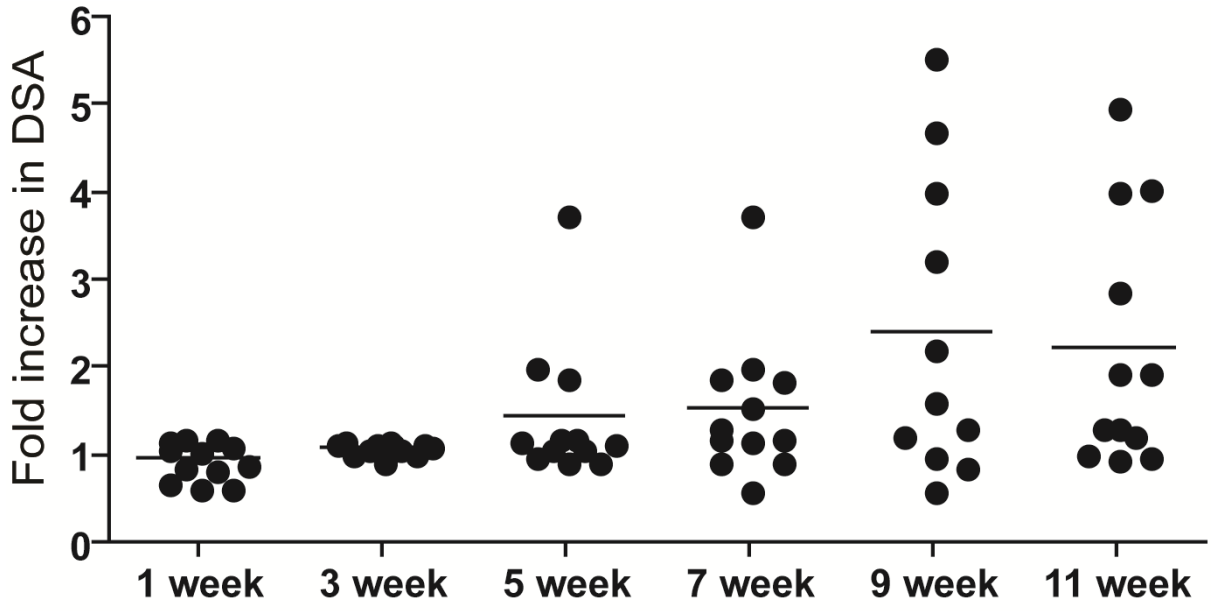

Figure 6. Kinetics of donor-specific alloantibody (DSA) repopulation
Alemtuzumab-treated recipients' peripheral blood was collected to determine alloantibody levels bi-weekly until week 11. The alloantibody levels progressively increased.

Although none of our recipients (n=12) showed acute rejection by day 100 with help from alemtuzumab treatment, we decided to track the subtle response of alloantibodies in the recipients and observe the humoral response of a higher resolution. The same method of DSA detection was performed on peripheral blood collected from treated mice bi-weekly until the 11th week after transplantation. Starting from the fifth week, the recipients showed increased levels of DSA in the collected serum. On average, the fold increase was 1.902 ±0.81 and the number of recipients that showed more than 100% increase in DSA compared to pre-transplantation level was three. The average level of DSA and the number of recipients that showed significant increase (>100%) progressively increased 4.037±1.35 fold and 6 fold respectively by the eleventh week. The powerful effect of alemtuzumab to maintain DSA levels to the pre-transplant level wore off as time progressed and humoral immune response intensified, becoming more apparent in many of recipients.

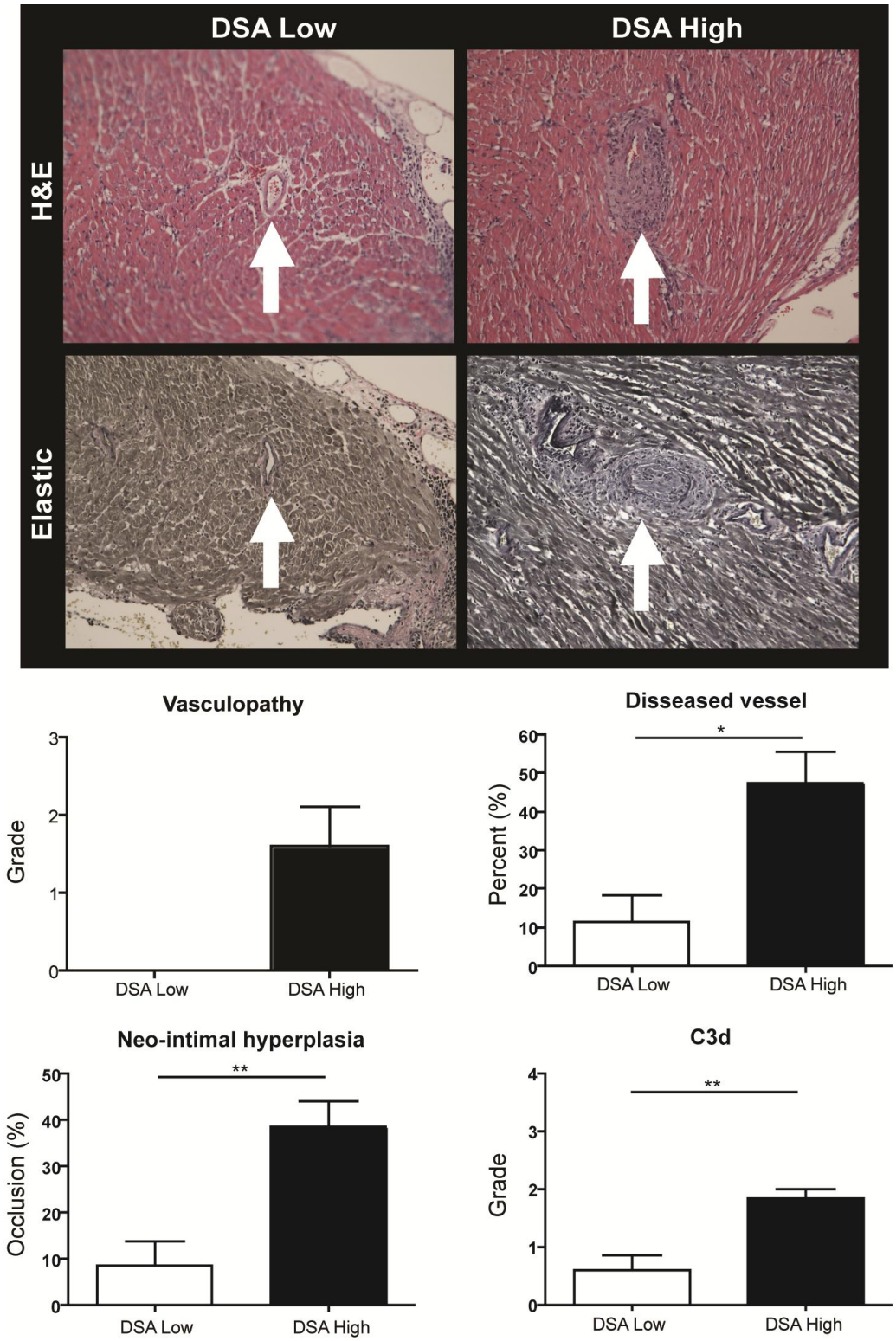

Figure 7. Histological analysis of representative graft tissue of DSA high and DSA low recipients.

Our histological data showed that DSA high recipients showed more significant symptoms of chronic allograft rejection

Alemtuzumab-treated recipients did not show compromised graft function as shown by the examination of beating quality (Figure 4). However, to determine the histologic status of allografts and to collect quantifiable data of chronic rejection, allografts were explanted 100 days after transplantation. The recipients that showed more than 100% increase in DSA compared to pre-transplantation levels were labeled as DSA-high and the other mice were labeled as DSA-low. Chronic allograft vasculopathy (CAV) is a form of coronary disease affecting both coronary arteries and veins and is generally translated as one of the distinctive characteristics of chronic rejection. Interestingly, DSA-high recipients showed more significant symptoms of chronic allograft vasculopathy (CAV). At day 100 post-transplantation, higher degrees of vasculopathy were identified in the grafts from DSA-high recipients compared to DSA-low recipients (1.60 ± 1.14 vs. 0.00 ± 0.00, $p < 0.01$). Vasculopathy was scored on elastic staining as 0, none; 1, mild, 0-10% of the luminal area compromised; 2, moderate, 10-50% of the luminal area compromised; and 3, severe, greater than 50% of the luminal area compromised.

Also, we scanned images of all vessels and analyzed and measured them with computer-based software (Aperio Technologies) to calculate the number of diseased vessels and the percentage of Neointimal hyperplasia from captured vessels for each graft. Neointimal hyperplasia is the response of a vessel to immune response injury that leads to a thickening of the vessels of the epithelial tissue that encroaches on the vessel lumen and serves as a crucial marker for chronic rejection. Vessels showing more than 20% of Neointimal hyperplasia were counted as diseased vessels and more vessels in DSA-high recipients were categorized as diseased compared to recipients of the DSA-low group (47.58 ± 7.89 %vs. 11.50 ± 6.88%, $p<0.05$). The degree of luminal occlusion (the percentage of overall Neointimal hyperplasia) was 38.48 ± 5.5% for DSA-high recipients and 8.432 ± 5.3 % for DSA-low recipients ($p < 0.01$). C3d

immunohistochemistry was scored semi-quantitatively as 0, absent; 1, staining 1-10% of small capillaries; 2, staining 10-50% of small capillaries; and 3, staining > 50% of small capillaries. Grading for C3d deposition showed increased levels of C3d pathological deposition in DSA-high recipients compared to their counterpart (0.60 ± 0.50 vs. 1.83 ± 0.41, $p<0.01$). In conclusion, recipients who showed significant increase in DSA (100%> compared to pre-transplant levels) displayed higher degrees of vasculopathy, diseased vessels, Neointimal hyperplasia, and C3d deposition in the allografts.

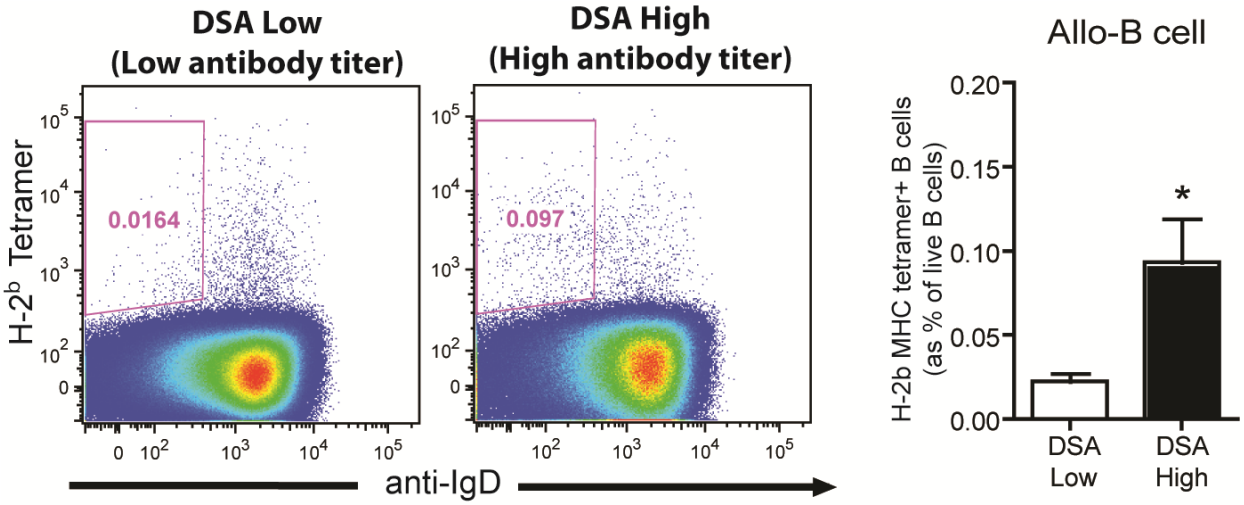

Figure 8 Analysis of allo-reactive B cells in alemtuzumab-treated DSA low and DSA high mice (100 days after transplantation)
Dot plots of live B cells were depicted and show the IgD (x-axis) profile of cells stained with donor MHC tetramer (y-axis). Increased allo-reactive B cells were identified from DSA+ recipients.

Although improvements in measuring DSAs, C3d, and Neointimal hyperplasia have helped to characterize chronic rejection of allografts, the allo-reactive B cell subset that governs the production of DSAs is poorly understood. We developed a novel tracking method for allo-reactive B cells using H-2b tetramers that bind to the donor-specific B cell antigen receptors (BCR). Close examination of these B cell subtypes and evaluation will enrich our understanding

of DSA production as well as antibody-mediated chronic rejection. To specifically calculate the number of allo-reactive B cells, B cells were gated against tetramer Tet B ($H\text{-}2^{b}$)high , IgD low, and $CD19^{+}$. CD19 is a glycoprotein surface molecule that is present on B cells and IgD is the surface immunoglobulin that at low expression indicates B cell activation. When B cells are activated, they are ready to take part in the defense of the body in the immune system or to produce donor specific antibody. As anticipated, the number of allo-reactive specific B cells correlated with the intensity of DSA detection; DSA-high recipients showed higher levels of allo-reactive B cells compared to DSA-low recipients. Out of live B cells gated with tetramer H-2b, the DSA-high recipients showed 0.018 ±0.018 % of allo-reactive B cells while the DSA-low recipients showed 0.134 ± 0.019 % of allo-reactive B cell. ($p<0.05$)

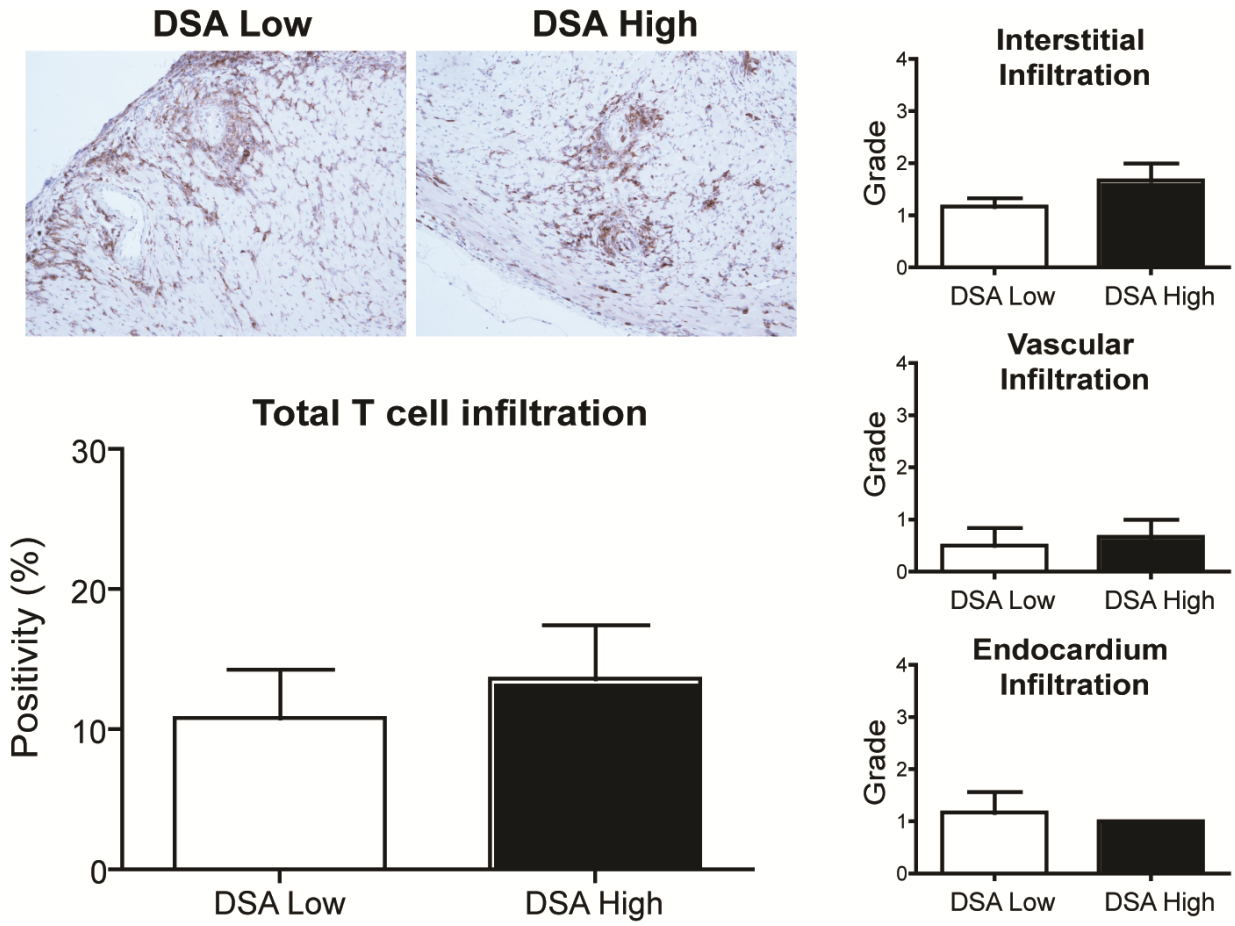

Figure 9 Allo-reactive T cells in alemtuzumab-treated DSA low and DSA high mice (100 days after transplantation)
The quantification of T cell infiltration showed similar levels of T cell infiltration in the allografts in both DSA high and low groups

The correlation among increased amount of DSAs, C3d, neointimal hyperplasia and allo-specific B cell pointed toward the conclusion that B cells are the ultimate effector that mediates chronic rejection after alemtuzumab was administered. However, according to a study by Kwun and Knechtle, the etiology of chronic rejection is described as "multi-factorial," not orchestrated by the action of the humoral immune response of our body alone (27). Before finalizing our conclusion, we decided to make sure that the repopulating T cells, as shown by Figure 3, were not involved in allograft injuries.

T cells only induce apoptosis via cell-surface interactions, so we checked the number of infiltrated T cells in the allografts to confirm whether T cells were involved in the development of chronic rejection. The T cell infiltration in the allografts was quantified by staining a whole slide of the allograft with CD3 markers and using the Image Scope positive pixel count algorithm. Morphometric analysis showed minimal differences between DSA-high recipients and DSA-low recipients in T cell infiltration throughout the entire graft tissue, except in the atrium (10.78±3.52 vs. 13.59 ±3.84%, $p>0.05$). Also, our data showed that there were insignificant differences of T cell infiltration in interstitial space, vessels, and endocardium area. Degrees of infiltration were scored semi-quantitatively as 0, absent; 1, staining 1-10% of small capillaries; 2, staining 10-50% of small capillaries; and 3, staining > 50% of small capillaries. In interstitial space, DSA-high and DSA-low groups showed 1.167 ± 0.167 and 1.667 ± 0.333 respectively ($p>0.05$). In vessels and endocardium area, DSA-high and DSA-low groups showed 0.500 ± 0.342 vs. 0.667 ± 0.333 and 1.167 ±0.983 vs. 1 ± 0, $p>0.05$.

IV. Discussion

4.1 Flow cytometry - effective system to track T cells and B cells

The system developed by our lab to study the immunosuppressive effect of alemtuzumab on cardiac allograft on CD52 transgenic mice allows for an in-depth and holistic analysis of the T and B cell response. The strength of our system stems from the use of flow cytometry, our most powerful technique. Flow cytometry allows for counting and examining microscopic particles. In our study, lymphocytes stained with fluorochrome were analyzed quantitatively as well as qualitatively. In terms of quantification, the flow cytometry machine allows us to count the number of cells that are stained with fluorochrome. For example, as shown in Figure 1, we were able to quantify how many T cells were present in the mice that were treated with alemtuzumab and in those that were not treated with alemtuzumab. This quantification of T cells in the two groups allowed us to confirm the T cell depleting ability of alemtuzumab.

The most important advantage of our equipment is that we can stain for as many as eight different fluorochromes per assay. By carefully designing our staining strategies we can gather valuable phenotypic information about lymphocytes from each marker we use. For example, if a lymphocyte population is expressing low surface IgD, it shows that B cells are activated and they are not naïve B cells. Another example is the staining of tetramer Tet B ($H\text{-}2^b$); the staining of this tetramer selects B cells that have donor-specific B cell antigen receptor (BCR). As used in our flow cytometry analysis, the combination of IgD, tetramer Tet B ($H\text{-}2^b$), and CD19 allowed us to track allo-reactive B cells.

4.2 Alemtuzumab's ability to deplete T cells in peripheral blood, the lymph nodes, and the spleen

This study used full MHC-mismatched cardiac transplantation in human CD52 transgenic mice to evaluate alemtuzumab's efficacy in prolonging allograft survival. In accordance with previous studies in humans (20), alemtuzumab delivered greater than 99.5% depletion of peripheral T cells after 2 administrations of 10 μg of alemtuzumab in transgenic mice (Figure 1). Before moving forward with our project, the confirmation that alemtuzumab depletes T cells in our transgenic mice was crucial as our goal was to simulate the immune-compromised status of heart transplanted patients after alemtuzumab treatment.

By using our mouse model, we were able to confirm the status of T cell populations in secondary lymphoid organs, such as the lymph node and spleen. Despite the fact that properties of alemtuzumab have been studied in vitro using human blood samples, more detailed human in vivo mechanism of action studies in lymph node and spleen tissue have been difficult to achieve. Our results provided insight into the in vivo activity of alemtuzumab in these organs. Although the depleting ability of alemtuzumab was extremely effective in immune organs, T cells in spleen and lymph nodes showed more resistance against alemtuzumab compared to those in peripheral blood (Figure 1).

One likely explanation for the lymph nodes' resistance is their immunological function in our body. To be more precise, the afferent vessels of the lymph node deliver unfiltered body fluid, which might contain micro-organisms, foreign antigens, and viruses, to the lymph node for filtration processing (6). Many immune-responsible cells are concentrated in this organ and lymph nodes are the major sites for initiation of immune responses. In a similar fashion to the lymph node, the spleen can be thought of as an immunological conference center. The organ captures foreign materials from the blood that passes through the spleen and initiates an immune

response when antigens are presented to T and B cells. We conjecture that less T cell depletion in the lymph node and spleen could affect T cell repopulation kinetics as well as T cell phenotypes during repopulation. Investigating how less depletion in these organs can affect T cell repopulation kinetics as well as T cell phenotypes during repopulation would be a rewarding future research topic.

4.3 Preventing acute rejection in the early phase of transplantation using alemtuzumab and repopulating T cell response against allograft.

Administration of alemtuzumab accomplished T cell depletion while leaving B cells, dendritic cells, and NK cells unaffected. Both T helper cells ($CD3^+$ and $CD4^+$) and T cytotoxic cells ($CD3^+$ and $CD8^+$) were depleted. Mice that were not treated with alemtuzumab rejected allografts within 8.31 ± 0.712 days following transplantation while those that were treated with alemtuzumab showed much-improved graft survival beyond 100 days after transplantation (Figure 4). As checked by daily heart palpitation tests, the functionality of the allografts remained constant and stable. The prolongation of graft survival through nothing more than T cell depletion in the early phase of transplantation was clinically relevant as many immunosuppressant drugs target T cell activation and signaling molecules. Our data re-confirmed that T cell management is integral in prolonging graft survival and preventing acute rejection in the early phase of transplantation.

The mice that were treated with alemtuzumab showed much less development of donor-specific alloantibodies (DSA) at 14 days after transplantation. On the other hand, untreated mice showed as much as 30 times higher DSA production compared to treated mice (Figure 5). The observation that T cell depleted mice produced much less DSA provided us with valuable suggestions that T cell and B cell activations are closely related and their activation is not

independent. As mentioned earlier, recent studies have shown that a subset of $CD4^+$ T cells, T follicular helper cells (T_{fh}), are responsible for aiding in B cell activation and humoral antibody response (25, 26). We believe that because most of $CD4^+$ T cells, including T_{fh} cells, have been depleted due to the antibody treatment, the activation of the humoral response takes much more time compared to untreated recipients. This observation provides vital information about the interconnectivity between B cells and T cells and T cell's role to initiate humoral response.

A previous in vivo study conducted in humans showed that a gradual repopulation of T lymphocyte began 1 month later after the last administration of alemtuzumab (28). However, Huang and many others' studies on alemtuzumab's effect was conducted on patients who had other autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. We do not have much information on how the immune system responds to the action of alemtuzumab alone in the presence of allografts. Furthermore, in human patients, alemtuzumab cannot be given alone to transplanted patients to prevent rejection (29). It can only be administered in conjunction with conventional immunosuppressant drugs such as tacrolimus. Thus, there is a lack of studies on the effect of alemtuzumab alone on the graft in humans. Our mouse model was able to isolate the effect of alemtuzumab on grafts alone.

Another interesting observation was the hypo-responsiveness of repopulating T cells against allografts. Despite T cell levels almost returning to pre-transplantation levels, T cells were not eliciting immune responses against the donor antigen (Figure 3 & Figure 4). This un-reactivity or hypo-responsiveness of T cells was observed in human renal transplant patients who were treated with alemtuzumab (29). However, as discussed earlier, the transplant patients never suppress their immune system with alemtuzumab treatment alone; the immunosuppressive regimen is orchestrated in conjunction with tacrolimus and other drugs. In our mouse model, we

were able to see this un-reactivity of repopulating T cells against allografts even when recipients were treated only with alemtuzumab. Although we do not have plausible mechanistic explanations as to why this phenomenon occurs, this significant discovery adds importance and valuable meaning to our research as alemtuzumab depletion induces more tolerogenic T cell repopulation.

4.4 Analyzing symptoms of chronic rejection and antibody-mediated injuries.

DSA detection was performed on peripheral blood every two weeks and alemtuzumab-treated mice started showing significantly increased levels of DSA (>100% than the pre-transplant level) by the fifth week (Figure 6). As time progressed, the humoral response against the allografts intensified. Not only did the alloantibody level increase, but also other histological markers for chronic rejection became more evident in the allografts, such as C3d deposition, vasculopathy, and neo-intimal hyperplasia (Figure 8). By merely looking at increased amount of DSA production, it is hard to conclude that more DSA translates into increased antibody-mediated chronic rejection. However, the correlation between alloantibody production and these histological markers for chronic rejection serves as proof that more DSA presence can be interpreted as a prelude to imminent chronic rejection.

As shown by figure 3, the T cell population was gradually increasing after initial depletion. Interestingly, the intensity of DSA detection correlated with the repopulating T cells. The more T cells became available out of the total number of lymphocytes, the more intense DSA production was observed. One plausible explanation of this phenomenon can be attributed to repopulating T follicular helper cells. As more T_{fh} cells became available, they were able to aid in the activation of B cells and accentuate humoral responses against allografts. In

accordance with our data about increased DSA level, more allo-reactive B cells were detected in the DSA-high group (Figure 8).

The next question we had to address was the possibility of repopulating T cells' involvement in causing allograft injuries. T cells only induce apoptosis or mediate graft injury via cell-surface interactions (3). In other words, T cells need to be physically present in the grafts in order to elicit immune response. Thus, if the T cell infiltration into the allograft can be quantified, we can calculate the involvement of T cells in chronic rejection. CD3 staining of allografts showed that there was a minimal difference in the number of infiltrated T cells in DSA-high and DSA-low groups (Figure 8). Our data successfully indicated that at the least, T cell graft infiltration is not a deciding factor for chronic rejection.

V. Future application of current results

Our CD52 transgenic heart transplanted model successfully served as a clinically relevant model for transplant patients who undergo alemtuzumab treatment. When alemtuzumab was used, mice showed T cell depletion in the spleen, the lymph node, and peripheral blood. In addition, T cell-mediated acute rejection was prevented and the treatment prolonged the graft survival. With more repopulating T cells, we observed more DSA production as well as other histological markers for chronic rejection. One possible explanation for this observation is the returning of T follicular helper cells. In order to confirm this T cell subtype's involvement in B cell response against allografts, we need to develop a way to track T_{fh} cells and analyze the proportion of this T cell subtype within repopulating T cells to see if there is any correlation with degrees of chronic rejection signs. Currently, CXCR5 is used as a marker for T_{fh}. We are planning to incorporate CXCR5 staining in our flow cytometry analysis.

Our results provide important insights into how T and B cells respond and explore the nature of the donor-specific humoral immune response after alemtuzumab administration. This project was able to isolate the effect of the monoclonal antibody against allografts, however, human transplant patients do not receive alemtuzumab alone to prevent their graft rejection. Generally, alemtuzumab will be used to deplete T cells as an induction therapy to decrease the amount of conventional immunosuppressive drugs such as tacrolimus, rapamycin, and cyclosporine. In future experiments, we are planning to add the aforementioned immunosuppressive drugs in conjunction with alemtuzumab induction therapy to mimic exact human patient settings. A study conducted by Knechtle et al, showed that when rapamycin was given together with alemtuzumab, there were more antibody-mediated rejections in human renal transplant patients (35). Using similar techniques and analysis methods to the ones we have

utilized in this study, we hope to shed some light on the mechanical understanding of this observation.

VI. Acknowledgements

I would like to thank all the people at the Emory Transplant Cetner labs-almost everyone here helped me in some way with the project, whether through showing me a new procedure, lending me pipettes, or providing advice. I am especially thankful for all of the help given to me by Dr. Jean Kwun, Adriana Gibby, Brett Mendel, Vinh Lu and Jinnie Kim without whom I would probably have been overwhelmed by the sheer number of mice involved in this project.

I also want to extend a special thank you to my adviser for this experiment, Dr. Stuart Knechtle for all of his guidance, and to the other members of my committee- Dr. Amanda Starnes, Dr. Kathleen Campbell, and Dr. Tracy Morkin

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