PRO-INFLAMMATORY CYTOKINE INCREASED IL-33 AND ST2 INDICATE PEDIATRIC ALLOGRAFT REJECTION

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ABSTRACT

Organ transplantation prolongs thousands of lives each year. While the evolution of post-transplant immunosuppressant therapies and better surgical techniques have improved short-term patient prognosis, these advancements have had little impact on long-term graft attrition and patient survival rates. Over-immunosuppression caused by anti-rejection therapies drugs increases recipient infections and cancer. Onerous, yet imprecise, surveillance procedures impose a burden on patients, but poorly detect nascent rejection. The identification of reliable biomarkers of rejection would enable earlier identification of rejection episodes and allow dynamic adjustment of immunosuppressant drug therapies. Interleukin (IL)-33 is a novel IL-1 family cytokine that is expressed or induced in several cell types (epithelial, endothelial, and myeloid cells, and myofibroblasts) and found in the tissues of commonly transplanted organs, including the heart, lungs, small bowel, and kidney. IL-33 has pleiotropic effects on the leukocytes and stromal cells expressing its receptor, Growth Stimulation Gene-2 (ST2). The effects of IL-33 are negatively regulated by a soluble decoy receptor, soluble ST2 (sST2). Several studies have demonstrated that mechanical stress or inflammatory stimuli, including IL-1β and TNFα, are able to induce both sST2 and IL-33 in cardiac myocytes and endothelial cells. Clinical measurements of circulating sST2 have been found it to be predictive of cardiovascular

disease risk and mortality in patients with a history of myocardial infarction (MI). Specifically, following MI, elevated sST2 indicates severe cardiac damage and predicts patient mortality. Increased levels of sST2 and IL-33 are observed in the sera of patients with inflammatory bowel disorders. Based on these reports, we *hypothesized* that IL-33 and ST2 expression are distinctly modulated in allografts due to pro-inflammatory cytokines secreted by infiltrating immune cells and that these proteins will serve as sensitive biomarkers of rejection. To test this hypothesis in a cohort of pediatric heart and small bowel transplant recipients, we examined levels of ST2/sST2 and IL-33 both locally in the allograft tissue and systemically during periods of rejection and quiescence. In endomyocardial biopsies (EMB) collected from rejecting heart transplant recipients, we found that both ST2 and IL-33 were increased significantly (p=0.0359 and 0.0049 respectively) compared to non-rejecting patients. Likewise, we observed similar increases in IL-33 and ST2 in patient sera (p=0.0006; sST2 and p=0.0010; IL-33). In small bowel patients undergoing rejection, biopsy samples displayed upregulation of ST2 gene expression (3.94-fold increase over non-rejection) and analysis of collected sera revealed an increase in ST2 levels (p=0.0306) in these patients. Our findings provide strong evidence that ST2 and IL-33 could function as tissue- and most importantly, serum-based biomarkers of rejection. This discovery would benefit the overall public health of allograft recipients by enabling earlier transplant rejection diagnosis, alleviate complications due to over-immunosuppression, and decrease reliance on biopsy procedures.

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ABBREVIATIONS

ACR	Acute Cellular Rejection
AMR	Antibody Mediated Rejection
IST	Immunosuppressant Therapy
DSA	Donor Specific Antigen
EMB	Endomyocardial Biopsy
HLA	Human Leukocyte Antigen
HTx	Heart Transplant
IFN-γ	Interferon λ
IL	Interleukin
IPA	Ingenuity Pathway Analysis
IST	Immunosuppressant Therapy
LPS	Lipopolysaccharide
POD	Post-Operative Date
Qdot	Quantum Dot Immunolabeling
SBTx	Small Bowel Transplant
sST2	Soluble ST2
ST2	Growth Stimulation Gene 2
Th2	T helper 2

TNFα	Tumor Necrosis Factor α
WSI	Whole Slide Imaging

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1.0 INTRODUCTION

1.1 ORGAN TRANSPLANTATION: EARLY HISTORY

Researchers first began experimenting with organ transplantation on both animals and humans in the 18th century. However, these procedures were plagued by surgical and immune complications. These barriers included functional failure of the allograft itself, infection, and immune-mediated destruction of the graft tissue, which was recognized as non-self, foreign material. Early attempts focused on the kidney and used pigs, sheep, and goats as a source of organs. However, none of the kidneys functioned for long, if at all, and most patients perished within a few hours after transplantation (1). Cadaver tissue was also a source of organs and was many times collected from prisoners following execution. However, even with compatible blood-type matching often times these poor quality grafts would only last a matter of days before failing (1). In Paris in 1953, Drs. Jean Hamburger and Louis Michon performed the first attempt at live donor kidney transplantation (mother-to-son). In the absence of immunosuppressants to stop destruction, or rejection of the graft tissue, the organ functioned for three weeks before succumbing (2). The same procedure was completed in 1954 by Drs. Joseph E. Murray and John Merrill at Brigham & Women's Hospital in Boston, Massachusetts (3). However, this would be the first successful renal transplantation because the graft was a highly viable kidney from a living monozygotic twin. Because the transferred kidney was genetically identical to that of the

recipient, the recipient immune system perceived the graft as self, and no agents were required to subdue immune-mediated graft rejection (3).

During the same period, others were beginning to unravel the immunology underlying allograft rejection and how to thwart it. In Nobel Prize winning work, Peter Medawar demonstrated that graft rejection is the result of an immunological event in the early 1940s (4). In the early 1950s, researchers began investigating the means to suppress the immune system by experimenting with adrenal cortical steroids and irradiation in laboratory animals (5, 6). Both prolonged graft survival marginally, but not to a significant degree. However, this set the stage for development of drug therapy as treatment for rejection. The first evidence of prolonging of allograft function due to drug therapy was demonstrated in 1960, when Willard Goodwin produced severe bone marrow depletion in a young female who had been transplanted with her mother's kidney by administering metho-trexate and cyclophosphamide (7). The patient subsequently developed rejection episodes attributed to recovery of bone marrow, but effects were temporarily reversed due to treatment with prednisone (7). Research using anti-leukemia drugs 6-MP and azathioprine showed promise by delaying rejection in rabbits in a dose dependent manner, demonstrating a mechanism of T-cell inhibition which provided better patient outcome than depletion a of bone marrow cells (8, 9). Over the last several decades, multiple classes of immunosuppressant drugs have been developed and due to emergence of these new therapies, prognosis following acute rejection has steadily improved.

1.2 ORGAN TRANSPLANTATION TODAY

Organ transplantation has now evolved into a procedure that consistently prolongs survival in both adult and pediatric patients undergoing end-stage organ failure (10, 11). In 2013 greater than 28,000 organ transplantation procedures were completed (optn.transplant.hrsa.gov). Currently, one year survival rates exceed 90% in kidney transplant recipients and are above 80% for heart, liver, lung, and intestinal allograft recipients (10). Improvements are attributed to advancements in surgical procedures and development of effective drug regimens. However, even with these improvements, barriers to successful patient outcomes still exist (12).

1.3 CURRENT BARRIERS TO SUCCESSFUL TRANSPLANTATION OUTCOMES

1.3.1 Acute Cellular Rejection

Acute cellular rejection (ACR) is most common within the first six months to one year of transplantation (13, 14). ACR is typically asymptomatic until diagnosed by biopsy or until graft dysfunction presents itself, such as arrhythmias or heart palpitations in the case of heart transplants (14). While the cellular and molecular events that precipitate ACR are complex, it is most notably characterized by infiltration of mononuclear cells, typically, CD3-positive T-lymphocytes as well as macrophages (14). ACR is observed due to the inflammation generated via T-cell responses directly to donor Human Leukocyte Antigens (HLAs) expressed on donor tissues and antigen presenting cells (APCs) in a process referred to as direct allorecognition. ACR can also be induced by indirect allorecognition, which occurs as a result of donor peptides

that are processed and then presented by recipient APCs in the context of self HLA (14, 15). This T-cell activity leads to their release of pro-inflammatory cytokines and cell lytic proteins, which can lead to tissue damage, necrosis, and graft loss of graft. The likelihood of a patient developing ACR increases with the number of HLA mismatches between donor and recipient, other factors include young age, female gender, and race (13). The current 'gold standard' for diagnosis of ACR within allografts involves biopsy of the transplanted tissue and examination by a transplant pathologist. For example, cardiac allografts are assessed by routine endomyocardial biopsy (EMB) (13, 14, 16). EMB samples are then pathologist scored and severity is graded on a scale determined by the International Society for Heart and Lung Transplantation (ISHLT) (**Table 1**) (12). A 0R grade denotes no evidence of cellular rejection while a grade of 3R is representative of severe ACR with diffuse cellular infiltrate, multifocal cellular damage, and vasculitis (12). Currently, death/graft loss due to acute rejection within the first year post-transplant is rare; however, episodes of ACR are common and typically can be remediated by treatment with immunosuppressant drugs.

Table 1: International Society for Heart and Lung Transplantation Grading Scale for ACR (2005)

(A) Acute cellular rejection	
2005	
Grade 0R	No acute cellular rejection
Grade 1R, Mild	Interstitial and/or perivascular infiltrate with up to one focus of myocyte damage
Grade 2R, Moderate	Two or more foci of infiltrate with associated myocyte damage
Grade 3R, Severe	Diffuse infiltrate with multifocal myocyte damage±oedema ±haemorrhage±vasculitis

Modified from Miller et al. 2013 ***Heart.***

1.3.2 Antibody Mediated Rejection

Antibody-mediated rejection (AMR) is not as easily characterized and diagnosed as ACR. Following heart transplantation, AMR is similar to ACR and can result in vascular damage, graft dysfunction and loss (13, 17). AMR occurs in about 10-20% of all heart transplant recipients and is associated with a higher risk of death than with ACR (13). Typically referred to as non-cellular or humoral rejection, AMR results from sensitization of the recipient to donor HLAs present in the allograft tissue (13, 17, 18). This sensitization results in the production of donor specific antibodies (DSA) (17). Another characteristic of AMR is complement binding, however, evidence now indicates the existence of a complement-independent mechanism as well (18). After solid organ transplantation, diagnosis of AMR is based on the detection of DSA in the serum and staining of the complement protein C4d in biopsied tissue. Complement activation induced by DSA can lead to endothelial activation within the graft which causes a harmful inflammatory response that in severe cases can lead to the recruitment of innate leukocytes such as activated macrophages that bind to the DSAs which can lead to targeted lysis of cells in the surrounding microvasculature resulting in tissue damage (17). Like ACR, AMR in heart transplant recipients is graded on a scale created by the ISHLT (**Table 2**) (14). Even with these criteria, AMR can be challenging to diagnose as pathology between cases can vary greatly (13). Common treatment regimens for AMR often include plasmapheresis coupled with intravenous immunoglobulin (IVIg), and administration of anti B-cell agents (19, 20).

Table 2: International Society for Heart and Lung Transplantation Grading Scale for AMR (2011)

(B) Antibody-mediated rejection	
2011	
pAMR0	Negative for pathologic AMR—both histologic and immunopathologic studies are negative
pAMR 1 (H+)	Histopathologic AMR alone—histologic findings present* and immunopathologic findings negative
pAMR 1 (I+)	Immunopathologic AMR alone—histological findings negative and immunopathologic findings positive†
pAMR 2	Pathologic AMR—both histological and immunopathologic findings are present
pAMR 3	Severe pathologic AMR‡

Modified from Miller et al. 2013 ***Heart.***

1.3.3 Current Immunosuppressant Therapy

Early immunosuppressants such as the ones mentioned earlier played an essential role in improving prognosis following transplantation. Tacrolimus is still commonly used today along with other therapeutic drugs such as alemtuzumab, rapamycin (sirolimus), and mycophenolate mofetil (MMF). These agents as well as other glucocorticoids and inhibitors work through various mechanisms that decrease T-cell activity and limit immune activation (12). Likewise, bortezomib, eculizumab, and anti-CD20 work to suppress B-cell, plasma cell, and complement activity associated with AMR (19, 21). Earlier methods of bone marrow depletion were found to be harmful and counteractive to better patient prognoses (1). While later immunosuppressant therapies (IST) supported improved graft attrition, hyperlipidemia, neurotoxicity, and diabetogenicity were all common side effects of these drugs (1). IST regimens today support fewer acute side effects, but their generalized suppressive function can also lead to infectious and malignancy related complications. Viral infections such as Epstein-Barr (EBV) which drives post-transplant lymphoproliferative disease (PTLD) and human cytomegalovirus (CMV) are

leading causes of morbidity in the immunosuppressed patient (22-24). In addition to these infectious complications, IST can also lead to the development of malignancies of the rectum, skin, and gynecological organs (12). Over-time IST can result in cumulative effects such as: renal toxicity, hypertension, and decreased bone density (12). While the benefits of IST are undeniable, these side effects can be taxing on transplant recipients, particularly pediatric patients who often times face a lifetime of treatment.

1.3.4 Diagnosis of Rejection

As acute rejection and other complications are common within the first year of transplantation, patients undergo routine surveillance to monitor graft function and to assess overall health (13). As mentioned earlier the standard of rejection diagnosis for most types of organ transplants is via tissue biopsy. Sampling usually occurs weekly during the first month post-transplantation, every two weeks for another two months, once monthly until the sixth month mark, then every three months until the end of the first year or anytime rejection is suspected (13). During biopsy procedures, several 1-2mm fragments of tissue are collected from the transplanted organ and placed in 10% formalin (25). The tissue is then embedded in paraffin, sectioned, and placed on glass slides. These slides are then stained and evaluated by pathologists. The typical histological staining of biopsied tissue is hematoxylin & eosin (H&E), which enables assessment of tissue and vascular architecture, and C4d staining, which evaluates AMR status (14, 25). Biopsies can indicate degree of tissue damage and determine the presence of mononuclear infiltrates, commonly T-cells and macrophages, which are indicators of ACR as described above. While this method is widely used it also has several shortcomings: it is costly, invasive, and provides a small spectrum of analysis given the small area sampled (13). In cardiac

transplant recipients this procedure also carries with it the risk of myocardial perforation, arrhythmia, access-site complications, and tricuspid regurgitation (14). In small bowel transplant recipients common complications are intestinal perforation and bleeding (26). The biopsy procedure, due to its invasive nature, is burdensome on high-risk patients who require frequent sampling, this often times leads to non-adherence of the patient to treatment and surveillance plans which could eventually result in development of rejection (27-29).

1.3.5 A Need for Innovations in Post-Transplant Care

Although IST is accredited with improving short-term patient outcomes, the continual evolution of IST has done little to improve long-term transplant recipient prognoses (10, 12). It is well appreciated that even the newest IST contributes to pathological conditions suffered by long-term survivors, such as hypertension and cardiac allograft vasculopathy (CAV) (10, 12). Biopsies, while the standard of diagnosis, are invasive, uncomfortable for patients, and not well suited for frequent sampling (27, 28). Thus, in order to improve long-term patient survival significant innovations in both post-transplant therapeutics and diagnostic care are required. The discovery of reliable biomarkers for rejection could be key in spearheading the way to a more targeted approach to post-transplant care. This advancement would limit complications from over-immunosuppression by allowing for adjustment of IST drugs and could also decrease dependence on biopsy procedures.

1.4 INTERLEUKIN-33 (IL-33) AND GROWTH STIMULATION GENE-2 (ST2) AS BIOMARKERS

1.4.1 Current Biomarkers of Rejection

Throughout the years, several tissue-based and circulating molecules have been proposed as biomarkers for rejection. Currently, the most common rejection markers are indicators of tissue necrosis, or in the case of heart transplant rejection, myocardial necrosis. Examples include: creatine kinase, cardiac troponin I, and C-reactive protein (13, 30). However, even though these markers correlate with rejection and are prognostic of outcome, they fall short in that they are typically only detectable after significant damage to the allograft has already occurred (13). Other circulating factors currently used to support a diagnosis of rejection are antibodies to HLAs and complement fragments. However, many other factors have been investigated, such as plasma cytokine measurements of IL-8, IL-6, and TNF-α, as well as activation markers such as CD69, and the soluble form of CD30 (31-33). While it is true that many of these molecules can be upregulated during acute rejection, they have been found to be either non-predictive of prognosis or lacking the specificity necessary to be used as accurate biomarkers. This highlights the importance of continued research to identify reliable serum and tissue-based biomarkers of rejection (13). Such a discovery would enable quicker diagnosis of rejection episodes which could lead to decreased accumulation of allograft damage over time and improvement of long-term patient prognoses. Likewise, the availability of a reliable early rejection biomarker(s) would enable dynamic regulation of IST to match current allograft conditions. This diagnostic capacity would reduce over immunosuppression and its related toxicities.

1.4.2 Why IL-33 and ST2?

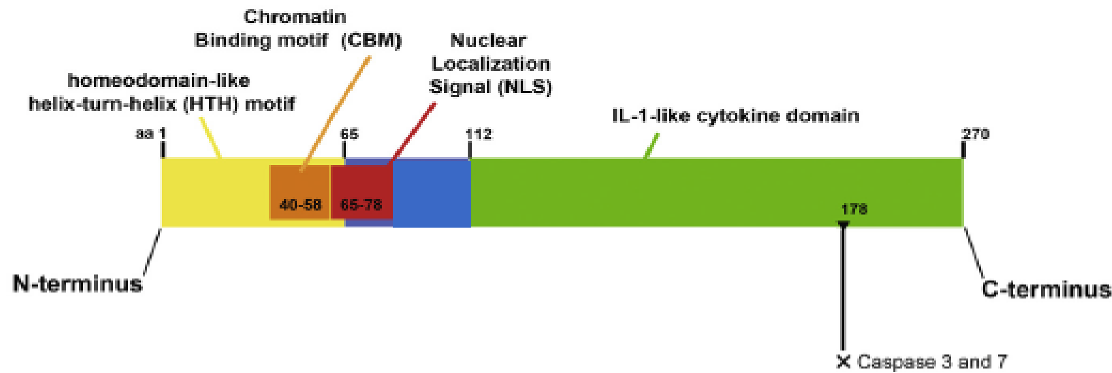
Since its discovery in 2005, interleukin-33 (IL-33), an IL-1 family cytokine, has been most commonly known for its ability to promote T helper 2 (T_H2) responses and association with asthma and autoimmunity (34, 35). Recently, IL-33 and its receptor Growth Stimulation Gene-2 (ST2) have emerged as indicators of cellular stress and inflammation (34, 36). In mice, it has been published that inflammatory stimuli, such as viral infections LCVM and influenza A, induce IL-33 in the lungs (37, 38). Also, the development of atherosclerosis in rodents has been associated with an increase in vascular tissue expression of this cytokine (39). Clinical measurements of the soluble form of the IL-33 receptor, sST2, have been noted to be predictive of mortality and cardiovascular disease risk in patients with heart failure or myocardial infarction (MI) (40-42). Interestingly, in cases of inflammatory bowel disorders such as Crohn's Disease and ulcerative colitis, both sST2 and IL-33 are found to be increased in serum (43-45). Another study showed that elevated levels of sST2 at time of diagnosis of graft-versus-host disease (GVHD) were prognostic of treatment-resistance and death (46). Accumulating evidence in both clinical and experimental models support the concept that increasing expression of IL-33 and/or ST2 could possibly serve as quantitative indicators of pathological inflammation.

The role of IL-33 during allograft rejection is currently unknown. Using a murine model of heart transplantation (HTx), we previously published that ST2 was increased locally, but not systemically, in allograft tissue during acute rejection (47, 48). In a separate article, it was described that sST2 was elevated in the sera of adult HTx patients during ACR (49). These data suggests that increases in ST2 in HTx EMB and/or serum may be a sensitive indicator of HTx

rejection. Also, given the observed systemic and local upregulation of IL-33 and ST2 during pathological inflammatory conditions of the vasculature and mucosa, it is possible that either factor could serve as biomarkers or sensitive indicators of rejection in allograft tissue (36).

1.4.2.1 Immunobiology of IL-33 and ST2

IL-33 is a recently identified member of the IL-1 family and is constitutively expressed in endothelium and epithelium, but can be induced in myofibroblasts and myeloid cells by inflammatory stimuli (34, 36). IL-33 is most commonly known for its role in asthma and autoimmune conditions (34, 50). IL-33 is generated as a biologically active cytokine that consists of a C-terminal cytokine domain, N-terminal nuclear localization sequence, and DNA binding domains that targets the protein to the nucleus (36). However, in contrast to other IL-1 family members, IL-33 is inactivated by cleavage with caspases 3 and 7 (51-53). Thus, the proteolytic mechanism that releases IL-33 from the nucleus for secretion is not well established. However, the active full length form IL-33 is thought to be released from the nucleus through tissue necrosis. Released IL-33 has also been proposed to act as an "alarmin" that alerts the immune system to pathology (51-53) and has pleotropic effects on adaptive and innate immune cells that express its receptor ST2. Specifically, by targeting leukocytes, including T cells, mast cells, eosinophils, basophils, innate lymphoid cells, and myeloid APC, IL-33 drives type 2 response dominated by IL-5 and IL-13 (35, 47, 54) Yet, IL-33 can also support immune regulation through the expansion of regulatory T and myeloid-derived suppressor cells (47). IL-12 induces ST2 on NK and $CD8^+$ T cells and subsequent exposure to IL-33 augments IFN-γ production by these cells (55, 56).



Modified from Liu and Turnquist, 2013 ***Cytokine.*** The IL-33 cytokine contains two domains: a homeodomain-like helix-turn-helix domain at the N-terminus and an IL-1-like cytokine domain located at the C-terminus. It also contains a nuclear localization signal and a chromatin binding motif which mediate association which histones and nuclear translocalization. Site where proteolytic cleavage and deactivation by caspase 3 and 7 occurs is marked by an X .

Figure 1: Human Interleukin-33 molecule

The IL-33 receptor, Growth Stimulation Gene-2 (ST2), which the literature sometimes denotes as IL-1 receptor-like 1 (IL1RL1), exists both as a transmembrane form and soluble decoy receptor, referred to as sST2. Both proteins are generated through alternative splicing of a single mRNA (57, 58). ST2 is a member of the Toll-like/IL-1 receptor (R) family and utilizes the IL-1R accessory protein to induce NF-kB and mitogen-activated protein kinases following IL-33 ligation (36). Both the soluble and membrane bound isoforms of this molecule are augmented in leukocytes and non-hematopoietic cells by inflammatory stimuli, including IL-1β, TNFα, or lipopolysaccharide (LPS), and well as mechanical stress (59).

2.0 STATEMENT OF THE PROJECT AND SPECIFIC AIMS

Innovation in post-transplant therapeutic care and rejection diagnostics is required as evolution of IST has failed to significantly improve long-term allograft survival. Currently, the standard for diagnosis of allograft rejection is pathologist-graded tissue biopsy, a costly and cumbersome methodology that is poorly suited for routine surveillance (14). This procedure is particularly burdensome on high-risk pediatric patients who require frequent monitoring (27, 28). The identification of a reliable graft/serum-derived biomarker will improve on long-term outcomes by enabling earlier diagnosis and treatment of rejection. It should also support precise adjustment of IST to lowest levels possible for effective rejection protection without repeated invasive biopsy procedures. This will be especially impactful for pediatric patients, who often require frequent monitoring (27, 28) and are most susceptible to the cumulative side effects of a lifelong IST (12). Upregulation of IL-33 and ST2 during pathological inflammatory conditions of suggest they could serve as biomarkers or sensitive indicators of rejection in allograft tissue (36). Thus, *we hypothesize that expression of both IL-33 and ST2 is distinctly modulated locally in allografts during rejection due to pro-inflammatory cytokines. We anticipate that these proteins can serve as sensitive and precise biomarkers of early transplant rejection.*

To test our hypothesis we proposed two specific aims:

1. **Determine if ST2 and IL-33 are modulated locally in allograft tissue by inflammatory cytokines.** In our rodent studies we demonstrate that IL-33 and ST2 is modulated in rejecting cardiac allografts. To substantiate this observation in a cohort of

pediatric heart transplant recipients, we utilized Quantum-dot immunolabeling (Qdot) coupled with Whole-Slide Imaging (WSI) to evaluate tissue expression of IL-33 and ST2 in patient EMBs. To quantitate the data derived from these methods we utilized NearCyte analytical software. To examine upregulation of ST2 and IL-33 in small bowel transplant recipients, gene expression in intestinal biopsies received from the University of Nebraska was measured as part of a 384 gene qRT-PCR array. Assessment of genes and associated networks was completed using Ingenuity Pathway Analysis (IPA) software.

2. **Define if systemic alterations in ST2 and IL-33 levels are indicative of transplant rejection.** To evaluate this aim, we measured levels of sST2 and IL-33 in sera collected from the same transplant groups described in Aim 1 using Enzyme-Linked Immunosorbent Assay (ELISA). Results were interpreted based on absorbance and compared between groups.

3.0 METHODS

3.1 PRELIMINARY RODENT STUDIES

Rodent Heterotopic Heart Transplantation*:* C57BL/6J ($H2^b$, B6) and BALB/c ($H2^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the specific pathogen-free facility of the University of Pittsburgh, School of Medicine used at 8–12 wk of age. Heterotopic (intra-abdominal) heart transplantation was performed using microsurgical techniques as described by Corry et al. (60) by Dr. Quan Liu of the Turnquist laboratory. Experiments were conducted under an institutional animal care and use committee-approved protocol and in accordance with National Institutes of Health guidelines.

Protein extraction and Immunoblotting*:* Total HTx protein lysates were generated in RIPA buffer supplemented with protease inhibitors. Lysates were cleared by centrifugation (13,000 rpm for 20 min) and the protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA). SDS/PAGE, transfer onto PVDF membranes and immunodetection were performed. For primary antibodies, anti-ST2 (Ab25877; Abcam) and anti-GAPDH (Sigma-Aldrich, St. Louis, MO) were used as described previously (61). The HRP-labeled secondary antibodies were used followed by detection with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Watham, MA). Western blot assays for rodent studies were completed by Dr. Quan Liu.

3.2 PATIENT POPULATIONS

Pediatric Heart Transplant Recipients: In collaboration with Dr. Brian Feingold we received samples collected from among 39 patients (demographics represented in **Table 3**) enrolled in an IRB approved (IRB# 0702122), prospective study of the prevalence of alloantibodies after heart transplantation at Children's Hospital of Pittsburgh of UPMC (median age 6.4 years). We identified acute cellular rejection (n=8 in 6 patients), antibody-mediated rejection (n=4 in 4 patients), and no rejection biopsies (n=13 in 11 patients) for which there was concurrent serum available to us for IL33/ST2 ELISA analysis. We also separately identified ACR events (n=7 in 4 patients), AMR events (n=7 in 5 patients), and no rejection events (n=7 in 6 patients) for which EMB specimens were available for quantum dot analysis. For each patient demographic and pertinent clinical data were obtained. For both analyses, patient events were matched by age, sex and time after HTx.

All patients received thymoglobulin induction therapy with subsequent tacrolimus-based immunosuppression plus adjunctive sirolimus or mycophenolate mofetil. Only patients with a positive, donor-specific cytotoxicity crossmatch or antibody-mediated rejection were treated with chronic steroids. All patients underwent serial post-HTx allograft surveillance EMB at 1-2 weeks, 2-4 weeks, 2 months, 4 months, 6-7 months, and 10-12 months. Patients also underwent EMB if rejection was suspected and to assess resolution following the rejection treatment.

Table 3: Patient Demographics of Pediatric Heart Transplant Recipients

Clinical Characteristics of Pediatric Heart Transplant Recipients			
Total number of patients	**39**		100%
Males	**23**		59%
Females	**16**		41%
Recipient age at transplant			
Mean	**8.0**	±6.4	
Median	**7.0**		
Recipient age at transplant <18y	**37**		95%
Recipient age at transplant ≥18y	**2**		5%
Etiology of heart failure			
Congenital heart disease (CHD)	**13**		33%
Cardiomyopathy (CM)	**22**		56%
Re-Transplantation (ReTx)	**4**		10%
Ave. Graft Survival (Years as of 11/18/2013)	**4.88**	**±1.56**	
T-cell crossmatch (TXM)			
Negative	**34**		87%
Positive	**2**		5%
Strong Positive	**3**		8%
B-cell crossmatch (BXM)			
Negative	**9**		23%
Postive	**3**		8%
Strong Positive	**2**		5%
Not Done	**25**		64%
Sex Matched Donor (Y)			46%
Ethnicity/Race			
White	**30**		77%
Arabic	**1**		3%
White/Asian	**1**		3%
White/Arabic	**1**		3%
Black	**5**		13%
Hispanic	**1**		3%

***Small Bowel Transplant Recipients**:* We obtained samples from 18 individuals (83% < 18 years old) (demographics represented in **Table 4**) undergoing isolated small bowel or multi-visceral transplantation from Dr. Ryan Fisher at the University of Nebraska Medical Center from 2004 to 2009. Samples were gathered from an established, institutional review board-approved tissue collection bank (IRB approval #417-02). Patients and/or parents of patients who underwent SBTx and who are scheduled to undergo surveillance intestinal biopsy, diagnostic

intestinal biopsy, or surgery involving the intestine were informed of the study. Sample tissue collected included additional biopsy specimens at the time of endoscopy, and the concomitant collection of extra blood. The intestinal tissue specimens were stored at -80°C in RNAlater (Applied Biosystems/Ambion, Austin, TX). The blood was centrifuged, and the serum stored at -80°C.

Table 4: Patient Demographics of Pediatric Small Bowel Transplant Recipients

Clinical characteristics of small bowel transplant recipients		
Total number of patients	**18**	
Male	**7**	*39%*
Female	**11**	
Recipient age at transplant < 18 y	**15**	*83%*
Recipient age at transplant ≥ 18 y	**3**	
Etiology of intestinal failure		
NEC	**1**	*6%*
Atresia	**3**	*17%*
Volvulus	**2**	*11%*
Gastroschisis	**3**	*17%*
Pseudo-obstruction	**3**	*17%*
MVID	**4**	*22%*
Hirschsprung	**2**	*11%*
Isolated bowel transplant	**3**	*17%*
Multivisceral transplant	**15**	

NEC = Necrotizing enterocolitis; MVID = Microvillus inclusion disease

3.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Levels of serum sST2 in transplant patients and normal controls were measured using commercially available ELISA kits (DuoSet, R&D Systems, Minneapolis, MN, USA). All serum samples were isolated at the time of collection, frozen the same day and stored at minus 80°C until use. On the day of assessment, samples were batch thawed and determinations of IL-33 and sST2 completed. All samples were analyzed in triplicate according to manufacturer specifications. Assay limits of detection are 31.25 pg/mL (sST2) and 23.44 pg/mL (IL-33). Absorbance was measured utilizing a Benchmark Plus™ plate reader (Bio Rad, Hercules, CA, USA) at a wavelength setting of 450nm.

3.4 QUANTUM DOT IMMUNOLABELING (Qdot)

Paraffin embedded patient EMBs were sectioned (4 µm) onto slides, which were then deparaffinized, steamed with antigen retrieval buffer pH 9.0 for 30 mins, and followed by blocking with avidin/biotin (Vector) and Serum Free protein block (Dako). Slides were then labeled overnight with rabbit anti-ST2 antibody (1:30, Sigma Aldrich, St. Louis, MO) and rat anti-IL-33 antibody (1:30, R&D, Minneapolis, MN). On day 2, after the slides were washed multiple times in PBS, anti-rabbit and rat biotinylated IgG secondary antibodies (1:200) were added consecutively and followed by respective streptavidin conjugated Qdot® 705 (1:50, ST2) and 655 (1:50, IL-33) (Life-Technologies) with avidin/biotin blocking between steps. Finally, slides were counter-stained with Hoechst nuclear dye and dehydrated by washing two times each

with 95 and 100% ethanol, and allowed to air dry. Slides were then coverslipped using Qmount™ Qdot® mounting media (Life-Technologies).

3.5 WHOLE-SLIDE SCANNING

Whole Slide Images (WSI) were produced by Dr. Kumiko Isse of the Demitrius laboratory and captured via a Zeiss Mirax MIDI scanner utilizing a Plan-Apochromat 40x/.95N.A. objective lens. AxioCam MRm digital CCD camera (Carl Zeiss, Jena, Germany) and specifically selected excitation/emission Qdot filters (Omega Optical, Brattleboro, VT) as previously described(62, 63).

3.6 IMAGE ANALYSIS

Pixel-based image analytics were performed on the WSI utilizing the internally developed-IAE-NearCYTE (http://nearcyte.org) imaging software developed by Andrew Lesniak. Manual outlining of EMB regions for each sample (See **Fig. 2**) were completed and negative areas (gaps, blood clots, tissue folds) excluded. Following the manual establishment of thresholds for fluorophore positivity, the software automatically quantifies the resultant fluorophore intensity and total expression area in the defined regions, and the data were exported to Microsoft Excel for numeric analysis.

3.7 QUANTITATIVE REAL-TIME PCR (qRT)-PCR

We also evaluated the tissue expression of IL-33 and its membrane-bound receptor ST2 mRNAs in intestinal allograft biopsies from patients with and without acute rejection by quantitative real-time (qRT)-PCR. Intestinal tissue mRNA expression for IL-33 and ST2 (among other genes) was measured using a 384 gene Inflammatory Cytokines and Receptors gene expression PCR array (Qiagen, Fredericksburg, MD). Fold-Change ($2^{-\Delta\Delta CT}$) was calculated as normalized gene expression ($2^{-\Delta CT}$) in the Test Sample divided the normalized gene expression ($2^{-\Delta CT}$) in the Control Sample. The p values were calculated based on a Student's t-test of the replicate $2^{-\Delta\Delta CT}$ values for each gene in the control group and treatment groups.

3.8 PATHWAY ANALYSIS

The 384 assessed genes were classified and associated networks were determined by Dr. Jeremy Lott, using IPA (Ingenuity© Systems, www.ingenuity.com), with stringency set using sample p-values with a cut off value of $p \leq 0.05$, up and down regulated genes were considered in the analysis using the Ingenuity Knowledge Base. The upstream regulators of altered genes were also analyzed and ranked based on both absolute z-score and p-value as determined by IPA software.

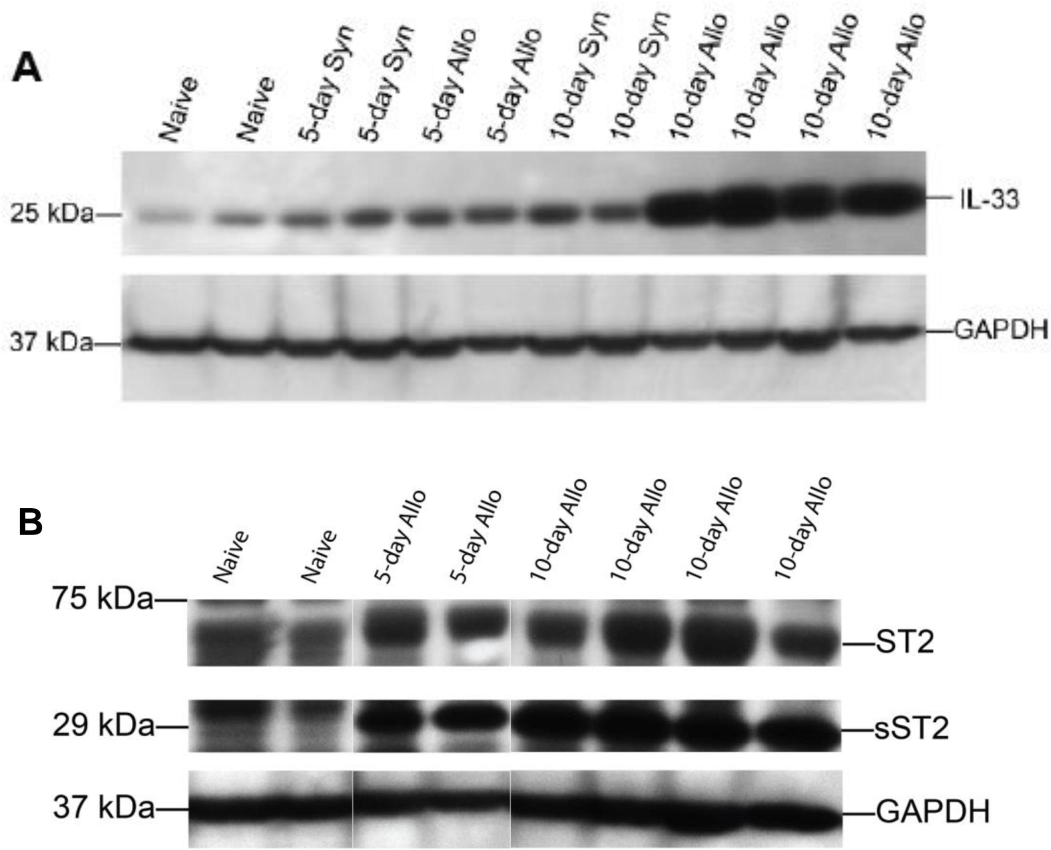
3.9 STATISTICAL ANALYSIS

Statistical methods to determine data significance were completed using Prism 5 (GraphPad Software). Differences between groups were determined using unpaired Student’s t-tests and Welch's correction when appropriate. Results were considered statistically significant if $p<0.05$.

4.0 RESULTS

4.1 PROOF OF PRINCIPAL STUDIES USING A RODENT HTX MODEL

To preface our studies in pediatric transplant recipients our group performed a number of experiments utilizing a mouse heterotopic transplant model of rejection. Using this approach, we published that ST2 increases within allograft heart tissue, an effect not observed in naïve hearts or in native heart of the recipients (47). These data indicate that this ST2 increases are most likely due to local alloimmune-based inflammation in the graft (47). More recently, using Western blot analysis testing lysates generated from allogeneic and syngeneic heart transplants excised at day 5 and 10 along with lysates from naïve hearts, we detected a dramatic increase in IL-33 in 10-day allograft tissue (**Fig. 2A**), an observation that had not been previously demonstrated in any transplant model. Our group next sought to use Western blot analysis and determine which isoform of ST2 is found in the rejecting allograft hearts. Utilizing an antibody that recognizes both ST2 isoforms and relying on differences in molecular weight to distinguish the transmembrane form (ST2; ≈60 kDa) from the soluble form (sST2; ≈30 kDa), we confirmed that both forms were increased dramatically in allograft hearts (**Fig. 2B**, top and middle panels). Naïve hearts displayed expression of the protein, but not to the degree of the rejecting allografts. These data indicate that both IL-33 and ST2 are increased in allografts during rejection.



C57BL/6J (B6) or BALB/cByJ hearts were heterotopically transplanted into B6 recipients and were explanted on the indicated day post-Tx. Following tissue disruption, total cellular lysates were probed with an antibody for IL-33 (R&D). (A) Western blot analysis shows a marginal increase of IL-33 in 5-day and 10-day syngeneic (syn) and 5-day allogeneic (allo) allograft hearts over naïve B6 hearts. A dramatic increase in IL-33 is observed in 10-day (allo) allograft hearts. (B) Western blot analysis for ST2 shows a notable increase in both the transmembrane form of ST2 (top panel) and in sST2 (middle panel) in day 5 and 10 allo hearts compared to naïve hearts. Following analysis, membranes were re-probed with an antibody for GAPDH (Sigma) following stripping, which was used as a loading control.

Figure 2: IL-33 and ST2 are profoundly modulated during rodent heart transplant rejection

4.2 AIM 1: DETERMINE IF ST2 AND IL-33 ARE MODULATED LOCALLY IN ALLOGRAFT TISSUE BY INFLAMMATORY CYTOKINES

To establish if expression of ST2 and IL-33 are modulated during clinical heart rejection (HTx) as observed in prior murine studies, we investigated IL-33 and ST2 staining patterns in EMBs obtained from a cohort of pediatric HTx recipients at the time of diagnosis of AMR (donor-specific alloAb^{+} and/or C4d staining) or ACR (ISHLT Grade $\geq$2R), as well as in 5 patients classified as non-rejectors (NoR) that remained free from either ACR or AMR (ISHLT 0-1R DSA-negative) in the first year following HTx, including the time of EMB collection/pathologist evaluation. Patient demographics are highlighted in, **Table 5**. Following Qdot-based multicolor immunostaining of EMBs with antibodies specific for human ST2 and IL-33, WSI analysis was utilized to quantitate expression levels of each of these molecules which enabled identification of target tissue regions and exclusion of areas of obstruction (clots and tissue damage). An example of the WSI analytical process is depicted in **Fig. 3A**. **Figure 3B** and displays a tissue representative of the non-rejecting group (post-operative date (POD) 331 and 26). In these EMBs there is limited observable expression of ST2 and nuclear IL-33. This is in stark contrast to EMBs of patients with ACR (2R ISHLT score; POD 25 & 308; **Fig. 3C),** which exhibit dramatic increases in nuclear IL-33 in endothelial cells of the microvasculature (indicated by yellow arrow in **Fig. 2C**) in addition to an abundance of ST2 in the HTx myocardium. EMBs collected from recipients with AMR (**Fig. 2D**, depicted at POD 11) display similar levels of diffuse ST2 staining throughout the myocardium and increased nuclear IL-33. These visual observations were reinforced following analysis utilizing the NearCYTE software.

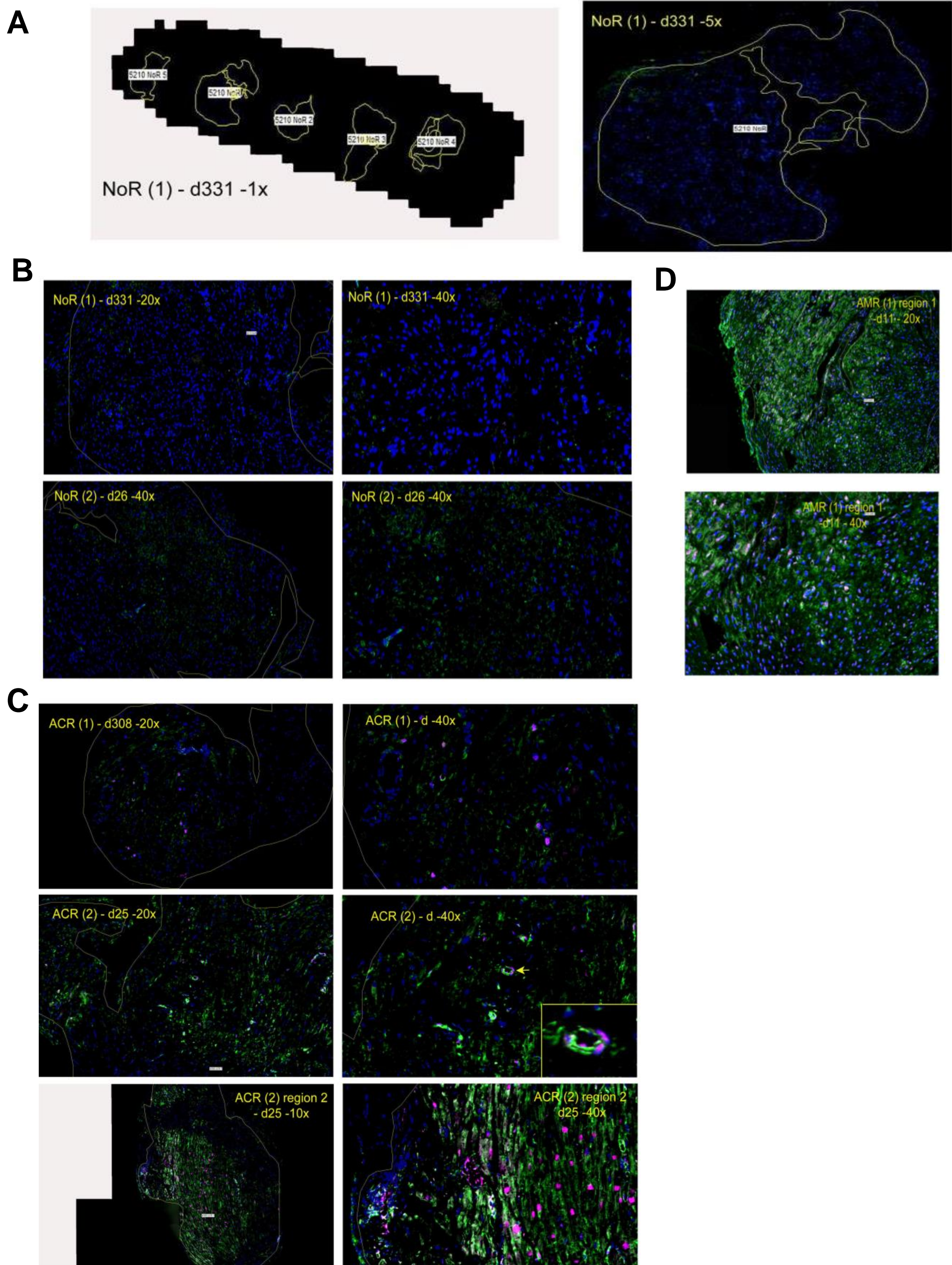


Figure 3: IL-33 and ST2 levels distinguishes pediatric HTx patients suffering rejection

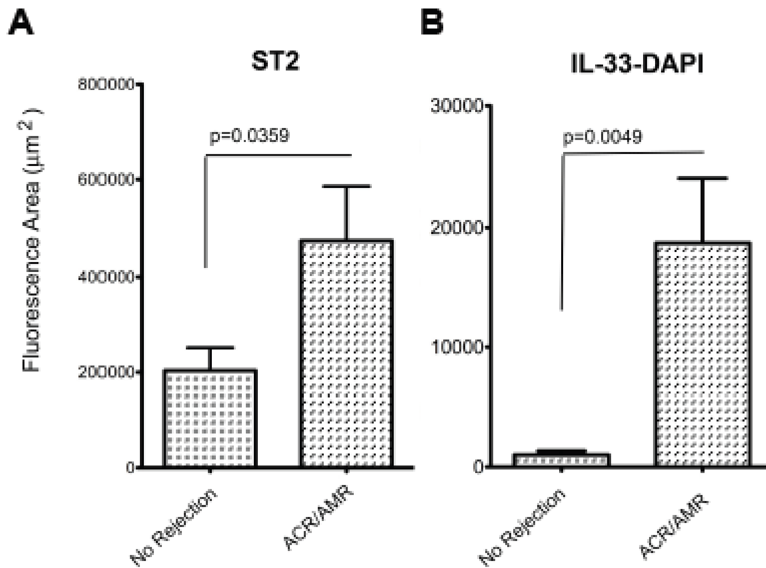
(A) Whole slide EMB analysis using NearCyte software as described in the methods. Multicolor immunostaining for IL-33 (Magenta), ST2 (Green) and DAPI (blue) was completed on ISHLT-graded pediatric HTx patient EMB. Following staining, slides were scanned with a Mirax MIDI (3D Histech, Budapest, Hungary). Images were generated with the NearCyte program and presented at magnifications of 1, 5, 10, 20, & 40X. (B) Top panels display a representative Non-rejecting group (NoR) sample at POD 331 and 26. NoR (n=6) display little IL-33 and confined areas of ST2 cells. (C) Displays sections of an EMB from a patient undergoing ACR (>3A ISHLT score) POD 305 and 25. ACR patient EMB (n=4) display distinct modulation of nuclear IL-33 and increased ST2. (D) AMR EMB (n=5) showed similar results as ACR (depicted at POD 11).

Figure 4 depicts the quantitative data derived using the NearCYTE program. The output data are presented as florescent area, which is a ratio of number of positive pixels over sum measurements of the selected tissue regions. We observed a significant increase in area staining positively for both ST2 and nuclear IL-33 (p=0.0359 and 0.0049 respectively) in rejecting EMBs compared to those from non-rejecting recipient samples. These data substantiate the observations presented in **Figure 3**.

Table 5: Characteristics of Heart Transplant Recipients used in Qdot Analysis

	n=	Males	Age at Tx	±STD	POD	±STD
No Rejection	n=5; 7 incidences	40%	5.54	4.78	63.71	118.11
ACR/AMR	n=9; 14 incidences	44%	7.22	5.26	73.29	96.69
	P=		*0.57*		*0.42*	

p-values indicate that is there is no significant difference in age or POD between individuals



Tissue analyses were completed as described in Fig 3. Graphs depict calculated Fluorescence Area ($microns^2$). At points of diagnosed ACR and AMR, levels of ST2 and IL-33 were significantly elevated compared to non-rejecting patients (p=0.0359, ST2 & p=0.0049, IL-33) at similar time points post-transplant (A & B). Statistical analysis was completed using a Student's t-test

Figure 4: Graphical representation of IL-33 and ST2 as measured by Qdot analysis

To investigate if ST2 and IL-33 levels have relevance to indicate rejection after transplant of other organs, we examined IL-33 and ST2 gene expression in small bowel transplant (SBTx) recipients. Specifically, we analyzed IL-33, ST2, along with 382 other immune related genes by qRT-PCR in samples from 7 patients at either times of rejection (n=9) or quiescence (n=10). Mean ST2 expression exhibited a significant 3.94-fold increase (p=0.0229) in rejection samples when compared to controls (**Fig. 5A**). IL-33 was also increased, however not to a significant degree. Thus, examination of biopsy mRNA expression supports distinct modulation of the ST2 during SBTx rejection. These data, with our above Western Blot data in rejecting rodent HTx or quantitative Qdot assessment of HTx EMB, provide further support for increases in local ST2 in allograft tissue during rejection episodes.

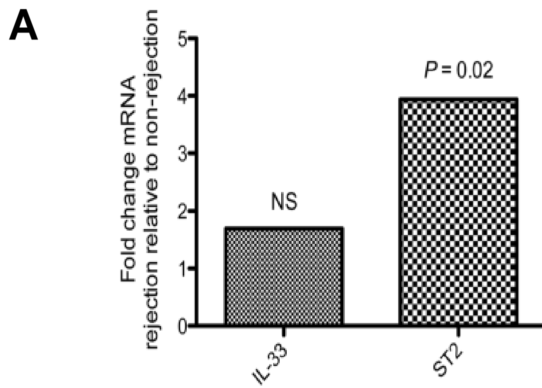
In addition to ST2 message, we were able to identify other genes whose expression was significantly modulated during SBTx rejection (Table 6). A total of 36 genes of the 384 assessed were modulated significantly during SBTx, 32 increased and 4 decreased when compared to non-rejection (Table 6 and **Fig. 5B**). In spite of increased nuclear IL-33 in rejecting HTx samples (Fig. 4 and 5), the message for IL-33 was not found significantly modulated (Table 6 and Fig. 5B). These data may suggest differences in tissue specific differences in IL-33 regulation. Alternatively, modulation of IL-33 mRNA may be a very early change during rejection and not evident at the time of diagnosed rejection.

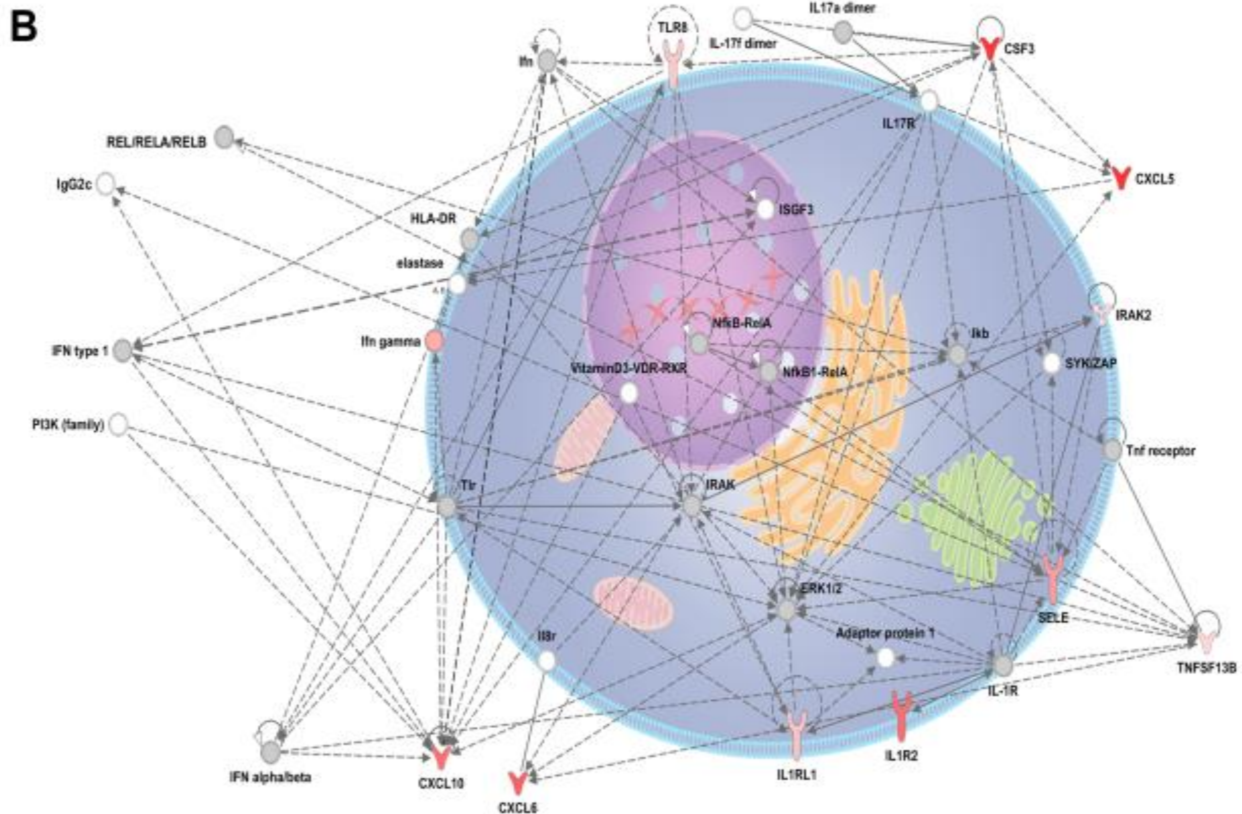
To further our understanding of the regulation of genes involved in SBTx rejection, these qRT-PCR data were probed using IPA pathway and network analysis. This generated associated pathways incorporating sets of both upregulated and downregulated genes. Based on fold-changes and *P*-values, the top modulated pathway contained ST2 and the representation of the gene-gene network is depicted in **Fig. 5B**. This information was analyzed once again using IPA to determine upstream transcriptional regulators that could explain the observed gene expression changes. IPA Upstream Regulator Analytic identified that TNF, LPS, IL-1β, and IFNγ were the predicted activators of this network containing ST2 (**Table 7**). These data are consistent with previous determinations that IL-1β, TNFα, or LPS cause production of sST2 in both cells and tissues (59, 64, 65). Likewise, these data indicate that local pro-inflammatory cytokines likely mediate the observed increase in allograft ST2 during rejection.

Table 6: Up and Down Regulated Genes Comparing Rejection to Control Group

Expression **Increased**	**Gene**	**Fold Regulation**	**p-value**
1	CSF3	14.7933	0.023996
2	CXCL5	13.2232	0.049074
3	CXCL6	10.4152	0.019259
4	CXCL10	9.3746	0.043514
5	IL1R2	9.3664	0.040937
6	CSF2	8.3832	0.025132
7	SELE	6.3121	0.014821
8	CTLA4	6.113	0.042203
9	MMP9	5.4335	0.020145
10	IFNG	5.237	0.007728
11	MADCAM1	4.9033	0.009603
12	BDKRB1	4.7097	0.00645
13	CD80	4.6207	0.019928
14	PLA2G7	4.2483	0.030589
15	**IL1RL1**	**3.9415**	**0.022935**
16	CCR4	3.8571	0.022242
17	CCL2	3.7402	0.012185
18	ICAM1	3.7257	0.014463
19	PTAFR	3.5569	0.027089
20	TLR8	3.5262	0.013663
21	OAS2	2.9562	0.018015
22	ITGAM	2.7774	0.025266
23	ITGB2	2.6459	0.017446
24	CD86	2.4816	0.035482
25	TNFSF13B	2.4666	0.021849
26	IRAK2	2.3559	0.03155
27	OASL	2.3497	0.032616
28	BDKRB2	2.1952	0.032777
29	ISG15	2.1538	0.043216
30	IFITM1	2.1519	0.021527
31	RIPK2	2.1306	0.044181
32	GBP1	2.104	0.046732
	IL33	**1.6942**	**0.755656**
Decreased			
1	PPARA	-2.084	0.026054
2	ACE	-3.6617	0.043689
3	IL5	-4.6147	0.021381
4	VIPR1	-7.2288	0.027744

Table was generated by IPA software comparing fold regulation between the two groups





Changes in expression of 384 immune-related genes, including *IL-33* and *ST2*, were determined by quantitative RT-PCR (qRT-PCR) in pathologist scored biopsies from SBTx patients at time points determined to be rejection-free (non-rejection; n=14) or undergoing active rejection (n=9). Presented data represent fold-change $2^{\wedge(-\Delta\Delta CT)}$ calculated as the normalized gene expression $2^{\wedge(-\Delta\Delta CT)}$ in the Test Sample divided by the normalized gene expression $2^{\wedge(-\Delta\Delta CT)}$ in the Control Sample. The p-values were calculated based on a Student's t-test of the replicate $2^{\wedge(-\Delta CT)}$ values for each gene in the control group and treatment groups. When compared to non-rejections, IL-33 mRNA levels from

rejections were not statistically different, however, levels of ST2 mRNA levels were increased in rejections (p=0.02) approximately 4-fold when compared to non-rejections (A). (B) To further understand the regulation of genes involved in small bowel transplantation rejection, fold-changes and p-values from genes were further analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems Software). Upregulated ST2 (IL1RL1) in network of significantly modulated genes. Depicted is a graphical representation of the highest-scored network generated from our data set and calculated on fold-change and associated p-values. Level of upregulation is indicated by intensity of red color at that node. Gray nodes are part of the network, but were not significantly modified between rejecting and non-rejecting samples. Solid lines indicate direct relationships, while dashed lines depicted indirect relationships.

Figure 5: Tissue expression of ST2 increases in patients undergoing acute rejection of SBTx

Table 7: TNF and IL-1β Amongst Top 20 Predicted Upstream Activators of ST2

	Predicted Activated Upstream Regulator	Molecule Type	Activation z-score	P-value of overlap
1	TNF	cytokine	5.12	2.30E-36
2	lipopolysaccharide	chemical drug	4.65	7.18E-30
3	IL1B	cytokine	4.19	1.75E-23
4	IFNG	cytokine	4.05	2.62E-30
5	poly rI:rC-RNA	chemical reagent	4.05	1.08E-19
6	MYD88	other	4.05	5.43E-22
7	IRF7	transcription regulator	4.05	1.39E-18
8	IL1	group	4.05	1.09E-19
9	TICAM1	other	4.05	1.25E-17
10	Interferon alpha	group	4.05	4.25E-21
11	TLR3	transmembrane receptor	4.05	3.77E-18
12	TLR4	transmembrane receptor	4.05	6.61E-22
13	NFkB (complex)	complex	4.06	1.28E-15
14	IFNL1	cytokine	4.06	1.10E-16
15	P38 MAPK	group	4.06	4.21E-18
16	IL6	cytokine	4.06	5.01E-16
17	E. coli B5 lipopolysaccharide	chemical - endogenous non-mammalian	4.06	4.61E-16
18	IL17A	cytokine	4.06	7.38E-15
19	E. coli lipopolysaccharide	chemical - endogenous non-mammalian	4.06	3.41E-15
20	ERK	group	4.06	1.62E-10

Table was generated by IPA software, data was derived from activation z-score that determines likely regulators based on p-value

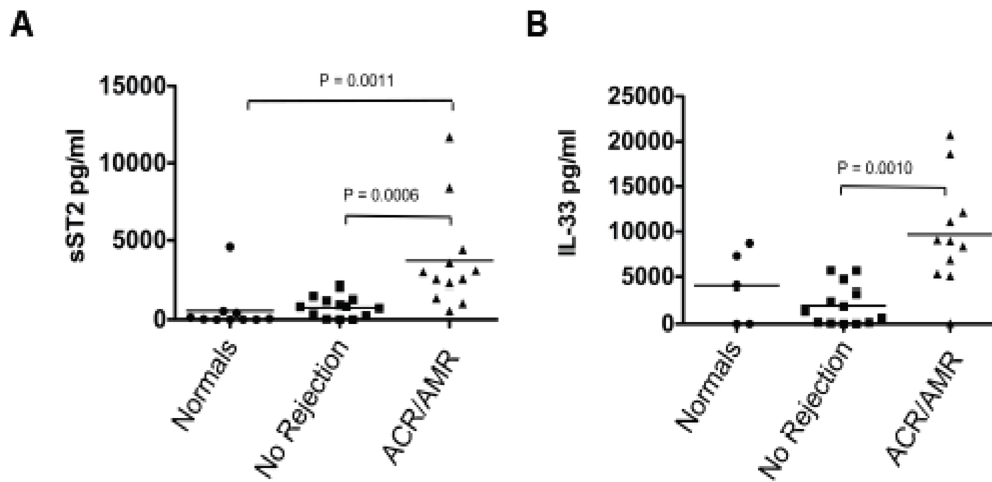
4.3 AIM 2: DEFINE IF SYSTEMIC ALTERATIONS IN ST2 AND IL-33 ARE INDICATIVE OF TRANSPLANT REJECTION

To determine if both IL-33 and ST2 are identifiable systemically during heart transplant rejection we performed ELISA assays on sera collected through routing surveillance of the HTx cohort as mentioned in Aim 1. **Table 8** summarizes recipient demographics. We observed that serum sST2 significantly increased in pediatric HTx patients at the time of acute rejection (**Fig. 6A**) this was in comparison to normal, healthy controls, as well as patients not classified as undergoing acute rejection (NoR) (p=0.0011 and p=0.0006). As with sST2 we observed a similar significant increase with IL-33 (p=0.0010) **(Fig. 6B)**. These data reinforce the Qdot observations in HTx EMBs in Aim 1, indicating that increased ST2 and IL-33 can be found both locally and systemically during diagnosed rejection episodes in pediatric HTx recipients.

Table 8: Characteristics of Pediatric Heart Transplant Recipient Samples used for ELISA

	n=	Males	Age at Tx	±STD	POD	±STD
No Rejection	n=11; 13 incidences	55%	6.1	6.0	32.3	7.3
ACR/AMR	n=10; 12 incidences	50%	8.1	5.2	28.6	7.3
	P=		*0.42*		*0.11*	

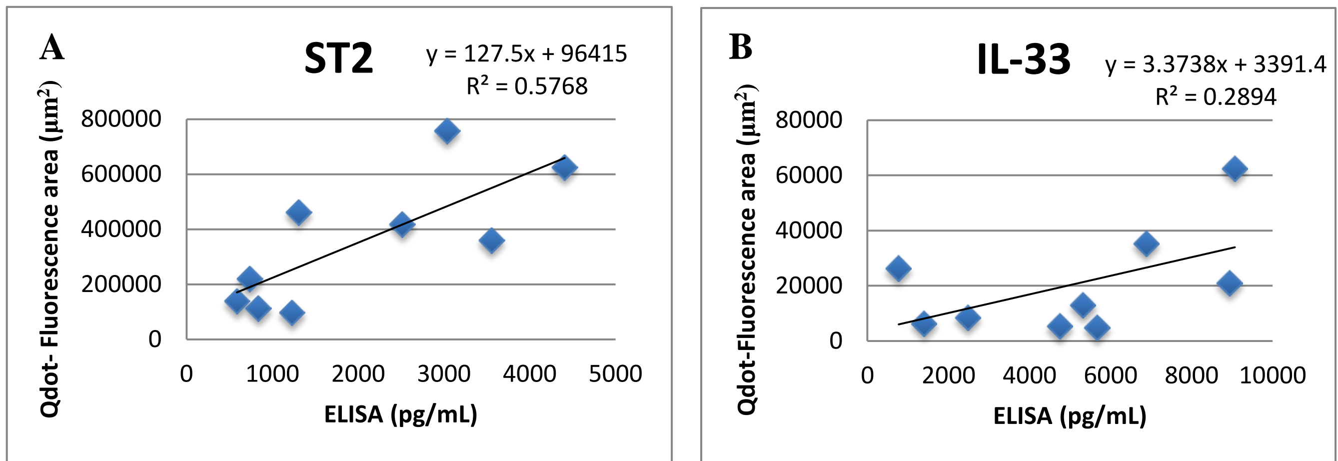
p-values indicate that is there is no significant difference in age or POD between individuals



Serum ST2 and IL-33 levels were assessed by ELISA in pediatric HTx patient sera during post-transplant day 21-50. Recipients were group during this period as either "Non-Rejection" patients, who were free from acute cellular rejection (ACR;>2 based on pathologist International Society for Heart and Lung Transplantation [ISHLT] scoring) episodes and antibody-mediated rejection (AMR; donor-specific alloAb^{+} and/or C4d staining^{+}) or those suffering one or more incidence of AMR/ACR during this period. HTx patients suffering ACR or AMR ("ACR/AMR"; n=10) displayed significantly increased levels of (A) sST2 and (B) IL-33 compared to those in the non-rejection group ("No Rejection"; n=11)and healthy controls (Normals; n = 9). Indicated significance levels were calculated via Prism 5 and a two-tailed, non-paired Student's 't' test.

Figure 6: Serum sST2 and IL-33 are elevated in pediatric HTx recipients suffering rejection episodes

To determine if local levels of IL-33 and ST2 correlate with systemic levels we compared quantitative data from Qdot staining and ELISA analysis derived from EMBs and sera collected at the same time-point from several individuals (n=9). We found that with ST2 expression there is evidence of correlation between serum levels and tissue expression **(Fig. 7A)**. However, there appears to be no real correlation between tissue and serum expression of IL-33 (**Fig. 7B**).



Expression of ST2 shows some correlation between high and low levels in the tissue and serum (A), however, tissue and serum levels of IL-33 do not seem to demonstrate this same trend.

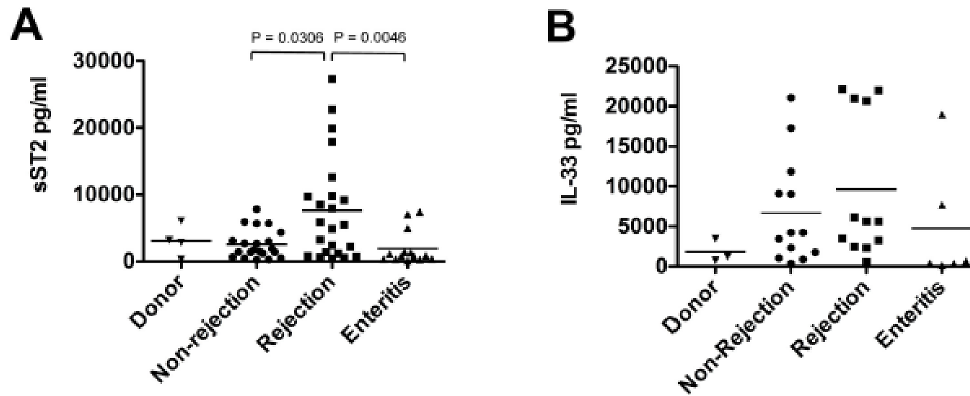
Figure 7: Co rrelative analyses of tissue and serum expression of ST2 and IL-33

To evaluate if these same results could be translated to rejection in SBTx, we assessed levels of IL-33 and sST2 in the sera of pediatric SBTx recipients during quiescence, rejection, or enteritis (demographics summarized in **Table 9**). As we observed in pediatric HTx recipients, serum levels of sST2 were significantly increased at the time of pathologist-diagnosed rejection (30 incidences) compared to time periods in the same patients when rejection was not evident (Non-Rejection; 24 incidences; **Fig. 7A**). Levels of IL-33, however, do not appear to be significantly altered during rejection compared to periods of non-rejection **(Fig 7B)**. Significantly, increases in sST2 (p=0.0306) (**Fig. 7A**) appear to be specific to rejection and differentiated from non-specific enteritis (p=0.0046) (11 incidences) in SBTx patients (**Fig. 7**). In all, these data demonstrate that serum sST2 is elevated during allograft rejection in pediatric SBTx recipients and thus provide further evidence to support systemic sST2 as a biomarker of allograft rejection.

Table 9: Characteristics of Pediatric Small Bowel Transplant Recipients used for ELISA

	n=	Males	Age at Tx	±STD	POD	±STD
No rejection (NR)	n=16; 27 incidences	38%	9.15	10.69	205.9	300.0
Rejection (R)	n=13; 29 incidences	38%	6.53	9.10	591.3	281.6
Non-specific enteritis (NE)	n=10; 11 incidences	30%	4.82	6.15	335.5	392.0
	P=	NR vs R	*0.62*		*1E-05 **	
		NR vs. NE	*0.37*		*0.369*	
		R vs NE	*0.71*		*0.29*	

p-values indicate that there are significant differences in age or POD between individuals within groupings due to data collected overtime from the same patients



(A-B) The level of circulating (A) sST2 and (B) IL-33 was assessed by ELISA in SBTx patient serum as above. Patient samples (n=15) were assessed at times of diagnosed rejection (25 incidences) and display significantly increased levels of sST2 compared to periods of non-rejection (n=18), patients diagnosed with enteritis (n=12), and donor serum samples (n=4). (B) IL-33 does not show a significant difference between non-rejection, enteritis, and rejecting patients. Indicated significance was calculated via Prism 5 and a two-tailed Student's 't' test.

Figure 8: Serum sST2 is elevated in pediatric SBTx recipients during diagnosed rejection episodes

5.0 DISCUSSION

To date the most significant advancements in post-transplant survival have occurred within the first year, due largely to the development of effective immunosuppressant therapies (12).While survival after the first year has been steadily improving within the last 10-20 years, it still follows a downward trend (10, 12). Complications suffered by long term survivors such as malignancy, infection, cardiac allograft vasculopathy (CAV), and renal toxicity, are directly or indirectly related to IST (10). Patients experiencing these complications often require reduced IST, which can precipitate into rejection episodes (22). Monitoring of rejection typically involves a pathologist-graded biopsy and patients who have or are at high risk of rejection often times require several of these a year. This procedure is painful, expensive, and can in itself cause organ dysfunction (14). Even with multiple samples taken per biopsy procedure, there is still limited tissue area available for analysis, which can lead to inaccurate diagnoses. These issues indicate a need for improved non-invasive rejection surveillance. Identification of a sensitive biomarker of early rejection would post-transplant care.

In this study, we have made several novel findings that strongly support the assessment of ST2 and IL-33 as means to aid diagnosis of pediatric transplant rejection. In both pediatric HTx and SBTx we reveal that quantifiable modulations of ST2 take place in the allograft during episodes of rejection. To the best of our knowledge, this represents the first demonstration of increased ST2 in rejecting clinical allograft samples. We also demonstrate that increased levels

of circulating sST2 occur at times of diagnosed rejection in HTx recipients. These findings are consistent with a recent report completed in a case-crossover study of sST2 levels in sera of adult HTx recipients (41). To expand on our findings in HTx recipients, we examined sera from pediatric SBTx recipients and found comparable increases in sST2. These data represent the first assessment of ST2 modulation following SBTx and support our original theory that serum increases in ST2 can be induced in association with inflammation caused by alloimmune responses, not only as a result of heart damage and myocardial stress as currently believed (49).

In addition to our findings in regards to ST2 expression in pediatric transplant recipients, we also acquired novel evidence of upregulation of the cytokine IL-33 in correlation with allograft rejection. IL-33 is associated with several inflammatory driven conditions. However, its presence during rejection has until now been unexplored. As with ST2, we identified increased nuclear IL-33 in rejecting tissues of HTx recipients as well as in the serum. In the case of SBTx, levels of IL-33 were less informative and appeared to be non-specific in differentiating between rejection and quiescent periods. This may be an inherent characteristic unique to the signature of small bowel transplants due to the complicated environment of the gut or evoked by increased inflammation and tissue trauma created by the surgery itself resulting in the release of IL-33 as an alarmin. Nevertheless, further examination is required to assess how IL-33 is modulated during SBTx.

Our recent observations support the concept that increases in systemic sST2 are driven by inflammatory events and are not unique to cardiovascular pathology. Likewise, they provide evidence that during inflammatory diseases, including graft-versus-host disease (GVHD), inflammatory bowel disease, ulcerative colitis, sepsis, arthritis, and now transplant rejection, circulating ST2 is associated with disease pathology. Further supporting this theory is that

increased ST2 observed during ulcerative colitis is decreased when patients are administered anti-TNF therapy (43, 44, 46).

Qdot-based, multicolor immunostaining of pediatric heart transplant EMBs revealed that expression of ST2 is minimally expressed in quiescent HTx biopsies, but is profoundly increased in the myocardium during ACR and AMR. This reinforces our previous observation that ST2 protein is upregulated in rejecting heterotopic murine HTx, but not endogenous recipient hearts (47). Levels of IL-33 were also increased in both groups of rejecting patients with similarly low expression in EMBs of patients not actively rejecting. Analysis of gene expression in biopsies of SBTx recipients revealed an analogous upregulation of ST2 mRNA in samples acquired during a period of rejection. ST2 was also determined to be amongst the most dominantly modulated gene networks and its activity is most likely regulated upstream by TNFα, LPS, IL-1β, and IFNγ.

Although our study has yielded many significant findings, it is not without limitations. One limitation was the inability of our biopsy-based Qdot assessments to distinguish between ST2 and sST2, due to utilization of probes recognizing shared regions of both. However, Western blot analysis performed during preliminary examinations using a model of murine HTx demonstrate that the increased levels of both isoforms of ST2 in the allograft during rejection. Moving forward it will be important to substantiate these rodent observations before regarding the allograft as a possible source of sST2 either by developing Qdot probes specific for the secreted isoform or similarly developing qRT-PCR primers that recognize isoform specific sequences.

Another issue centers around the limited sample size in both our HTx and SBTx cohorts. Since these are both relatively rare procedures, particularly in children, the availability of pediatric samples is limited, which forced us out of necessity to use a retrospective study design.

Likewise, in our examination of HTx recipients, limited serum samples and biopsies were available for assessment, resulting in small sample sizes for both ACR and AMR time points 20-100 POD. Thus, in our Qdot analyses of patient EMBs we chose to assess the groups together in a single "rejection" cohort. Even though both ACR and AMR are representative of alloimmune-mediated graft damage, we do appreciate that they are distinct forms of rejection with distinct characteristics. Nevertheless, the principal goal of our study was to establish if rejection, either ACR or AMR, is able to modulate expression of IL-33 or ST2, either locally in allograft tissue or systemically in the sera of pediatric Tx recipients. Likewise, the emergence of pro-inflammatory cytokines expected to drive ST2 and IL-33 is shared between both ACR and AMR. When ELISA data are assessed individually, both the ACR (n=6; 8 incidences) and AMR (n=4; 4 incidences) cohorts display a significantly increased level of circulating sST2 and IL-33 ($p \leq 0.05$; Unpaired Student *t* test) at POD 20-100 relative to quiescent patients during this timeframe (n=11; 14 incidences). This is in comparison to analysis of individual groups using Qdot analysis, in which each group only displays a trend towards increased ST2 and IL-33 expression ($p \geq 0.05$). To build on our data derived from both tissue and serum analysis, we wanted to determine if local and systemic expression of ST2/IL-33 correlate within a patient at a single time point. Once again, due to our limited sample size we were restricted to only a small number of patients who had both biopsies and serum collected on the same date available for study. Even so, we were able to see a trend where graft ST2 correlated with serum ST2 levels. IL-33 did not correlate between serum and tissue. This may suggest that distinct pathways for release of ST2 and IL-33 exist. Specifically, sST2 in the serum is expected to be secreted rapidly after induced by pro-inflammatory cytokines. Alternatively, IL-33, while increased in the graft may not become evident in the sera until released during tissue damage.

We encountered another limitation when assessing our SBTx studies. Due to the absence of rejection-free SBTx recipients and limited availability of control or donor tissue, we were required to compare episodes of rejection, quiescence, or diagnosed enteritis within the same patient. While this situation may have created difficulty in assessment of rejection vs. a true rejection free environment, it did provide us with a unique opportunity. It enabled us to gain insight into chronological changes in circulating sST2, and to a lesser degree IL-33, relative to quiescence, diagnosis of rejection, and start of rejection treatment. Similar to HTx data findings reported by by Pascual-Figal et al. (49), we also observed in our cohort of SBTx patients that 7 of 12 SBTx recipients assessed for circulating sST2 had a ≈2-fold or greater increase during emergence of initial rejection episode when compared to prior serum samples taken during quiescence (**Fig. 9, supplementary data**; Average fold change = 12.24±15.79; Max = 43.63; Min= 0.15). Likewise, rejection treatment tended to result in a decrease in sST2 levels (**Fig. 9, supplementary data**). While encouraging, these observations are complicated by the fact that sST2 levels were profoundly decreased even when rejection treatment was unsuccessful and resulted in loss of the allograft (n=3) (**Fig. 9, supplementary data**). Thus, while our data reveal that sST2 increases are associated with initialization of rejection episodes, further research in SBTx is necessary to define if differences in sST2 levels are acting as an indicator of successful rejection treatment or resulting from loss of sST2 producing cells during severe rejection.

Overall, we have completed the first simultaneous biopsy and serum based assessment of ST2 and IL-33 in two distinct pediatric transplant populations. We establish that both molecules are easily quantifiable in samples taken during routine surveillance protocols and increase significantly during episodes of rejection. Despite our sample constrains, our data examining circulating levels of sST2 and IL-33, as well assessing tissue expression of these proteins in both

patient EMBs and in murine allograft heart, in total make a convincing case for potential use of a combination biopsy/serum based assay of ST2 and IL-33 to assess rejection. Our study lays the groundwork for future verification of ST2 and IL-33 as robust biomarkers of rejection. This discovery would help to lessen the burden of IST related complications by allowing doctors to maintain recipients at a lower level of IST given that they could rapidly detect early rejection and address before damage to graft results. This targeted adjustment of IST regimen would decrease accumulative IST side effects over time. Reliable biomarkers would also revolutionize transplant diagnostic procedures, enabling a less invasive approach to rejection diagnosis. Both improvements would lead to better adherence to treatment and screening protocols. In time, this should improve graft attrition rates and long-term patient survival to the benefit of public health.

6.0 FINAL SUMMARY

Our study findings are summarized in a diagram below (**Fig. 9**). Given our IPA data suggesting that TNFα, IL-1β, and IFNγ are the dominant drivers of ST2 during rejection, we believe that the inflammatory cytokines generated by graft-infiltrating immune cells during ACR and AMR trigger upregulation of ST2 and sST2 in cardiac myocytes and fibroblasts. These cytokines include IFNγ from infiltrating activated T-cells and TNFα and IL-1β from inflammatory macrophages. Accordingly, sST2 would be released into the circulation and ST2 would be readily detectable during early AMR and ACR. Our Qdot data demonstrates nuclear expression of IL-33 is also upregulated at this time, however we anticipate that release of IL-33 will result from tissue damage. Thus, we believe sST2 would be most useful as a biomarker for early rejection and unlike previously tested biomarkers which are indicators primarily of necrosis, we expect sST2 to be measurable before significant damage has occurred. As for IL-33, we propose that it could serve as a tissue biomarker, detectable by biopsy early in rejection, however, it presence in the serum suggests that it is being released most likely due to tissue damage or necrosis. This suggests that IL-33 may be an effective marker of advanced rejection and could be included in a panel with other markers of necrosis such as cardiac troponin I and creatine kinase.

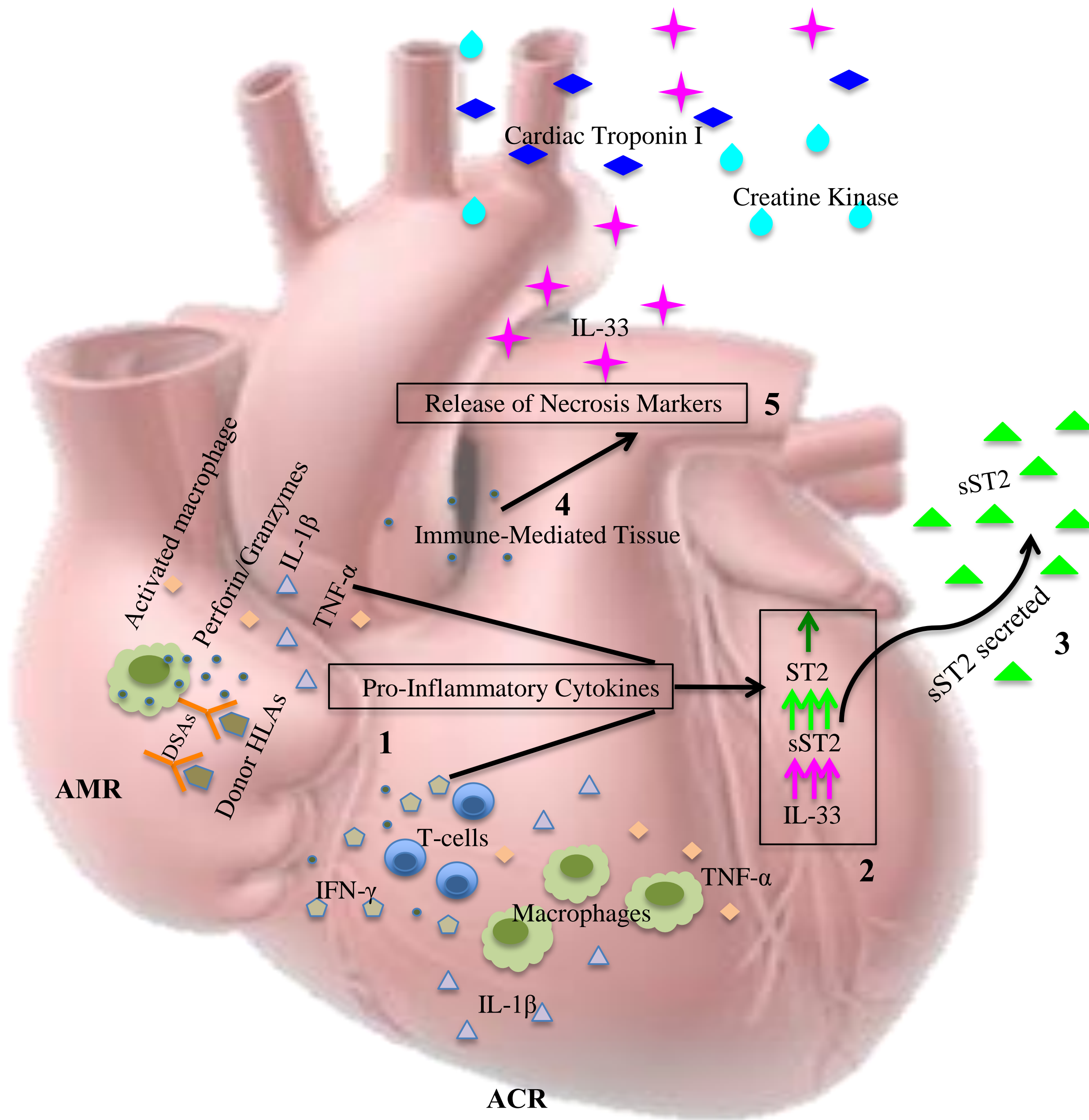


Figure 9: Schematic representation of increased IL-33 and ST2/sST2 expression and release during allograft rejection (ACR/AMR)

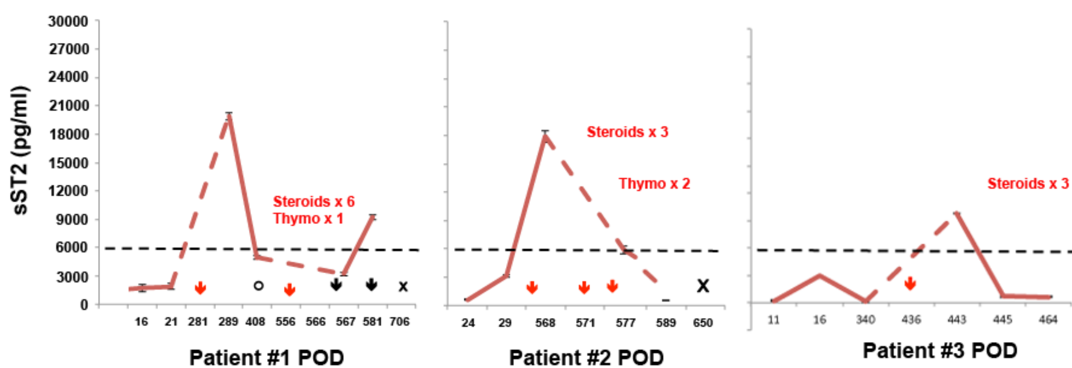
Inflammatory cytokines are generated during ACR and AMR by infiltrating mononuclear cells (macrophages/T-cells) within the allograft (1). These cytokines induce upregulation of ST2, sST2, and IL-33 in cardiac myocytes and fibroblasts (2). Secretion of sST2 is triggered which allows for quantitation of this molecule in the serum (3). Advanced rejection can result in tissue damage/necrosis, which can be mediated by targeted lysis of cells by activated macrophages and cytotoxic T-cells due to granzyme/perforin activity (4). IL-33 along with other markers of necrosis such as cardiac troponin I and creatine kinase are subsequently released into the serum as a result of this damage (5), thus indicating that IL-33 may be more useful as a marker of necrosis rather than for rejection.

7.0 FUTURE DIRECTIONS

The limitations within our study highlight the necessity of additional research in order to further reinforce our conclusions. Expanding our HTx population, especially in the AMR group, would add to the impact of our current findings. Currently our Qdot analyses only display a trend towards increased IL-33 and ST2 in AMR. We expect that by adding additional patient samples to this group, observed differences will reach statistical significance. Also, growing this population would also enable us to examine a wider range of time points which would allow us to observe if POD plays a role in appearance of these markers in relation to time of rejection diagnosis to start of treatment and rejection resolution. For example, in a perfect scenario we would expect to observe a measurable increase in sST2/IL-33 prior to diagnosis of rejection, similarly high levels in both tissue and serum at time of rejection, and a subsequent decrease in IL-33 and ST2 post successful treatment and resolution of rejection. In addition, we would also like to build on our tissue/serum correlative studies in order to see a solid relationship between circulating levels of IL-33 and sST2 and tissue levels of the molecules. Due to the unsuccessful Qdot staining resulting from the poor condition of the small bowel biopsies that we received from our collaborators in Nebraska, we lacked a Qdot assessment for this population. In the future, we are anticipating receipt of chronologically obtained SBTx biopsies from Children's Hospital of Pittsburgh, which should allow us to complete Qdot-based staining and provide a basis of comparison with our HTx study findings.

Also, given that infections, particularly viral, are common in immunosuppressed transplant recipients, it is essential that a biomarker of rejection be exclusive to rejection and not influenced by inflammatory markers induced by viral infections. Inflammatory cytokines such as IL-6, IL-12, TNFα, and IFNγ, identified during episodes of PTLD which is driven by EBV, have also been detected in rejecting patients, making them poor candidates as biomarkers. In our study, we found in SBTx assessments that levels of sST2 and IL-33 were significantly higher at time of rejection than during diagnosed enteritis, which can have several causes including viral infection (66). This suggests that elevated measurements of ST2 and IL-33 are unique to rejection. However, further assessment will be necessary to determine if this is in fact the case. To expand on this observation we plan to assess IL-33/sST2 along with a panel of other inflammatory cytokines in the sera of pediatric HTx recipients who will be grouped based on EBV status, which is determined through routine surveillance, along with rejection status. Through these studies we hope to gain knowledge of how expression of these molecules is modulated in a rejection/infection system.

APPENDIX: SUPPLEMENTARY DATA



Red arrows represent times of severe rejection, while black arrows indicated time points of mild to moderate rejection. Open circles represent incidence of enteritis and X indicate loss of graft. Black dashed line depicts normal levels observed in donor serum + 2SD. Purple dashed lines indicate manually connected time-points were no serum samples were available for evaluation. POD = post-operative day.

Figure 10: Changes over time in serum levels of sST2 in three representative SBTx recipients

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