Published in final edited form as:

Crit Rev Immunol. 2017 ; 37(1): 23–37. doi:10.1615/CritRevImmunol.2017024944.

Lung Injury and Loss of Regulatory T Cells Primes for Lung-Restricted Autoimmunity

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Abstract

Lung transplantation is a life-saving therapy for several end-stage lung diseases. However, lung allografts suffer from the lowest survival rate predominantly due to rejection. The pathogenesis of alloimmunity and its role in allograft rejection has been extensively studied and multiple approaches described to induce tolerance. However, in the context of lung transplantation, dysregulation of mechanisms which maintain tolerance against self-antigens can lead to lung-restricted autoimmunity which has been recently identified to drive the immunopathogenesis of allograft rejection. Indeed, both preexisting as well as *de novo* lung-restricted autoimmunity can play a major role in the development of lung allograft rejection. The three most widely studied lung-restricted self-antigens include collagen type I, collagen type V, and k-alpha 1 tubulin. In this review, we discuss the role of lung-restricted autoimmunity in the development of both early as well as late lung allograft rejection and recent literature providing insight into the development of lung-restricted autoimmunity through the dysfunction of immune mechanisms which maintain peripheral tolerance.

Keywords

lung transplantation; self-antigens; graft dysfunction; transplant tolerance; exosomes; autoantibodies

I. INTRODUCTION

Lung transplantation (LTx) is a life-saving therapy for several end-stage lung diseases including idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, pulmonary hypertension, cystic fibrosis, alpha-1 antitrypsin deficiency, bronchiectasis, interstitial lung disease, and congenital lung diseases.^{1,2} However, lung allografts suffer from the lowest survival rate due to the development of allograft rejection (www.ishlt.org and <https://optn.transplant.hrsa.gov/>).^{3–6} Alloimmunity has been traditionally postulated to play the predominant role in the pathogenesis of lung allograft rejection.^{7–12} The pathogenesis of

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alloimmunity has been extensively demonstrated in many studies and multiple approaches have been presented to induce tolerance to the graft.^{13,14} Through the alloimmune response, recipient T cells recognize donor major histocompatibility complex (MHC) antigens or its human counterpart, the human leukocyte antigen (HLA), by two distinct pathways of direct and indirect allorecognition.^{15–17} After LTx, presence of ‘shed’ donor HLA antigens in the bronchoalveolar lavage (BAL) fluids, are processed and presented to T helper cells, which are engaged in indirect recognition pathway that can trigger the growth and maturation of antigen specific B cells resulting in antibody production to mismatched HLA antigens. Furthermore, it has been demonstrated that anti-HLA can activate human airway epithelial cells (AEC) resulting in the production of several growth factors including fibrinogenic growth factors.^{8,10,18} The CD4⁺ T-helper cells recognize processed forms of soluble HLA in the context of self MHC and these T cells are likely involved in the development of humoral immune responses to donor HLA. In addition to this, experimental models have proposed that the alloimmune response, in particular the isotype switch from IgM to IgG, is dependent on the CD4⁺ T-helper mediated indirect recognition pathway, which is tightly regulated by both T-cell costimulatory signals and recognition of cognate antigen.^{19,20}

However, despite significant advances in the immunosuppressive protocols directed at ameliorating alloimmunity, the incidence of acute as well as chronic lung allograft rejection remains high.²¹ Intriguingly, published studies have also provided conflicting evidence by demonstrating that the degree of HLA mismatching between donor and recipient may not impact lung allograft rejection.^{22–26} This indicates the possibility of alternate immune mechanisms in mediating lung allograft rejection.^{27,28} Emerging data from our laboratories and others have implicated the role of autoimmunity against lung-restricted self-antigens in the development of rejection following lung transplantation.^{3–6,29–35} The three most widely studied lung-restricted self-antigens include collagen type I (Col-I), collagen type V (Col-V), and α 1 tubulin (KAT).^{3,5,36,37} In this article, we discuss the evidence supporting the role of lung-restricted autoimmunity in the development of lung allograft rejection and provide insight into the development of tissue-restricted autoimmunity through dysregulation of regulatory T cells.

II. LUNG-RESTRICTED AUTOIMMUNITY CAN MEDIATE LUNG ALLOGRAFT REJECTION

Autoimmunity can contribute to primary lung allograft dysfunction as well as organ failure due to non-immune etiologies and has been associated with “bystander” activation of organ specific, autoreactive T cells and production of autoantibodies.³⁸ Regardless of the etiology, autoimmunity present prior to transplantation has the potential to recur following re-exposure to the autoantigen after transplantation and thereby, participates in allograft injury.^{39–41} Associative evidence in human transplant recipients indicates strong associations between autoimmunity and post-transplant injury in human transplant recipients.^{27,42–44}

A. Hyperacute Rejection Mimicking as Primary Graft Dysfunction

Primary graft dysfunction (PGD) affects over 50% of lung transplant recipients within 72 hours and is characterized clinically by hypoxemic respiratory failure and histologically with

extensive neutrophil infiltration and diffuse alveolar damage.^{4,45} It remains the leading cause of early post-transplant mortality.^{46–49} PGD has been associated with ischemia-reperfusion injury and neutrophil infiltration has been shown to be necessary for the pathogenesis of PGD.⁵⁰ We have recently demonstrated that donor-derived pulmonary intravascular non-classical monocytes recruit recipient neutrophils in a toll-receptor dependent manner through the production of CXCL2 and mediate PGD.^{51,52} Intriguingly, ischemic time may not always correlate with development of PGD.⁴⁵ Severe PGD can develop in lung allografts despite very short ischemia. Additionally, the characteristic histological features of PGD such as neutrophil infiltration, alveolar edema, and capillaritis are also observed in antibody-mediated rejection (AMR), raising the suspicion that pre-formed antibodies may lead to allograft rejection which may mimic PGD. Some patients with PGD demonstrate C4d deposition on the allograft even in the absence of any donor HLA specific antibodies which also suggests activation of antibody-antigen complexes. Supporting this notion, we found that a form of hyperacute rejection, classically mediated by pre-formed antibodies against donor HLA class I antigens, can occur in human lung transplant recipients with pre-formed antibodies against lung-restricted self-antigens, namely collagen type V and α -tubulin.^{53,54} Unlike histocompatibility antigens, the self-antigens are conserved within the species. Ischemia-reperfusion injury can reveal epitopes of self-antigens which serve as structural proteins in the lung.^{3,55} Therefore, pre-formed autoantibodies present in the recipient can bind their cognate antigens in the lung allograft following ischemia-reperfusion. In murine models we found that pre-formed lung-restricted autoantibodies against self-antigens can induce PGD in syngeneic lung grafts and broke tolerance after allogeneic lung transplantation, without affecting other organs.³⁷ Further, any single pre-formed autoantibody led to epitope spreading and propagation of autoimmunity against other lung-restricted antigens but not those absent in the lung tissue. Other investigators have supported the role of pre-formed lung-restricted autoantibodies in the development of PGD as well.^{56,57} Development of PGD associated with preexisting LRA has also been demonstrated in the rat LTx model. In a study by Iwata et al., administration of Col-V Abs in rats prior to syngeneic graft transplantation, rats who received the Col-V Abs developed a syndrome of PGD.⁵⁶ The authors showed that lung allografts with PGD associated with Col-V Abs demonstrated both Ab and complement deposition. In a prospective clinical study, we previously observed that about 30% of lung transplant recipient had one or more pre-formed autoantibodies which strongly predisposed to PGD.⁵ Furthermore, PGD was associated with a very significant increase in pro-inflammatory cytokines which augmented development of donor-specific alloimmunity and chronic rejection.³³ Together, these data indicate that the syndrome of PGD may represent two distinct forms of injury related to ischemia-reperfusion and pre-formed lung-restricted antibodies.

B. Acute Antibody Mediated Rejection

We have reported the development of AMR in lung allograft recipient from lung-restricted autoantibodies developing *de novo* following transplantation.⁵³ The patient we reported developed new antibodies against lung-restricted autoantibodies collagen type V and α -tubulin which were associated with AMR. No donor specific HLA antibodies were detected and the patient was successfully treated using antibody-directed therapy and clearance of autoantibodies. While the association between autoantibodies and lung AMR

still remains under-investigated in the clinical arena, there is growing recognition of this association using the murine models. Further prospective clinical studies are required to establish treatment thresholds for these autoantibodies.

C. Chronic Allograft Rejection.

Chronic lung allograft rejection is the predominant cause of poor long-term survival following lung transplantation.^{28,36,58} Over time, all patients are expected to develop chronic rejection. Our prior prospective clinical study also established the pre-existing autoantibodies remain one of the most important risk factors for chronic lung allograft rejection.^{5,6,37,53} Interestingly, over 90% of patients who do not have pre-existing autoantibodies at the time of transplant will develop lung-restricted autoantibodies within 3 years which predisposes to chronic rejection. Therefore, both pre-existing and *de novo* lung-restricted autoantibodies lead to chronic rejection. While several risk factors have been identified for chronic rejection, lung-restricted autoimmunity has emerged as an important common terminal pathway leading to chronic rejection from a variety of these risk factors. For example, alloimmunity predisposes to *de novo* lung-restricted autoimmunity and chronic rejection. Clinical studies demonstrated that therapy directed against alloimmunity only reduced the incidence of chronic rejection if autoantibodies were depleted.^{59,60} Abrogation of alloimmunity alone was not associated with amelioration of chronic rejection. Similarly, gastroesophageal reflux which has been associated with chronic rejection is now known to induce *de novo* lung-restricted autoimmunity.^{61–63} The lung-restricted antigens, in particular Col-V, is a structural protein most abundant in the peri-capillary and peri-bronchiolar tissue where chronic rejection is observed.³

Using murine models, we have demonstrated that both pre-existing or *de novo* autoantibodies against Col-V and KAT can prevent the development of allotolerance as well as break tolerance established by co-stimulate blockade. The MHC class I-related chain A (MICA) is another non-histocompatibility antigen associated with chronic lung allograft rejection. Anti-HLA precedes *de novo* development of anti-MICA with peak titers of anti-MICA detected during chronic lung allograft rejection.⁶⁴ Taken together, lung-restricted autoimmunity, both preexisting or *de novo*, are associated with lung allograft rejection.

III. PATHOGENESIS OF LUNG-RESTRICTED AUTOIMMUNITY

A. Importance of Regulatory T Cells in Transplant Tolerance.

In autoimmune conditions, activation of autoreactive T cells upon encountering cognate autoantigen occurs due to the alteration or even breakdown of the immune tolerance mechanisms, which result in consequential tissue and organ damage.^{16,65} The thymus is responsible for the deletion of self-reactive lymphocytes during the development phase.^{66–69} However, recent data shows that self-reactive lymphocytes against several organs, including lungs, escape the thymic deletion and are dynamically suppressed by the CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs).^{29,30,36,70}

Tregs are known for their role in mediating immune tolerance to self-antigens and have been characterized by the high and stable expression of surface interleukin-2 receptor α -chain

(IL2R α , CD25^{hi}).⁷¹ In the periphery, they constitute approximately 1–3% of the circulating CD4⁺ T cells.⁷² Tregs can be found in many different tissues where they play an important role in suppressing inflammation and autoimmune responses.^{73,74} In the context of cell and organ transplantation, Tregs are capable of controlling alloimmune responses and maintaining operational tolerance.^{75–81} Studies including our own group's results have demonstrated a crucial role of Tregs in suppressing both auto- and allo-immunity following lung transplantation.^{31,37,82,83} T cell receptor (TCR) stimulation of Tregs by cognate antigens during an active immune response augments Treg suppression by increasing their steady-state functions and inducing an expanded array of suppressive mechanisms, including production of the immunosuppressive molecules IL-10, IL-35, TGF- β , and cAMP; expression of ectoenzymes CD39 and CD73 to degrade extracellular ATP; and expression of granzymes and perforin for direct killing of APCs.⁸⁴ Thus, Tregs prevent expansion of conventional T cells (Tconvs) and prevent their acquisition of effector function.⁸⁰ Once activated, Tregs traffic to sites of inflammation, where they suppress immune cell effector functions and limit collateral tissue destruction.^{85–87} Depending on the context of their activation, Tregs develop the ability to suppress certain effector functions. For example, in the context of a Th1-mediated immune response, Tregs acquire a Th1-like phenotype by expression of the archetypal Th1 molecules IFN- γ and CXCR3, allowing them to suppress the function of Th1 and CD8⁺ effector T cells (Teffs). Similarly, Tregs can specialize to suppress Th2, Th17, and T follicular helper cells.⁸⁰

In certain, pro-inflammatory conditions, T cells have the ability to differentiate into Th17 cells, and this process is independent of Th1 or Th2 cell development.^{88,89} Th17 polarization requires the presence of IL-1 β , IL-6, IL-21, and IL-23, cytokines which induce the activation of the transcription factor signal transducer and activator of transcription 3 (STAT3). Generally, Th17 cells have a major function in combating against pathogens, they recruit neutrophils and macrophages to the site of inflammation, they are crucial in the initiation of inflammation, mostly against extracellular pathogens.⁹⁰ However, the persistent secretion of IL-17 promotes chronic inflammation and can contribute to the pathogenesis of inflammatory and autoimmune diseases.

Adoptive transfer of *in vitro* differentiated Tregs inhibit the differentiation of Th17 cells and increased IL10 production leading to alleviating the pathology of OB in the mouse orthotopic lung transplantation model.⁹¹ This approach of Tregs transfer was also successful in a mixed chimerism model of heart and bone marrow transplantation (BMT) preventing chronic rejection of heart allografts comparing to BMT and heart allograft group only.⁹²

While polyspecific Tregs can be used to prevent graft-versus-host disease in lymphopenic hosts after hematopoietic stem cell ablation,⁹³ polyspecific Tregs are usually insufficient to prevent organ rejection in nonlymphopenic hosts after organ transplantation.^{77,94} To overcome this hurdle, graft-specific tolerance was achieved,^{95–97} and most recently with highly specific chimeric antigen receptor (CAR) that recognizes donor HLA molecule and completely prevent rejection of allogeneic target cells and tissues in immune reconstituted humanized mice in the absence of any immunosuppression.⁹⁸ Together, these studies indicate that Tregs are crucial for transplant tolerance.

B. Epitope Spreading

Epitope spread is the phenomenon in which diversification of immune recognition occurs from the initial dominant epitope to related epitopes either within the original or different proteins. Classic immune response against a self or foreign antigen (Ag) usually occurs on one or two dominant epitopes within that Ag. In all possible scenarios, infection, organ transplantation or autoimmunity (organ-specific or systemic), epitope spreading initiated as a result of tissue damage is thought to play an active role in ongoing disease pathology. However, there is some evidence that epitope spreading is an important component of protective immune response, as seen in tumor clearance, and as a mechanism to downregulate immune responses, such as those occurring in autoimmunity.⁹⁹

Epitope spreading is a well-recognized phenomenon in solid organ transplantation,¹⁰⁰ and it has been proposed that epitope spreading against alloantigens may play an important role in allograft rejection and also in other autoimmune diseases, including multiple sclerosis, type 1 diabetes and myasthenia gravis.⁹⁹ Alloimmune responses to the transplanted organ are initiated as a result of direct cytotoxic T cell (CTL) recognition of MHC class I molecules expressed on donor APCs. Subsequently, the indirect helper CD4⁺ T cell recognition of recipient MHC class II molecules plus alloantigenic peptide directed alloimmune response can spread to additional determinants within the primary target antigen, in intramolecular epitope spreading.^{101,102} Similarly, the indirect recognition pathway can also promote the development of autoimmune T and B cells that contribute to the rejection process.¹⁰³ The indirect alloresponse triggers autoimmunity after transplantation presumably via antigen mimicry between autoantigen peptides and donor MHC peptides.¹⁰⁴ Once autoreactive T cells are generated, chronic stimulation of these cells can lead to epitope spreading, which has been reported to contribute to the generation of autoantibodies.⁵⁴

Despite traditional postulation of self-reactive lymphocytes deletion in the thymus, recent data indicate that nonubiquitous antigens present in organs such as the lung, pancreas, and small intestine are not expressed on the thymocytes, and lymphocytes specific to these self-Ags do not undergo thymic deletion.⁷⁰ Tregs dynamically suppress these self-reactive lymphocytes against tissue-restricted self-Ags.³¹ Because the self-Ags are normally sequestered, activation of self-reactive lymphocytes is further prevented. LTx recipients and patients with end-stage lung disease undergo many injury-repair cycles that create an inflammatory milieu, which can lead to the expansion of autoreactive lymphocytes. Various mechanisms including molecular mimicry and bystander activation,¹⁰⁵ have been proposed for this phenomenon in lung transplant include release of the sequestered self-Ags, lowering of activation thresholds of self-reactive lymphocytes,¹⁰⁶ and epitope spreading. Cellular immune responses against an alloantigen have also been shown to spread to additional epitopes within the parent or other self-proteins, a phenomenon termed as intramolecular and intermolecular epitope spreading, respectively.¹⁰⁷

In a recent study, we tested whether preexisting Abs to lung self-Ags and PGD are mechanistically linked using the murine model of unilateral LTx, and recipients were passively given one or more Abs to lung-restricted antigens before transplantation of syngeneic lung grafts. Each of the Abs demonstrated a dose-dependent graft dysfunction of

the syngeneic grafts. Interestingly, preexisting LRA led to epitope spreading wherein administration of Col-V Abs induced de novo $\alpha 1$ T Abs after LTx and vice versa.⁶

Various mechanisms including molecular mimicry, bystander activation, release of self-antigens and epitope spreading have been hypothesized to play a role in the development of immune responses to self-antigens, however, contrary to the published results. In another study, sequential analysis of antibody development against Col-V following lung transplantation demonstrated 'restrictive epitope shift' mechanism, where the shift of immunodominant epitopes of Col-V plays a crucial role in defining the T helper cell phenotype switch, and the ensuing induction of cytokines specific to Col-V leads to immune responses to Col-V, leading to chronic lung allograft rejection following human LTx. Based on these findings, we postulate that during high turnover of the extracellular matrix in the lungs the 'cryptic' antigenic determinants of procollagen is most probably exposed, which is antigenic and stimulates both humoral and cellular immune responses to this self-antigen, thereby triggering an autoimmune process at the graft site.⁵⁵

A more refined epitope analysis of collagen V-specific CD4⁺ Th17 cells in patients with bronchiolitis obliterans syndrome (BOS) showed HLA-restricted epitope binding of $\alpha 1$ (V) in association with the HLA-DR15 genotype.¹⁰⁸ Interestingly, an HLA-DR15 negative recipient who received a transplant from a HLA-DR15 positive donor also responded to DR15-restricted Col-V epitopes after LTx, suggesting determinant spreading from recipient DR-restricted epitopes to donor HLA-DR restricted epitopes.^{108,109} During progression of chronic rejection in heart and kidney transplant recipients, 'inter-molecular epitope spreading' has been described for indirect pathway responses to donor alloantigen.¹¹⁰

However, in allograft recipients with recurring episodes of rejection or in patients in which chronic rejection was at its onset, recipient T-cell reactivity could spread to other epitopes within the allogeneic MHC molecule, as well as to other alloantigens expressed by the graft tissue. In an early study, recipients of heart allografts were monitored for reactivity against donor human leukocyte antigen (HLA)-DR peptides before and up to 36 months after transplantation.¹¹¹ In patients who received a doubly mismatched transplant (HLA-DR disparity at both loci), chronic rejection was significantly more likely when intermolecular epitope spreading occurred than if reactivity was directed only to the initially targeted HLA-DR molecule.¹⁰⁷

There are also evidences that epitope spreading may be important for immunoregulation. A study examining skin-graft tolerance after donor-specific transfusions found that tolerance induced by transfusions mismatched at one MHC I locus actually spread to skin transplants that were mismatched at two MHC I loci or at one MHC I locus plus multiple minor histocompatibility antigens.¹¹² This is suggested to be the result of epitope spreading of tolerance.¹¹³ It is plausible, therefore, that acquisition of a donor class II-restricted response to self-antigens is yet another mechanism that contributes to chronic rejection. This hypothesis has important clinical implications for the development of autoimmunity post-transplantation and underscores the importance of identifying the specific autoantigens and their HLA-restricted epitopes. Ultimately this information might guide the allocation of lung transplants and the development of therapeutic strategies to prevent chronic rejection.

C. Role of Exosomes in Lung Transplantation

Intercellular communication is vital for the regulation and coordination of many different processes. Emerging evidence has highlighted the role of extracellular vesicles (EV) in cell-to-cell communication between neighboring cells or remote cells or tissues.¹¹⁴ Exosomes are extracellular nano-vesicles, 30–120 nm in size, that are released into the extracellular space by reverse budding of multi-vesicular bodies containing intraluminal vesicles that can bind to the plasma membrane of the acceptor cell and be internalized through either endocytosis or micropinocytosis and secreted into body fluids.^{115–119} Extracellular microvesicles facilitate cellular communications through delivery of their functional cargo in health and disease.^{120–123} Exosomes contain membrane, cytosolic proteins, messenger RNA (mRNA) and miRNA.^{124–126} Previous studies have also demonstrated their role in pathogenesis of cancer.^{127,128} MHC classes I and II have been detected on tumor cell exosomes and can induce antitumor immunity.¹²⁸ Exosomes from epithelial cells can induce activation of macrophages during lung inflammation, suggesting that exosomes can trigger airway inflammation,¹²⁹ and exosomes with MHC class II and co-stimulatory molecules are reported to be present in human BAL fluid which might be derived from APCs with a regulatory role in local immune response in the respiratory system.¹³⁰

In a recent study, Gunasekaran et al. showed that exosomes containing self-Ags are detectable in both the BAL fluid and sera of LTx recipients with acute rejection and BOS but not in stable patients. Exosomes isolated from BAL fluid and sera showed nanovesicular structure by electron microscopy visualization (EM) and expression of exosome-specific surface marker CD63 in exosomes derived from LTx recipients with AR and BOS.¹³¹

Exosomes have also been reported to transfer functional MHCs to dendritic cells, which can express internalized protein and initiate antigen presentation to CD4⁺ T cells, dendritic cells (DCs) secrete exosomes, that bear MHC and T cell costimulatory molecules.¹³² In lung transplant setting, exosomes originate from the transplanted organ after immune injury. The mismatched donor HLA and self-Ags are expressed on exosome surfaces and that exosomes contain miRNAs known to induce inflammation, endothelial activation, antibody-mediated chronic rejection and Th17 differentiation.¹³¹ In addition to carrying antigen, exosomes promote the exchange of functional peptide-MHC complexes between DCs. Such a mechanism may increase the number of DCs bearing a particular peptide, thus amplifying the initiation of primary adaptive immune responses.¹³² The mechanism of exosome contribution to acute rejection and chronic rejection after LTx need more comprehensive investigation.

Gunasekaran and colleagues conducted a case-control observational study in patients who underwent bilateral LTx, they proposed the possibility that exosomes expressing donor HLA, self-Ags and immunoregulatory miRNAs released from the donor lungs during rejection may support the immune responses that ultimately lead to allograft rejection and BOS.¹³¹ An in-depth analysis of the BAL fluid exosomal shuttle RNA population after lung transplantation and evaluation for differential expression between acute rejection and quiescence could validate BAL fluid exosomal shuttle RNA as a source for understanding the pathophysiology of acute rejection and for biomarker discovery in the lungs.¹³³

D. Role of Regulatory T Cells in Lung Transplantation

Many of these general principles of Treg function in controlling immune responses apply to Treg function in the context of alloimmune responses and development of chronic lung allograft dysfunction (CLAD). Recently, in a long-term follow up study, higher frequencies of specific Treg subpopulations starting to increase as early as 3 weeks after lung transplantation have shown to be protective against CLAD development at 2 years.¹³⁴

As such, lung allografts are vulnerable to many forms of injury that can result in local inflammation.^{28,36,38} In a murine model of orthotopic tracheal transplantation, we previously reported that respiratory viruses can upregulate FasL on infected airway epithelial cells, triggering the loss of Tregs³⁵. The development of lung-restricted immunity in lung recipients^{27,31,135} in the setting of tissue injury therefore suggests concomitant loss of self-tolerance maintained by Tregs.

The loss of Tregs is associated with lung allograft rejection in both murine and human transplantation, suggesting that maintenance of the Treg function is important for downregulating immune responses against alloantigens and self-Ags.^{83,136} We found that lung transplant recipients develop expansion of self-reactive T cells during chronic lung allograft rejection³¹ are associated with an increase in CD30, a T cell activation marker.³⁴ Using both human and murine studies we found that respiratory viruses were strongly associated with obliterative airway disease, a hallmark of chronic lung rejection.^{5,137,138} While this was a transient effect with the levels of Tregs ultimately being restored as the infection cleared, we found that the loss of Tregs lead to expansion of autoimmunity against lung-restricted autoantigens. This might perhaps explain how respiratory viruses might predispose to chronic lung allograft rejection. Using the orthotopic tracheal transplant model, we validated this and reported that respiratory viruses increased the intramural fibrosis of the tracheal allografts, a marker of chronic rejection. We found that the respiratory viruses infected tracheal epithelial cells and induced apoptosis of Tregs through Fas-dependent mechanisms. Blocking the Fas-FasL interactions between infected tracheal epithelial cells and Tregs prevented Treg apoptosis. Since, Tregs are responsible for dynamic suppression of tissue-restricted lymphocytes which escape thymic deletion, this explains how respiratory viruses lead to expansion of tissue-restricted autoimmunity and chronic lung allograft rejection.

E. The “Two-Hit” Hypothesis for Development of Lung-Restricted Autoimmunity

In prior studies we observed that lung transplant recipients developed periodic nadirs in the levels of circulating Tregs. When we analyzed the clinical events surrounding these nadirs, we observed that the majority were associated with microbiologically proven lower respiratory infections from influenza virus, parainfluenza virus, adenovirus, and respiratory syncytial virus. We also observed that respiratory viral infections induced Treg apoptosis in the draining lymph nodes of orthotopic tracheal grafts which was associated with increased intramural fibrosis.³⁷ Immunization of the recipient with inactivated parainfluenza virus conferred protection against the Treg apoptosis after inoculation of the same levels of virus into the recipient. Using *in vitro* tracheal epithelial cell lines, we observed that the virus can infect these cells, upregulating Fas and inducing apoptosis in Tregs which could be

abrogated using anti-FasL antibodies. Clinically, we observed that the respiratory viral infections and nadirs in the Treg levels were associated with increased autoantibody development. These findings from both human and murine studies led us to further hypothesize that Tregs were responsible for maintaining dynamic tolerance against self-Ags present in the lung tissue. However, loss of Tregs alone might not be sufficient to induce lung-restricted autoimmunity since the frequency of self-reactive lymphocytes is expected to be very low and if there is no injury mechanism to stimulate their expansion, autoimmunity might not develop. Hence, we proposed a “two-hit” mechanisms in which both loss of Tregs and a lung injury mechanism were necessary at the same time to induce lung-restricted autoimmunity.

Using a variety of murine models, we tested this hypothesis. In the wild type animals, we injected anti-MHC class I antibodies intratracheally to induce lung specific injury. Concomitantly, we induced loss of regional Tregs by infecting the hosts with sendai (murine parainfluenza) virus. We observed development of both humoral and cellular autoimmunity against lung-restricted self-antigens Col-V and KAT but not Col-II which is not present in abundance in the lungs. Unrelated MHC class I antibodies in combination with sendai virus infection could not induce lung-restricted autoimmunity and MHC class I antibodies against the host alone could not induce autoimmunity in the absence of viral infection. To selectively deplete the FoxP3⁺ Tregs we injected anti-MHC class I antibodies along with diphtheria toxin to FoxP3-DTR transgenic mice and noted development of lung-restricted autoimmunity. Similarly, when we injected hydrochloric acid, to mimic gastroesophageal reflux, in combination with diphtheria toxin we noticed development of lung-restricted autoimmunity. We did not elicit responses to non-lung antigens and either diphtheria toxin or hydrochloric acid alone did not induce lung-restricted autoimmunity. Since patients with lung failure likely have ongoing lung injury resulting from the primary disease and are known to be susceptible for respiratory infection our studies might explain why such a large proportion of patients undergoing LTx have pre-existing autoantibodies. Following transplantation, HLA antibodies as well as gastroesophageal reflux are common³ and can serve as the injury mechanism and if these patients develop loss of Tregs, for example due to respiratory viral infection, they can develop lung-restricted autoimmunity.³⁷ Indeed, when we followed patients with post-transplant gastroesophageal reflux only those who developed respiratory viral infections and loss of Tregs demonstrated development of *de novo* development of lung-restricted autoimmunity.

IV. CONCLUSIONS

Published literature strongly supports the role of autoimmunity in the development of allograft rejection. Ongoing efforts to translate this information into clinical practice has the potential to significantly improve survival following solid organ transplantation.

ACKNOWLEDGEMENTS

We thank Ms. Elena Susan for formatting and submitting this manuscript. AB is supported by National Institutes of Health HL125940 and matching funds by the Thoracic Surgery Foundation.

ABBREVIATIONS:

LTx	Lung transplantation
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
BAL	Bronchoalveolar lavage
AEC	Airway epithelial cells
Col-I	Collagen type I
Col-V	Collagen type V
KAT	k-alpha 1 tubulin
PGD	Primary graft dysfunction
AMR	Antibody-mediated rejection
MICA	MHC class I-related chain A
Tregs	Regulatory T cells
TCR	T cell receptor
Tconvs	Conventional T cells
Teffs	Effector T cells
STAT3	Signal transducer and activator of transcription 3
BMT	Bone marrow transplantation
CAR	Chimeric antigen receptor
Ag	Antigen
CTL	Cytotoxic T cell
BOS	Bronchiolitis obliterans syndrome
EV	Extracellular vesicles
mRNA	Messenger RNA
EM	Electron microscopy visualization
DCs	Dendritic cells
CLAD	Chronic lung allograft dysfunction

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