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Regulatory T cells in lung transplantation—an emerging concept

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Abstract

Lung transplantation represents an option for patients with a variety of end-stage lung diseases. While surgical advances have led to improvements in short-term survival, long-term survival is limited by chronic rejection termed bronchiolitis obliterans syndrome (BOS). A growing body of work is devoted to determining why some patients develop BOS. One avenue of interest that has emerged recently is the role that regulatory T cells (Tregs) may have in protection from BOS. In this review, we will discuss the evidence that Tregs are relevant to outcomes following transplant. We will discuss the relevant animal models, in vitro assays, and human observational studies that support a role for Tregs. We will also explore the interplay between injurious T cells such as Th17 cells and Tregs as well as the effect that additional cell types and chemokines have on the balance between inflammation and regulation. Finally, we will review emerging therapies which may harness the ability of Tregs to lessen the effects of BOS.

Keywords

Lung transplantation; Bronchiolitis obliterans; Tregs; FoxP3; Th17

Introduction

Lung transplantation has emerged over the past decade as a viable therapy for patients with end-stage lung disease with a world-wide experience of more than 21,000 cases. For many patients, lung transplantation has led to increased quality of life and improved longevity. Nevertheless, outcomes following lung transplantation among patients are quite variable. Greater than 80% of patients live at least 1 year, but beyond that, outcomes following lung transplantation lag considerably behind liver and kidney transplantation with below 50% survival at 5 years and below 20% survival at 10 years [1]. The major cause of mortality in lung transplantation is bronchiolitis obliterans (OB), which appears histologically as fibroproliferation of the terminal bronchiole. Because the detection of confirmed OB by lung biopsy is often difficult to document even when it exists, a surrogate marker for histologic OB has been developed, termed bronchiolitis obliterans syndrome (BOS). A patient is designated to have BOS when their forced expiratory volume by spirometry drops below 80% of their best post-transplant value and no other etiology for airflow obstruction can be identified.

An emerging area of interest is elucidating the mechanisms underlying long-term survival in lung transplantation, with the hopes of trying to replicate these concepts widely. In experimental animal models of solid organ transplantation, multiple approaches have led to sustained allograft acceptance without the need for immunosuppression. Many of these approaches take advantage of simultaneous dampening of alloreactive T cell responses and corresponding augmentation of regulatory T cell frequency/function (reviewed in [2]). Because of the role for regulatory T cells (Tregs) in animal models of transplant, a growing body of work has focused on determining a role of these cells in the maintenance of graft function in clinical transplantation. This role has been illustrated in liver transplant, in which a significant subset of patients achieves a state of immunosuppression—free stable allograft function. In fact these drug-free tolerant patients may constitute up to 20% of long-term liver transplant recipients [3]. An emerging concept in liver transplant is that these fortunate individuals experience an active Treg involvement as part of the mechanism for their graft acceptance [4].

In contrast to liver transplantation, no such patients exist in lung transplantation. Perhaps this is due to the much higher immunologic hurdle of transplantation of an organ which actively participates in host–pathogen defense. Hence in lung transplantation, when assessing why some patients do well and some patients do poorly, we are limited to analysis of patients with less clear cut endpoints such as protection from ischemia reperfusion injury, extent of acute rejection early after transplantation, and the degree of physiologic derangement in airflow years after transplantation. Here, we will review the evidence of a role for Tregs in lung transplantation in both animal models and in human recipients, with the relevant endpoints being protection from immunity to cryptic autoantigens, the association of Tregs and acute rejection, and the interaction of Tregs and freedom from BOS.

Essential to understanding the role of regulatory cells in lung transplantation is a concise definition of what constitutes a Treg. In broadest terms, Tregs are cells which suppress the activation and proliferation of other immune cells including antigen presenting cells (APCs), CD4+ and CD8+ T lymphocytes. Tregs express high levels of surface CD25, the alpha chain of the interleukin (IL)-2 receptor. In fact, the earliest reports of Tregs in transplantation were limited to only CD4 and CD25 as phenotypic markers. More recently, Tregs have also been defined as cells which bear the intracellular transcription factor, foxP3, a protein that was initially described as essential to the prevention of severe autoimmunity [5]. Once it became known that foxP3 was essential to both the generation and function of Tregs, the inclusion of foxP3 as an essential marker for “Tregs” became canonized [6, 7]. Further work has confirmed that Tregs function by both cell–cell contact and through the secretion of paracrine factors such as IL-10, IL-2, and TGFβ (reviewed in [8]). Finding a marker to define Tregs had both a beneficial and detrimental effect in clinical transplantation. On one hand, it gave investigators a useful tool to track Tregs without the confounding effect of other recently activated T cells, as CD25 expression is also seen in the transition of naïve CD4 T cells into effector cells. On the other hand, the finding that foxP3 was the marker for Tregs focused attention away from other cell types which may have “regulatory functions”. Among these important non-Treg regulatory cells are Tr1 cells, which generate IL-10 and Th3 cells which generate TGFβ. The relevance of these non-Treg regulatory cells is that these cell types (and others) likely directly participate in the conversion of foxP3- cells into foxP3+ cells in peripheral tolerance mechanisms [9]. In this review we will stick to the convention of labeling Tregs as CD4 cells that express foxP3, but will point out where other regulatory cell populations are relevant.

Tregs in inflammatory lung diseases

The relative rarity of lung transplantation makes understanding the role of T cell regulation on graft function difficult to study. Hence it is informative to generate hypothesis on how regulatory T cells may improve graft function by understanding how these cells have a role in other forms of lung disease. Asthma and acute respiratory distress syndrome represent two distinct types of lung disease with a relatively high prevalence in which T cell regulation has been studied. Asthma, while clinically heterogeneous across patients, is characterized by the unifying features of airway smooth muscle hyperresponsiveness, increased secretion of mucous in the small airways and recruitment of inflammatory cells into the airway including eosinophils and neutrophils. The end result of chronic asthma is irreversible airway narrowing which is pathologically similar (but not identical) to the small airway luminal airway obliteration seen in OB. The general consensus with asthma is strongly associated with exuberant Th2 responses in the airway, although there is evidence to suggest a role in Th17 pathways as well [10–13]. In children with asthma, the frequency of CD4+ CD25+ cells was decreased in the bronchoalveolar lavage fluid (BALF) compared with healthy controls. Furthermore, both the percentage of CD4+ CD25+ cells and foxP3 mRNA in the BALF correlated with the trajectory of disease course; children with higher levels of BALF Tregs demonstrated a less precipitous decline in airflow measured by serial spirometry [14]. Notably, and potentially germane to both the management of asthma and prevention of lung transplant rejection, treatment of these children with inhaled corticosteroids for 4 weeks resulted in increased frequency of CD4+ CD25+ in the BALF.

The role of Tregs in the murine models of asthma has been extensively studied. In one model, mice are sensitized to chicken ovalbumin protein (OVA) and then rechallenged with inhaled OVA. Adoptive transfer of transgenic CD4 T cells specific for OVA peptide results in an exuberant inflammatory bronchiolitis and increased airways resistance. Adoptive transfer of CD4+ CD25+ OVA specific into these mice has been shown to abrogate airway hyperresponsiveness, an effect which was dependent on the induction of IL-10 by recipient (but not transferred) Tregs [15]. Furthermore, in a longitudinal study in which mice were repeatedly challenged with inhaled allergen, CD4+ CD25+ cells could prevent smooth muscle hypertrophy and new deposition of collagen, but could not reverse airway remodeling once it had already occurred [16].

Acute respiratory distress syndrome (ARDS) is the term applied to the rapid onset of pulmonary infiltrates of noncardiac origin that occurs in response to a variety of lung specific and lung extrinsic triggers. Examples of these triggers include bacterial pneumonia, aspiration of gastric contents, pancreatitis, and trauma. Pathophysiologically, ARDS is characterized by a cascade of pulmonary capillary damage, neutrophil influx, and proinflammatory cytokine production [17]. The study of T cell regulation on ARDS as a factor implicating outcomes is feasible given an incidence of nearly 200,000 cases in the USA annually [18]. The first description of BALF Tregs in humans followed only two patients with evolving acute lung injury. Both patients demonstrated relatively low absolute numbers of CD25+ FoxP3+ CD4 at the time of diagnosis of ARDS, but tenfold increase in BALF Tregs by day 7 after the onset of their lung injury [19]. Undoubtedly these findings will be followed with future studies in which the Treg dynamics are assessed as a prognostic factor for resolution or mortality from ARDS. Studies in an animal model of ARDS already suggest that Tregs may be protective. Both wild-type and RAG knockout (lymphocyte deficient) mice exposed to intratracheal lipopolysaccharide (LPS) demonstrate features analogous to human ARDS including infiltration of lymphocytes into the lung, increased BALF protein, and lung histology showing alveolar damage. FoxP3+ Tregs were found to be increased as early as 1 day post-LPS treatment, and peaking at day 7 in wild-type mice. The treatment of both RAG knockout and wild-type mice with adoptive intravenous transfer

of CD4⁺ CD25⁺ cells dramatically reduced all indices of lung injury in these mice [19]. Interestingly, multiple mechanisms appear to be at play for the protective effect of these cells. Among these, Tregs diminished the ability of LPS to induce TNF α by activated macrophages, induced the production of activated TGF β by macrophages, and increased the apoptosis of neutrophils. Using the intratracheal LPS model a recent study has demonstrated a significant increase in both IL-17 in the BALF of treated mice and surface expression of the costimulatory molecule CTLA4 on CD4⁺ cells recovered from the BALF. Treatment of these mice with anti-CTLA4 (which blocks interactions between CD80 and CD86 on APCs and CD28 and CTLA4 on T cells) resulted in both a significant decrease in BALF IL-17 and a reciprocal increase in CD4⁺ foxP3⁺ cells. Treatment with anti-CTLA4 also led to decreased BALF albumin, presumably indicating a protective role in dampening lung injury by maintaining integrity of the alveolar epithelium. Taken together these findings indicate that Th17/Treg balance may be an important determinant of course in acute lung injury and that the relationship between these two cell types may be modifiable with pharmacologic approaches.

In vitro modeling of human Tregs at the interface of allo and auto immunity

An emerging notion in lung transplantation is that repetitive injury of the terminal bronchiole leads to the eventual fibroproliferation of this airway, a phenomenon termed bronchiolitis obliterans. One mode of injury is thought to be direct allorecognition of allo-MHC epitopes by host T cells. But lung allografts may experience multiple insults which do not directly result from allo-MHC: host T cell interactions. These insults include gastroesophageal reflux, ischemia reperfusion injury, and direct denudation of the pulmonary epithelium by viral injury (panel A of Fig. 1). One consequence of these injurious processes is the unmasking of neo-self antigens which were not previously subjected to self-deletion in the thymus. Perhaps the best studied of these epitopes in lung transplantation is that of type 5 collagen (ColV) [20–22]. ColV is a relatively rare collagen in humans, represented less than 5% of total collagen, but it becomes exposed on the basement membrane of small airways when it is cleaved from type I and III collagen in by Matrix metalloproteinases (MMP) 2 and 9 [23]. Importantly, MMP9 elevations have been described in response to both ischemia reperfusion injury and in patients with BOS [24–27]. In a study of human lung transplant recipients, co-culture of CD4⁺ T cells with autologous APCs and ColV lead to production of soluble IL-10. The induction of IL-10 production was dependent on a cell population distinct from CD25⁺ CD4⁺ Tregs, but was dependent on these cells for IL-10 production. Removal of CD25⁺ CD4⁺ cells from the in vitro culture lead to decreased IL-10 production and increased IFN γ production [28]. When four patients with BOS were studied, there was a significant reduction of α 4 β 7 integrin positive CD25⁺ cells, which is interesting in light of the finding that this subpopulation of Tregs is thought to represent the group of Tregs capable of inducing IL-10 on bystander CD4 cells [29]. In a similar study, IL-10 production in response to ColV was compared in eight patients with BOS and eight healthy lung transplant recipient controls. There was a markedly diminished IL-10 production in vitro in patients with BOS. The production of IL-10 in response to ColV was dependent on a population of CD4⁺ CD25^{hi} cells [30]. Collectively, these findings support the notion that at least in vitro two distinct CD4 populations are necessary for inhibition of type-1 responses: a population of CD25⁺ Tregs and an additional population of foxp3[–] CD4⁺ cells capable of producing IL-10 (Fig. 1, panelB).

Evidence for Treg involvement in animal models

Based on the concept that ColV-specific Tregs can suppress in vitro responses to ColV by non-tregs, several groups have developed rat models of lung transplantation which take advantage of ColV-derived regulatory cells. In this model, minor histocompatibility-

mismatched single lung allografts demonstrate rapid cellular infiltrates by 2 weeks and obliteration of terminal airways by 10 weeks. Importantly when rats are fed human placenta derived ColV prior to transplant, they remain relatively resistant to the development of OB [21, 22]. CD4 spleen cells taken from an orally tolerized rat can confer protection from OB when adoptively transferred into another rat prior to transplant. In MLR studies from the tolerized mice, it turned out that the CD4⁺ CD45RChi cells were responsible for in vitro suppression of allospecific T cell response, an effect that could be abrogated by blocking TGF β . Further, the suppressive CD4⁺ CD45RC cells had diminished expression of Smad7, the Smad family molecule which strongly blocks TGF β signaling [31]. Clearly, these cells were not phenotypically classic Tregs, but rather conformed more closely to Th3 cells (reviewed in [32]). It is unknown whether these tolerizing cells expressed foxP3. In contrast, in the setting of a fully MHC-mismatched rat lung transplant model, ColV oral tolerance was insufficient to prevent significant acute rejection and graft failure. Yet when ColV oral tolerance was combined with low-dose standard immunosuppression with cyclosporine, long-term graft survival was achieved [33]. The survival of these grafts was associated with many of the usual suspects seen in other tolerance studies: an increase in perivascular foxP3, intragraft expression of IL-10, and suppression of IL-17 responses.

Anti-type V collagen T cell responses in human lung transplant recipients

Because immune effector and immune suppressor responses to ColV are associated with OB and tolerance in the rat model of lung transplant, longitudinal studies have been carried out in humans to determine if anti-ColV responses correlate with increased susceptibility to BOS. Burlingham and colleagues have studied this using the novel trans-vivo DTH assay [34]. In this assay, human T cells and the antigen of choice are injected into the footpad of a SCID mouse. The immune response is evaluated as the degree of induration measured in the footpad of the mouse. What these investigators have shown is that lung transplant recipients make elevated DTH responses to ColV, but not other types of collagen. Importantly, patients with Goodpasture's syndrome, a renal disease associated with antibodies against renal basement membrane constituents make DTH responses to Col(II), but not ColV. Collectively, these findings point to immunity to cryptic self as a potential mechanism for injury within a particular tissue compartment. When this assay system was applied to patients after lung transplant, it was the patients with preexisting BOS who had the most exuberant anti-ColV responses [34]. When this in vitro system was further dissected by removal of cell subset or by blocking antibodies to various cytokines several striking features were observed. In particular, footpad swelling was not dependent on either CD8 cells or INF γ , but was dependent of CD4 cells capable of producing IL-17 and CD14⁺ monocytes capable of producing IL-1 β and TNF- α . These findings hence strongly implicated a role for so called Th17 cells working in concert with inflammatory monocytes as being responsible for the trans-vivo response associated with BOS.

How does this association of elevated Th17 responses and adverse clinical outcomes in lung transplantation relate to the frequency or function of Tregs? First it is known that TNF α can diminish the suppressive function of Tregs [35], and this monokine was critical for the trans-vivo responses to ColV in humans. Similarly, IL-1 β has been shown to promote autoimmunity in NOD mice in part through inhibiting the function of CD4⁺ CD25⁺ FoxP3⁺ cells [36]. Next, multiple investigators have shown that Th17 commitment is reciprocally linked to Treg development: in mice CD4 cells exposed to TGF β alone convert into foxP3⁺ Tregs, while in the presence of TGF β plus IL-6 convert into Th17 cells [37–39]. In humans, Treg vs. Th17 commitment may not represent total lineage commitment either, as a number of groups have shown that human Tregs can be converted into Th17 cells with the addition of IL-1 β [40–42]. Collectively, these findings suggest that elevated Th17 responses may correlate with impaired Treg responses in response to ColV, a concept that is theoretically

testable in future work. In this regard, it is noteworthy that in a murine model of asthma, in which ovalbumin (OVA)-specific Th17 cells induce marked bronchiolar inflammation (similar histologically to OB) in response to inhaled OVA that the addition of antigen specific Tregs by adoptive transfer can markedly inhibit the cellular infiltrates [43]. Hence, similar to other disease states, freedom from BOS may ultimately hinge on the balance between Th17 cells and Tregs.

Tregs as a biomarker—on the road to mechanism

In clinical lung transplantation, the assessment of Tregs has emerged first as a tool to predict which patients are at high vs. low risk for progression to BOS. One of the first systematic analyses of peripheral T cell phenotypes published to date has implicated a subset of memory CD4 cells as a biomarker for subsequent BOS. Studer and colleagues have shown that patients with CD4 cells demonstrating low levels of surface CD28 are at risk of progressing to BOS [44]. Furthermore, the extent to which CD28 is diminished in the peripheral blood of these patients did not seem to be associated with age, acute rejection, or viral infection with CMV. Germane to the previously described in vitro studies [28], CD28null cells made significantly less IL-10 in comparison to CD28+ cells after anti-CD3 activation. While not the primary focus of their analysis, these investigators found that the CD28null cells expressed lower levels of surface CD25 and intracellular foxP3. Implicit in this finding is that the expansion of CD4 cells which have the hallmark features of extensive antigen exposure and proliferation is associated with a corresponding decreased frequency of peripheral Tregs. What these data cannot tell us is whether the expansion of the CD28null cells is a consequence of low Treg numbers/function or if the expansion of these cells is happening in spite of normal peripheral Tregs. Finally, it is possible that CD28 frequency and the development of Tregs are directly linked; recent data has shown that foxP3 expression by thymocytes is dependent on signaling through CD28 [45].

Meloni and colleagues reported the first case series to specifically assess Treg frequencies in the blood of patients with and without BOS [46]. Their analyses were performed before commercially available antibodies to detect intracellular foxP3 were available. Hence they defined peripheral Tregs as being cells which were CD4+ CD25+ and CD69-, a marker of early T cell activation. They showed that patients with BOS had significantly lower absolute numbers of CD4+ CD25+ cells compared with stable lung transplant recipients (20 cells/ μ L vs. 10 cells/ μ L). Interestingly, the absolute number of Tregs was higher in normal control subjects compared with either stable or chronically rejecting lung transplant patients. This later point may be a reflection of the effect of immunosuppression including calcinurin inhibitors. The development of Tregs relies in part on the presence of IL-2; calcinurin exposure hence can inhibit Foxp3 gene expression [47]. Because this early study was cross-sectional in design, it could not account for the fact that patients with BOS may have lower Treg counts as a direct consequence of higher cumulative and mean levels of calcinurin exposure. These findings have subsequently been confirmed by another group in which foxP3 expression was compared in autologous dendritic cell/peripheral blood mononuclear cells (PBMC) co-cultures in patients with BOS and patients with stable lung function [48]. Induction of foxP3 and production of IL-10 was observed in stable patients, whereas these markers were decreased after co-culture in patients with BOS. When the phenotype of the dendritic cells (DCs) used in these in vitro assays was assessed, stable patients DC's had a more immature phenotype which included increased expression of indolamine oxidase (IDO), the enzyme responsible for catalyzing the conversion of tryptophan to kynurenine. IDO activity has been linked to tolerance mechanisms in part through direct inhibition of T cell proliferation [49]. Further studies to define the effect of IDO levels on clinical stability of lung transplant recipients have generated surprising results. Elevated plasma ratios of kynurenine to tryptophan—reflecting increased IDO expression—were observed in patients

with BOS compared to stable patients [50]. Taken together, these studies of peripheral blood indicate that Tregs may be diminished in patients with BOS and that the levels of these cells may depend on continued interaction with other circulating cells (e.g., DCs). Yet these studies still cannot answer the question of whether BOS occurs because of low Tregs, or whether BOS leads to Treg depletion.

One of the limitations of using peripheral blood cell populations as a biomarker is that this cellular compartment may not indicate what is happening at the cellular level in the allograft itself. Because lung transplant patients are subjected to repeated surveillance bronchoscopy, they are an interesting study population in solid organ transplant patients in that their allograft can be sampled repeatedly over time. Bronchoalveolar lavage sampling involves placing a bronchoscope into a distal airway, placing saline into that segment of lung, and sampling the aspirated fluid for both cellular and protein constituents. One of the first studies to assess T cell subsets within the lung and correlate them to rejection found that patients with acute rejection had lower frequencies of CD3+ cells that expressed foxP3 compared to non-rejectors [51]. This study was limited by small sample size (20 patients) and cross-sectional study design in which patients were compared at different time points post-transplant. Furthermore, it is relevant that their definition of Treg frequency used all CD3+ cells as the denominator, and they found a significant association between elevated CD8 cells (which do not express foxP3, but are CD3+) and acute rejection. Our group has reported on a systematic study of lung transplant recipients who underwent serial surveillance fiberoptic bronchoscopies in the first post-transplant year [52]. We first identified patients who had acute rejection on their first post-transplant biopsy and compared the frequency of CD4+ cells which were positive for foxP3 in those patients to the same frequency in patients who were initially quiescent. We found that patients with early acute rejection actually had roughly twice the frequency of foxP3+ cells compared to quiescent patients. When we assessed patients over an entire year period, we found a strong correlation between cumulative rejection score—the sum of all rejection episodes by histologic grade—and mean BAL FoxP3+ frequency. These findings are not particularly surprising given recent reports in renal transplantation in which urinary sediment rich in foxP3 mRNA was associated with acute cellular rejection, but at the same time predicted response rates to typical augmentation of standard immunosuppression [53, 54]. Hence, for the case of acute rejection (and similar to the lung findings in murine ARDS), increased Tregs within the allograft may serve as a lagging rather than leading indicator of immunologic events. It is also possible that increased foxP3 expression is not only an indicator of Treg involvement, but also a feature of transient activation of non-regulatory cells. From single cell studies, we now know that low levels of foxP3 expression are transiently observed in vitro in sorted CD4+ CD25– cells subjected to nonspecific activation [55]. Furthermore, CD4+ CD25– cells which have been activated and produce foxP3 protein are functionally distinct from bona fide Tregs in their lack of suppressive capacity and their increased production of IFN γ and TNF α [56]. These findings highlight the fact that at least in humans, foxP3 may serve as a relevant biomarker for predicting outcomes without necessarily highlighting a mechanism for its actions. Perhaps future studies will take advantage of newer technologies such as assessing foxP3 promoter methylation status as a means to distinguish true Tregs from transiently activated non-Tregs [57].

While it appears that lung Tregs may be acutely activated in acute rejection, less is known about the prospects of these cells to control chronic rejection, nor is it known whether these cells can be a predictive biomarker for BOS. One published study has attempted to address this by dichotomizing post-lung transplant patients with BOS into two categories: “stable BOS” and “evolving BOS.” Stable BOS patients were defined as meeting the criteria of having obstructive airflow disease that did not change over 1 year's time while evolving BOS patients had declining spirometry on sequential studies. When divided in this manner,

patients with stable BOS had higher frequencies of CD4⁺ CD25⁺ CD69[−] cells compared with patients with evolving BOS (22% vs. 16%, $p=0.06$), but both populations had higher levels of these cells compared with “healthy” lung transplant recipients [58]. This study is important because it lays the groundwork for future studies which prospectively follow lung transplant patients over time and assess lung Tregs with respect to progression to BOS. Nevertheless, it is likely too early to draw firm conclusions from this study. Patients with “stable BOS” vs. “evolving BOS” crossed over between groups during the study. Additionally, there was no attempt made to account for time post-transplant as a variable which may modify Treg frequency. Hence if there are differences between stable and evolving patients they may be due more to the natural progression of lung immunity post-transplant rather than a driver of relative immunologic quiescence.

Going forward, the use of lung-specific sampling through BAL may benefit from not only cellular analysis of lavage fluid but also of the chemokines milieu within that fluid. For example, the above studies utilized flow cytometry of the cellular fraction of lung lavage fluid in which fewer than 10% of the recovered cells are lymphocytes, and among that, only several percent of the cells are Tregs. Hence important decisions regarding the relevance of Tregs relies often on sampling very rare events (sometimes less than 500 events per sample). It is well known that chemokines within the lung lavage fluid can be readily detected down to picograms per milliliter range and strongly correlate with the presence or absence of BOS. For example, the CXC chemokines CXCL9, CXCL10, and CXCL11 have been demonstrated to be elevated both in BOS and in a murine tracheal transplant model of BOS with strong specificity and sensitivity when comparing BOS from non-BOS patients [59]. Hence it is plausible that if Treg migration into the lung as a mechanism for protection of BOS is relevant, that stable patients would demonstrate elevated concentrations of chemokines important for Treg trafficking in their BAL fluid. One example of this is the chemokines CCL22 which has been shown in tumor models to be important for Treg recruitment into sites of malignancy and hence prevention of tumor killing [60, 61]. In fact, in a preliminary analysis Bhorade and colleagues found that CCL22 was detectable in six of nine stable lung transplant patients vs. only one of six patients with BOS (S. Bhorade in press, *Transplantation*). If these findings are validated, they could be applied to a larger cohort of patients prospectively in parallel with cellular analyses of Tregs which bear CXCR4, the receptor for CCL22.

Applying new knowledge into future study

The studies reviewed here reveal targeted areas for future pragmatic clinical studies. For example, it appears likely that a signature in both the PBMC and BAL component related to either Tregs or their relevant chemokines will identify patients at risk for BOS. These findings deserve prospective validation by additional centers. Once validated, a key question becomes what to do with the knowledge that a patient, based on a particular Treg phenotype is at high or low risk for development of BOS? An important related question is when does a Treg signature of good risk vs. bad risk emerge? Supposing we can identify such Treg signatures early—the first 6 months post-transplantation—then the question of what to do with a good risk signature is straightforward. These are the patients that should have protocol-driven de-escalation of immunosuppression. A key concept is that unlike liver transplant recipients, these good risk lung patients are unlikely to come off all immunosuppression. Nevertheless, any decrease in immunosuppressive exposure in the correct patient is likely to improve the survival curve post-transplant by decreasing the incidence and severity of issues such as coronary artery disease, metabolic syndrome, and PTLD.

On the other hand, what to do with patients with a poor risk signature is much more controversial. The majority of lung transplant patients are treated with three drug immunosuppression, typically a calcinurin inhibitor, cell cycle inhibitor, and prednisone. Further dosages of the calcinurin inhibitor target trough levels of drug typically higher than that used in kidney or liver transplantation. Hence, additional conventional immunosuppression is likely not the answer as it comes at the price of increased risk for infection and end-organ damage attributable to the systemic effects of drugs. Perhaps then, patients with Treg signatures deemed poor risk would be candidates for less conventional therapy. Examples of such unconventional therapy include extracorporeal photopheresis (ECP) and the use of novel biologic agents such as the CTLA4-ig fusion protein LEA29Y (belatacept).

ECP involves treatment of PBMC with DNA-intercalating agents followed by passage of cells through a circuit that applies UV light. These apoptotic cells then are reinfused into the recipient where they have been shown to induce Tregs [62]. The mechanism(s) whereby ECP induces immune regulation are still unclear but likely involve the release of IL-10 by treated leukocytes [63], the inhibition of IL-6 and TNF α production [64], increased TGF β production [65], and stimulation of CD39 which converts ATP to adenosine [66]. When applied to clinical situations, the use of ECP has led to modest benefit in graft vs. host disease (GVHD) following allogeneic bone marrow transplantation. It deserves to mention that histologically GVHD in the lungs appears almost identical to obliterative bronchiolitis in lung transplantation. Very early work assessing ECP in lung transplantation has shown in an uncontrolled observational study that patients exposed to ECP experience a diminished progression of BOS [67]. Furthermore, in an additional observational study ECP was associated with stabilization of peripheral CD4⁺ CD69⁻ CD25^{hi} cell counts in four of six patients with BOS who also demonstrated stabilization of spirometry [68]. Both of these studies are limited in that their study design does not allow comparison between treated and untreated patients, and by the fact that it is unclear if the effects seen are due in part from Tregs. Nevertheless, they form the scientific rationale for prospective clinical trials which can answer the question of whether ECP improves BOS and whether peripheral Tregs represent a relevant biomarker for study.

Costimulatory blockade with biologic agents is a potential new modality in solid organ transplantation. The concept of costimulatory blockade is to interrupt signals needed by T cells for optimal activation. Blockade of the CD28 pathway results in anergy and tolerance in animal models of transplantation [69]. Nevertheless, Treg function may also be dependent on the CD28 pathway [70, 71]. Hence attempts at costimulatory blockade might prove unsuccessful if such therapies lead to declines in Treg function and or frequency. In experimental lung transplantation in mice, the use of CTLA4-Ig in a fully allogeneic mismatch model was associated with significantly improved lung histology at 7 days coupled with the accumulation of CD4⁺ FoxP3⁺ T cells within the graft which had suppressive function against alloreactive CD8 cells in vitro [72]. In a human study of renal transplant recipients enrolled in a clinical trial of the second-generation CTLA4-Ig fusion protein known as belatacept (also known as LEA29Y), there were no differences in short- or long-term circulating Treg frequencies or function when compared to patients receiving conventional calcinurin-based therapy. Yet patients who received belatacept and experienced rejection had higher numbers of intra-graft Tregs compared to patients on conventional therapy with similar degrees of rejection [73]. It is noteworthy that kidney recipients who received belatacept in the phase III clinical trial experienced a higher rate of rejection, but overall enjoyed superior graft function [74]. Collectively these studies suggest that while costimulatory pathways may be important in the generation of Tregs, within the context of transplantation, targeted costimulatory blockade may result in a net benefit in the balance between T effector and T reg cells.

While ECP and costimulatory blockade represent novel high-tech approaches to altering immune responses, it is possible that the application of simple preexisting therapies may have a role in enhancing T cell regulation in lung transplant recipients. The use of inhaled corticosteroids is one example of such an approach. Inhaled corticosteroids are widely used for the treatment of asthma and bronchitis. As a result, there is a significant body of work detailing their effects on local T cell activation. For example, in vitro studies of patients with atopy and asthma have demonstrated an effect of fluticasone propionate on enhancing CD4⁺ CD25⁺ function [75]. Preincubation of CD4⁺ CD25⁺ cells with fluticasone increased the ability of these cells to secrete IL-10, and augmented the suppressive effects of the CD4⁺ CD25⁺ cells on CD4⁺ CD25⁻ cells that were exposed to allergens. This suppressive effect was blocked using antibodies to IL-10. Glucocorticoids have been shown to increase gene expression of foxP3 in the CD4⁺ cells of patients with asthma [76]. In a similar model, the combination of fluticasone and the β 2 agonist salmeterol significantly enhanced IL-10 production and decrease IL-13 production in a co-culture system in which allergen-specific CD4 T cells lines were exposed to their specific allergens [77]. These findings are relevant given that combination therapy with both inhaled corticosteroids and β 2 agonists are now considered the mainstays of clinical asthma therapy, and the use of these agents is neither burdensome nor particularly risky. Additional studies of refractory asthmatics whose CD4⁺ T cells do not produce IL-10 in vitro in response to steroids, could be rescued from this phenotype with the addition of vitamin D3 [78], the biologically active version of vitamin D which is widely available as a nutritional supplement. Finally, it is possible that even more “low tech” approaches could be used to increase the proportion of biologically relevant lung Tregs. One such approach is aerobic exercise. Prior studies have demonstrated that patients with type 2 diabetes demonstrate increased circulating foxP3⁺ cells in response to moderate exercise [79]. In parallel, in the murine OVA asthma model, repetitive aerobic activity was associated with increased lung foxP3⁺ cells in parallel to diminished BALF IL-17 levels [80].

Given that severe chronic asthma and OB share similar histologic features, namely, chronic small airway remodeling, it is conceivable that these standard asthma interventions may have clinical benefit in lung transplant recipients. One hint that the combination of inhaled corticosteroids plus β 2 agonist may be helpful comes from the allogeneic bone marrow transplantation literature. As in lung transplant, OB is a potential complication in the setting of bone marrow transplant presumably related to a graft vs. host phenomenon occurring at the terminal bronchiole. Histologically, OB following bone marrow transplant is indistinguishable from that occurring following lung transplant. Similar to lung transplantation, there is no effective systemic therapy for OB following bone marrow transplant. Bergeron and colleagues have tested the ability of combination inhaled therapy in 13 bone marrow recipients with irreversible airflow obstruction. Following initiation of therapy there was an improvement in forced expiratory volume in 1 s (FEV1) in 11 patients, and stabilization of airflow in two patients [81]. A similar small pilot study has been performed in lung transplant recipients with airflow obstruction and lung biopsy specimens demonstrating lymphocytic bronchitis (histologically the precursor to full-blown OB). In these 14 patients treated with inhaled corticosteroids alone, there was a small but significant improvement in FEV1 following initiation of inhaled corticosteroids [82]. Importantly, neither of these studies systematically examined the BALF fluid before and after initiation of inhaled therapy. A current randomized clinical trial of inhaled fluticasone (Clinical trial NCT00656916) will hopefully confirm the utility of this agent in bone marrow transplant recipients with OB. It is likely that future studies will systematically assess whether the combined treatment of inhaled steroids plus β 2 agonists does indeed result in greater recruitment of airway Tregs in transplant. The vast majority of lung transplant recipients are maintained on life-long oral corticosteroid therapy. Hence, the finding of increased BALF Tregs in the setting of add-on therapy with inhaled corticosteroids would suggest that the

mode of drug delivery (inhaled vs. oral) may be essential to modifying leukocyte populations which have already trafficked to the site of injury.

Concluding remarks

While outcomes in lung transplantation remain suboptimal with respect to other solid organ transplants, there exists a great deal of disparity within lung transplant recipients with respect to survival, with some patients enjoying durable adequate lung function. These patients represent an opportunity to understand the pathogenesis of BOS. Emerging data suggest that part of this beneficial effect is related to augmented immunologic regulation. This concept of regulation is likely not the result of actions of a particular cellular subset, but rather the cumulative effect of collaboration between Tregs, APCs, and non-Tregs which have accessory regulatory features such as the release of IL-10 or TGF β . While this field is relatively young, it appears that the balance between Th17 and Tregs may predict which patients are at risk of developing BOS. Many factors may contribute to this balance (see Fig. 1), including the relative concentration of pro-Treg (CCL22, IL-10, TGF β) vs pro-Th17 (IL-6, CXCL10, TGF β) present in the local environment. Once the mechanisms underlying regulation are better delineated, perhaps these processes can be augmented in all lung transplant recipients as part of a broader novel immunologic approach to transplantation.

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Fig. 1.

A schematic representation of bronchiolar injury and Treg/TH17 balance. **a** Multiple distinct processes lead to damage of the bronchiolar epithelium. **b** In the aftermath of damage, there is exposure to cryptic self antigens and release of proinflammatory chemokines such as CXCL9 and CXCL10. Simultaneous increases in production of IL1 β , TNF α , IL-6, and IL-23 by cells such as activated T cells, macrophages, and dendritic cells leads to exuberant Th17 responses. Recruitment of Tregs by CCL22/CCR7 interaction, and by elaboration of TGF β and IL-10 by accessory cells such as Th3 cells leads to increased numbers of Tregs. **c** The relative balance between Th17 cells and Tregs determines whether bronchiolar injury develops into OB or resolves.