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Updates on the controversial roles of regulatory lymphoid cells in idiopathic pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is the most common and severe form of pulmonary fibrosis, characterized by scar formation in the lung interstitium. Transforming growth factor beta (TGF-β) is known as a key mediator in the fibrotic process, acting on fibroblasts and mediating their proliferation and differentiation into myofibroblasts. Although the immune system is not considered responsible for the initiation of IPF, markers of tolerogenic immunity define the pro-fibrotic microenvironment in the lungs. In homeostatic conditions, regulatory T cells (Tregs) constitute the main lymphoid population responsible for maintaining peripheral tolerance. Similar to Tregs, regulatory B cells (Bregs) represent a recently described subset of B lymphocytes with immunosuppressive functions. In the context of IPF, numerous studies have suggested a role for Tregs in enhancing fibrosis, mainly via the secretion of TGF-β. In humans, most studies show increased percentages of Tregs associated with the severity of IPF, although their exact role remains unclear. In mice, the most commonly used model involves triggering acute lung inflammation with bleomycin, leading to a subsequent fibrotic process. Consequently, data are still conflicting, as Tregs may play a protective role during the inflammatory phase and a deleterious role during the fibrotic phase. Bregs have been less studied in the context of IPF, but their role appears to be protective in experimental models of lung fibrosis. This review presents the latest updates on studies exploring the implication of regulatory lymphoid cells in IPF and compares the different approaches to better understand the origins of conflicting findings.

1 Introduction

IPF is a progressive interstitial lung disease (ILD) characterized by thickening and scarring of lung parenchyma (1). It histologically presents as usual interstitial pneumonia (UIP) (2), with symptoms related to impaired gas exchange, including dyspnea, dry cough, and fatigue (3). The prognosis is poor, with a median survival of 3-5 years after diagnosis (4). Dominant hypotheses suggest that IPF results from genetic predisposition added to external factors, such as smoking, pollution, and viral infections, mainly in elderly males (5). Repetitive epithelial injury in the alveoli leads to an abnormal healing process involving senescence, epigenetic reprogramming, aberrant epithelial-mesenchymal crosstalk, and inflammatory cell recruitment (6–8). Fibroblasts and recruited fibrocytes differentiate within the injured areas into myofibroblasts, which become resistant to apoptosis, proliferate, and exhibit uncontrolled production of extracellular matrix (ECM) components, notably collagen (9). Resident and recruited immune cells can polarize towards a pro-fibrotic phenotype and directly enhance the accumulation of ECM via the secretion of pro-fibrotic mediators, such as TGF-β, interleukin (IL-) 10, tumor necrosis factor (TNF), and matrix metalloproteinases (MMP) (5). Current treatment relies on nintedanib and pirfenidone, two anti-fibrotic drugs (10). Pirfenidone slows collagen deposition and suppresses TGF-β activity (2), but its exact mechanism of action has not yet been fully elucidated (10). Nintedanib is a tyrosine kinase inhibitor that downregulates the production of ECM proteins and inhibits TGF-β-induced myofibroblast differentiation (11). Both drugs have significant adverse effects and fail to stop the progression of the disease toward its fatal outcome (12). For the time being, lung transplantation remains the only curative treatment (2), therefore, a deeper understanding of the mechanisms underlying IPF initiation and development is fundamental to unveil targeted and efficient therapies.

For years, the hypothesis of the inflammatory origin of IPF has seen an exploration of different immune cells and mediators (13). However, the non-response of IPF patients to immunosuppressive therapeutic strategies has reduced the interest in targeting the immune system. The fatal blow to this hypothesis came from the PANTHER-IPF trial (14). A combination of prednisone, azathioprine, and N-acetylcysteine resulted in increased risks of death and hospitalization in IPF patients. The failure of these trials not only suggests a limited secondary role for inflammation in IPF but also hints at the immune system potentially playing a critical regulatory role in this disease. This could explain the detrimental outcomes observed with its suppression. Therefore, instead of outright suppression, modulating the immune system may prove beneficial in managing IPF (15). For most myeloid immune cell subsets, namely neutrophils, macrophages, fibrocytes, and monocytes, a pro-fibrotic role has been reported. Concerning the adaptive immune response, T and B lymphocytes may play a protective or detrimental role depending on the subpopulation involved (16).

Among these subpopulations, regulatory B and T cells, namely Bregs and Tregs, are crucial in dampening the immune response and preserving immune homeostasis (17, 18). Aberrant activation of these subpopulations might be implicated in fibrotic conditions. While a wide amount of work exists on the role of Tregs in IPF,

although contradictory, Bregs have not yet been extensively studied and appear to have a protective role. This review summarizes the conflicting findings from the past five years regarding the roles of Treg and Breg subsets in IPF development.

2 Regulatory T cells

Tregs are mainly identified as CD4+ CD25+ FoxP3+ T cells and are known to be essential in maintaining peripheral immune tolerance, thereby preventing aberrant activation of the immune system and the development of autoreactive cells (17). Once activated, Tregs are a major source of immunosuppressive cytokines, mainly IL-10, TGF-β, and IL-35, that limit the differentiation, proliferation, and activation of effector CD4+ T cells. Although Treg activation is antigen-specific, bystander suppression could be exerted on neighboring T cells independently of antigens (19). Other direct mechanisms to suppress effector T cells were described, including the blockade of dendritic cell (DC) maturation, either through lymphocyte-activation gene 3 binding to the major histocompatibility class II molecules, or through cytotoxic T-lymphocyte antigen 4 (CTLA4)-CD80/CD86 interactions. This leads to the secretion of indoleamine 2,3-dioxygenase and consequent inhibition of DCs' activity (20).

Given the crucial role of Tregs in dampening the immune response and thereby maintaining homeostasis, dysregulation of their activity can result in various diseases, such as autoimmune diseases, allergies, cancer, and chronic infections (21). Though studies remain controversial, Tregs are also implicated in various fibrotic conditions, such as hepatic fibrosis (22) and systemic sclerosis (23).

2.1 Tregs in murine models of pulmonary fibrosis

The secretion of IL-10 and TGF-β by Tregs would have a deleterious effect on the mechanisms involved in the pathophysiology of PF (24). However, it appears that depending on their suppressive phenotype and the stage of the disease, regulatory T cells could also play a protective role in both human and murine models of PF (25–27). The bleomycin model is widely used in mice studies. It is characterized by two distinct phases: the inflammatory phase usually occurs during the first week after bleomycin injection, while the fibrotic phase takes place starting from the second week. In a study conducted by Takao et al., PF was induced in mice through oropharyngeal administration of bleomycin, and it was demonstrated that human bone marrow-derived serum-free mesenchymal stromal cells (SF−MSCs) administrated intravenously 4 days after bleomycin treatment have a beneficial effect on the disease. This protective role is exerted through the induction of regulatory T cells, whose numbers are increased in the blood and lungs of mice on the 2nd day after SF−MSCs treatment. Indeed, in blood and lungs, SF−MSCs lead to increased TGF-β1 and decreased IL-6 levels at day 7 and 14, respectively. Consistently, Treg depletion causes a loss of

the antifibrotic effects of SF-MSCs (26). Furthermore, it was demonstrated in mice that the adoptive transfer of Tregs 14 days after bleomycin treatment, reduces IL-10 and FGF9 levels in plasma, and subsequently decreases fibrotic lesions at day 28 (25). Given the established link between the aryl hydrocarbon receptor (AhR) activation and Treg generation (28), Takei et al. demonstrated that AhR engagement after bleomycin administration increases the number of pulmonary Tregs and alleviates fibrosis development (27). Zhang and colleagues showed that from days 3 to 28 after bleomycin instillation, Treg percentages are increased in both peripheral blood and spleen. Additionally, the proportion in the blood is positively correlated with the fibrotic score. From day 3 to day 14, Th1 responses are prevalent, while a switch towards Th2 polarization starts from day 14. Tregs participate in this Th2 polarization, as their co-culture with spleen lymphocytes leads to decreased interferon (IFN-) γ and increased IL-4 secretion. Additionally, the adoptive transfer of Tregs in bleomycin-treated mice during the inflammatory phase worsens fibrotic lesions, while preventive transfer leads to decreased hydroxyproline (HP) content in the lungs (29) (Figure 1). When PF is induced in mice through overexpression of TGF-β, the levels of regulatory T cells in the lungs and pro-fibrotic markers correlate with the tissular sodium level, a detrimental factor in a fibrotic environment (30). In a recent study, Liu et al. showed that the percentage of Tregs from CD4+ T lymphocytes increases from day 3 to day 14 after bleomycin administration, while it decreases from day 14 to day 28. Also, they showed that anti-fibrotic drugs such as pirfenidone, prednisolone, neferine, and isoliensinine, can alter Treg dynamics across the different phases of lung injury, highlighting therefore a new factor that could explain data variabilities across the different studies (31). Interestingly, Zhao et al. showed that intermittent fasting attenuates bleomycin-induced PF and decreases the percentages and counts of pulmonary Tregs. The authors therefore suggested that the reduction of Tregs through intermittent fasting dampens PF development (32).

In the context of the acute exacerbation (AE) of PF, Tregs' role remains also controversial. In a pulmonary fibrosis model induced by adenoviral vector-mediated overexpression of TGF-β1 (AdTGF-β1) and exacerbated with S. pneumoniae infection, Treg numbers were shown to be significantly increased in both bronchoalveolar lavage (BAL) and lungs at day 14 compared to days 7 and 21. While depletion of Tregs worsens the exacerbation, expansion with IL-2/anti–IL-2 monoclonal antibody (mAb) complexes leads to its inhibition. The levels of TNFα, IL-6, and TGF-β1 are decreased 14 days after the injection of mAb complexes, suggesting that Tregs modulate T cell cytokine responses and dampen PF development (33). Contrarily, Tao et al. showed that Tregs are involved in the macrophage inducible C-type lectin (Mincle)-mediated AE of PF in the bleomycin mouse model. Mincle deficiency results in a smaller Treg population in BAL, improved survival, and milder fibrotic lesions. Thus, the authors propose a detrimental role for Tregs in the AE of PF (34).

2.2 Tregs in IPF patients

Although mechanistic data concerning the role of human Tregs in IPF in the last 5 years seems to be lacking, the reported proportions in patients compared to controls are not always consistent from one study to another. In particular, peripheral blood, BAL, and lung tissue do not always follow a common thread among IPF patients, and inconsistency is also encountered among samples of the same nature. A protective role for Tregs in humans has been suggested and appears to be tightly regulated by the expression of immune receptors. Decreased surface expression of the inducible T-cell costimulator (ICOS) on CD4+ T lymphocytes, a receptor responsible for the maintenance of
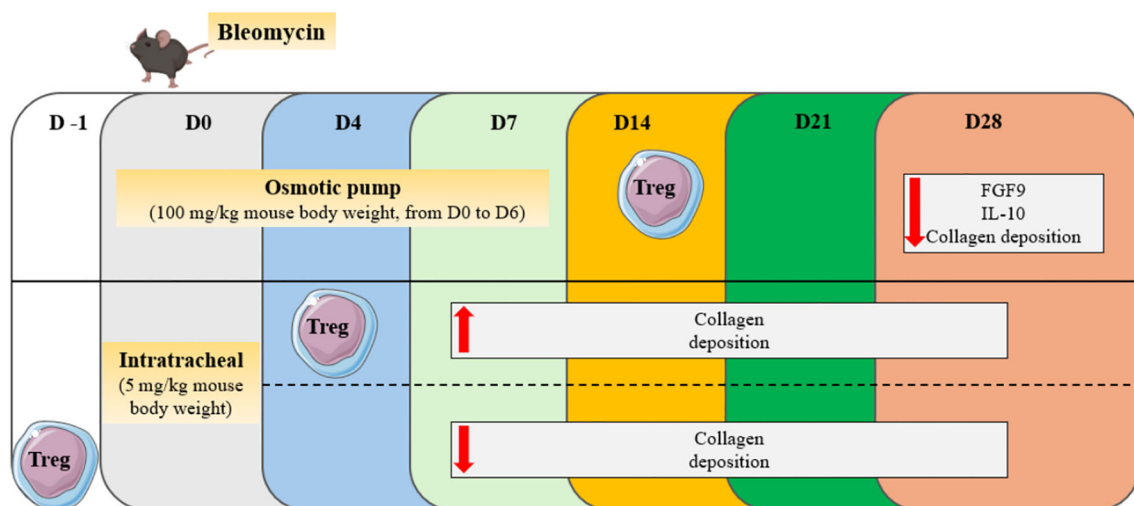

FIGURE 1
Effect of Treg transfer in the bleomycin model of PF. Depending on the adoptive transfer day (D-1, D4, D14), Tregs can play a protective or detrimental role across the different phases of bleomycin-induced lung injury.

immunosuppressive Tregs, is significantly correlated with decreased lung function, while a high expression results in improved survival (35). Conversely, Programmed cell death protein 1 (PD-1) blockade on myofibroblasts leads to increased Treg differentiation and decreased collagen levels, suggesting a protective role for this T cell population in IPF (36). Yin et al. used a risk assessment model of inflammation-linked genes to distinguish two groups of IPF patients based on their overall survival. Cell infiltration analysis revealed that the infiltration of Tregs is higher in the BAL of the high-risk group as compared to the low-risk group (37). Percentages from CD4+ T cells are also higher in BAL of IPF patients compared to sarcoidosis patients (38) and connective tissue disease-associated ILDs (39). Percentages of Tregs from blood CD4 + T cells are instead lower in IPF than in sarcoidosis patients (38). Compared to healthy controls, some authors reported that the percentages of Tregs from CD4+ T cells in the blood of IPF patients are higher (40), while others showed a decreased percentage (36). Moreover, it seems that activated fibroblasts do not enhance the Treg population, as co-culture of TGF-β-stimulated human fibroblasts with CD4+ T cells does not cause them to differentiate into Tregs (36). The recruitment of Tregs in IPF patients compared to healthy controls was also assessed by Wu et al. using publicly available datasets. The infiltration status of 22 types of immune cells was analyzed using a CIBERSORT algorithm (41). While some studies report no difference between Treg cell percentages in lungs from IPF patients compared to controls (42), Dai et al. found decreased percentages of Tregs in IPF tissues (43). A recent study by Unterman et al., based on transcriptomic data, demonstrated different data in the blood (44). Single-cell RNA sequencing was performed on peripheral blood mononuclear cells (PBMCs) from healthy subjects and stable or progressive IPF. It was shown that Tregs are increased in PBMCs from patients presenting with the progressive form of the disease compared to the stable group, and in combined IPF patients compared to controls. However, no difference was noticed between stable IPF patients and healthy controls. The authors therefore proposed that the discrepancy in Treg variation reported by the literature may be due to a lack of distinction between patients at different stages of the disease. Results also suggested that Treg recruitment implicates the interactions of the receptors CCR4 and CCR8 on Tregs with their respective ligands CCL22 and CCL18. Both chemokines are known to be secreted by DCs and macrophages and show higher levels in plasma from IPF patients compared to controls. Moreover, Tregs are the only lymphocyte population that increases in the lungs (44). In murine models of PF, the fibrotic response originates from strong inflammatory processes that develop following bleomycin injection (45). It is therefore coherent that Tregs may be protective at least during the inflammatory phase but then switch roles to contribute to fibrosis by secreting pro-fibrotic mediators. However, the cause of human IPF is unknown, and it remains unclear whether inflammation participates in the initiation of the disease or is a collateral consequence of a fatal pathophysiological process. Therefore, an understanding of how Tregs mechanistically participate in IPF development is still lacking, especially since the

intrinsic immunosuppressive role of Tregs might not be sufficient to point out their pro-fibrotic contribution.

3 Bregs in PF

Besides their classical functions, B cells and plasmablasts can differentiate into regulatory B cells. Induction of these cells can occur following stimulation with LPS, cytokines such as IL-1β, IL-2, IL-6, and IFN-α, or contact with T cells. Plasmacytoid DCs have also been demonstrated to induce the differentiation of B cells into Bregs through binding of CD40 and secretion of IFN-α. Additionally, Immunoglobulin-A, PD-L1, TIGIT, and Fas-L expressing Bregs can be generated through the binding of proliferation-inducing ligand (APRIL) to naive B cells (46). Bregs secrete IL-10, IL-35, and TGF-β (47), and can express PD-1 ligand, Granzyme B, CD39, CD73, and the AhR (46), thereby contributing to the dampening of the immune reaction to prevent chronic inflammation and the development of autoimmune conditions (47). Indeed, Bregs inhibit the differentiation of pro-inflammatory lymphocytes, such as Th1, Th17, and CD8+ T cells, while promoting the activity of immunosuppressive Tregs and inducible NK cells (47). The phenotypic characterization of Bregs remains debatable, even though secretion of IL-10 has been commonly referred to as the "hallmark of Breg suppression" for years (48). Although the involvement of IL-10 in the pathophysiology of IPF is well known, it being a pro-fibrotic cytokine, the role of Bregs remains yet to be clarified, with works mainly focusing on quantitative assessments in patients' samples. A study by Asai et al. revealed that the proportion of Bregs in blood among total CD3-CD19+ B cells is lower in IPF patients compared to healthy controls, with lower numbers in patients presenting a more severe form of the disease. This suggests a protective role of Bregs in this context (49). D'Alessandro et al. also described lower percentages of Bregs from total B cells in the peripheral blood of IPF patients compared to the healthy control group. Interestingly, nintedanib treatment can restore Breg numbers and consequently redirect the inflammatory responses (50). Another study reported that in the bleomycin or adenoviral TGF-β-induced models of pulmonary fibrosis, Bregs are less than 1% of total B cells. In both models, Breg percentages from total B cells are higher in fibrotic lungs than in controls, but B cell depletion does not affect fibrosis development (51). Breg's intrinsic immunosuppressive role may suggest novel perspectives regarding the modulation of the immune response in IPF. Therefore, their involvement in IPF deserves to be further elucidated.

4 Discussion

While Bregs are relatively new to the field of IPF research, the role of Tregs in this pathology remains a topic of considerable debate, and robust strategies targeting these populations have yet to be developed. Most human studies focus on variations in Treg percentages in the blood and BAL, resulting in inconsistent findings (Table 1). Many factors might underlie this discrepancy starting

TABLE 1 Variations in the Treg and Breg populations in murine models of PF and IPF patients.

MOUSE					
Reference	Model of PF/Species	Treg variation	Specimen	Effect on fibrosis development	Treg role
(26)	Bleomycin mouse model – SF-MSCs administration at D4	Increased counts	Blood and lungs D6	Antifibrotic effect of SF-MSCs exerted through Treg induction. Increased TGFβ at D7, decreased IL-6 at D14	Protective
(25)	Bleomycin mouse model – Treg transfer on D14			Reduced fibrotic lesions. Decreased IL-10 and FGF9 in plasma at D9	Protective
(27)	Bleomycin mouse model – AhR ligand treatment at D0, D1, D2	Increased counts	Lungs	Dampened inflammatory phase and thereby fibrosis	Protective
(29)	Bleomycin mouse model	Increased percentages	Peripheral blood and spleen	Positive correlation with the fibrotic score	Deleterious
		-adoptive transfer of Tregs during the inflammatory phase		Worsening of the fibrotic lesions	Deleterious
		-preventive transfer of Tregs		Decreased hydroxyproline content in the lungs	Protective
(30)	TGF-β1 transgenic mouse model	Increased percentages	Lungs	Positive correlation with Na+ levels in the tissue	Deleterious
(31)	Bleomycin mouse model	Increased percentages (day 3 to 14)	Peripheral blood		
		Decreased percentages (day 14 to 28)			
(32)	Bleomycin mouse model - intermittent fasting	Decreased counts and percentages	Lungs	PF attenuation	Protective
(33)	AdTGF-β1 mouse model, S. pneumoniae-induced exacerbation	Increased counts, with the peak on D14	Lungs and BAL		Protective
		Depletion		Worsened exacerbation	
		Expansion		Inhibited exacerbation	
(34)	Bleomycin mouse model, Mincle-mediated AE	Decreased counts following the silencing of Mincle-coding gene	BAL	Improved survival and milder fibrotic lesions	Deleterious
Reference	Model of PF		Breg variation		Specimen
(51)	Bleomycin/AdTGFβ1 mouse model		Higher percentages		Lung tissue
HUMAN					
Reference	Treg variation		Specimen		Control
(37)	Increased percentages in high-risk patients		BAL		Low-risk group
(38)	Increased percentages				Sarcoidosis
	Decreased percentages		Peripheral blood		
	Increased percentages		BAL		CTD-ILDs
(40)	Increased percentages		Peripheral blood		Healthy
(36)	Decreased percentages				
(42)	No difference		Lungs		Non-fibrotic
(43)	Decreased percentages				
Reference	Breg variation		Specimen		Control
(49)	Decreased % from total B cells		Peripheral blood		Healthy
(50)	Decreased % from total B cells, restored with Nintedanib		Peripheral blood		Healthy

with the heterogeneity of the disease itself and the different stages of progression. Indeed, various mechanisms, which may differ from patient to patient, could initiate IPF and uniquely affect the balance between immune cell populations (52, 53). To partly address this challenge, a thorough stratification strategy of IPF patients according to various epidemiological and clinical parameters might be essential to detect variations between subgroups. This aligns with the recent study by Unterman et al., which underscores the importance of considering the disease's progressive nature when assessing Treg quantitative variations (44). Additionally, basic demographic factors like gender and age are not to be neglected in the stratification strategy. It is known that males generally have more Tregs than females and that natural Tregs accumulate with age. Therefore, these factors should be considered when assessing variabilities across studies. Since older males have a high IPF prevalence, it is tempting to speculate that Treg accumulation in the lungs increases the risk of developing IPF.

Another critical point is the heterogeneity of Tregs themselves and their potential plasticity in IPF. Various Treg subpopulations may emerge and diminish throughout the disease progression, making it less informative to focus solely on total Treg numbers. Instead, examining distinct Treg profiles at different stages and severity levels of IPF might provide more valuable insights into the potential existence of pro- and anti-fibrotic subsets of Tregs in IPF. For instance, different Treg subsets can be identified according to their chemokine receptor expression, such as CXCR3+ Tregs and CCR6+ Tregs, highlighting their functional diversity. More transcriptomic studies will also be required to understand the specific gene expression patterns associated with Treg subsets and their functions in IPF.

Beyond understanding the global protective or deleterious role of Tregs in IPF, it is crucial to determine if these cells are antigen-specific and activated to increase tolerance toward specific antigens. Identifying the self- or non-self antigens that trigger Treg recruitment to the lungs can lead us to a better investigation of the factors triggering IPF development. If the activation is antigen-independent, understanding the mechanisms behind this activation is equally important.

In mice, studying Tregs in the context of bleomycin-induced models presents significant challenges. Unlike bleomycin-induced lung injury, where fibrosis occurs as a consequence of acute lung inflammation, IPF is characterized by chronic and progressive scarring of lung tissue. This fundamental difference undermines the utility of bleomycin models for accurately studying the role of Tregs in IPF. In particular, depletion and adoptive transfer experiments do not account for the plasticity and heterogeneity of Tregs across the disease stages.

While novel technologies have revolutionized research in the field of immunology, the study of Tregs and Bregs in IPF still has much ground to cover and warrants deeper investigation into its underlying mechanisms. However, it's important to acknowledge the many limitations, such as the difficulty in accessing human samples and the relatively limited numbers of these cell populations. Additionally, the lack of relevance of mouse models to human disease poses further challenges to achieving a comprehensive understanding. Moving forward, addressing these challenges with collaborative efforts and innovative approaches could be beneficial for improving therapeutic outcomes for IPF patients.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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