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The number of regulatory T cells in transbronchial lung allograft biopsies is related to FoxP3 mRNA levels in bronchoalveolar lavage fluid and to the degree of acute cellular rejection

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ABSTRACT

Background: The transcription factor Forkhead Box P3 (FoxP3) is a marker of regulatory T cells (Tregs) — a subset of T cells known to suppress a wide range of immune responses. These cells are considered to be pivotal for the induction of tolerance to donor antigens in human allografts. We aimed to correlate the number of lymphocytes expressing FoxP3 in transbronchial biopsies from lung allografts with the FoxP3 expression in bronchoalveolar lavage fluid (BALF). In addition, we aimed to correlate the number of FoxP3+ cells in transbronchial biopsies with the degree of acute cellular rejection in lung allografts.
Materials and methods: The expression of FoxP3 was evaluated using immunohistochemical staining in 40 lung allograft biopsies obtained from 23 patients. The number of Tregs was related to the FoxP3 mRNA levels as determined using qRT-PCR in corresponding BALF samples from the same patients. Furthermore, the number of Tregs was related to the degree of acute allograft rejection (according to ISHLT criteria, A0–A4).
Results: Regression analysis showed a significant concordance between the number of Tregs in lung tissue and the level of FoxP3 mRNA relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels in BALF ($n = 40$, $p = 0.0001$). In addition, we found a significant increase in the number of Tregs during acute allograft rejections of grades A2 and higher (median: 32.6 Tregs/mm^2) when compared to those of grades A1 and A0 (median: 4.9 Tregs/mm^2) ($p = 0.0002$).
Discussion and conclusion: The association between the distribution of Tregs in transbronchial biopsies and the level of FoxP3 mRNA in BALF indicates that assessment of FoxP3 mRNA in BALF is a reliable non-invasive method for evaluating the number of Tregs in lung tissue. Furthermore, the association between the number of Tregs in lung tissue and the degree of acute cellular rejection shows that Tregs are recruited to the site of inflammation and may be involved in the regulation of acute rejection. Thus, Tregs may play a role in the cellular processes that affect lung allograft outcome.

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1. Introduction

Over the last two decades, lung transplantation has evolved as an accepted treatment of end-stage pulmonary failure in patients who are unresponsive to other therapies. Advances in immunosuppression treatment and surgical techniques have greatly improved short-term survival for this patient population; however, long-term outcome is still poor, with 5- and 10-year survival rates in our center of 63% and 36%, respectively [1]. The poor outcome is mainly due to chronic allograft rejection with fibrous obliteration of the small airways [2–4]. This condition affects an estimated 48% of patients within 5 years of transplantation [2]. The immunological background for chronic rejection is poorly understood, but acute cellular rejection is the most important known risk factor for the development of subsequent chronic allograft rejection [5]. Therefore, the study of inflammation during acute rejection episodes might offer insights into the mechanisms leading to chronic rejection.

In this study, we focus on a subset of CD4+ T lymphocytes known as regulatory T cells (Tregs). These cells play an important role in maintaining immune homeostasis, and they are considered to be pivotal for the induction of tolerance to donor alloantigens in different human allografts by suppressing a wide range of immune responses [6–12]. The balance between alloantigen reactive Tregs and T-effector cells is thought to determine rejection outcome for the graft [13]. Several subsets of T cells with regulatory function is known, e.g., some CD8+ T cells, CD3+CD4−CD8− double negative cells and CD4+ IL-10 producing Tr1 cells, but the most extensively studied type is CD4+FoxP3+ T cells. This group of cells comprises the “natural” Tregs developed in

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Table 1
Demographic characteristics of the study population.

Patient number	Gender	Age at Tx	Type of Tx	Primary diagnosis	Samples available (months)
1	M	38	S	A1AT	24
2	M	60	S	A1AT, EMF	24
3	M	28	D	CF	24
4	F	34	D	CF	24
5	M	56	S	COPD, EMF	12
6	F	28	D	CF	3
7	F	58	S	COPD	12
8	F	59	D	COPD	6
9	M	56	D	A1AT, EMF	6
10	F	31	D	CF	6
11	M	21	D	CF	3, 6
12	F	55	D	NSIP	6
13	M	56	D	UIP	1/2, 1 1/2, 3
14	F	33	D	CF	1, 1 1/2, 3
15	F	51	S	A1AT	1/2, 1, 1 1/2, 3
16	F	59	S	COPD, EMF	1/2, 1, 1 1/2, 3
17	F	39	S	EMF	1 1/2, 3
18	F	23	D	CF	1/2, 1, 1 1/2
19	F	23	D	CF	1/2, 1, 1 1/2
20	M	58	D	SARC	1 1/2
21	M	58	S	UIP	1, 1
22	M	58	D	A1AT	1 1/2
23	M	56	S	COPD	1 1/2

Patients included in the study.
Abbreviations used: Tx: Transplantation, A1AT: Alfa 1 antitrypsin deficiency, EMF: Pulmonary emphysema, CF: Cystic fibrosis, COPD: Chronic obstructive pulmonary disease, NSIP: Non-specific interstitial pneumonia, UIP: Usual interstitial pneumonia, SARC: Sarcoidosis.

the thymus as well as the "induced" Tregs developed peripherally. Both cell types mediate their effect by a diversity of mechanisms, e.g., by suppression of proliferation of T-effector cells. The transcription factor FoxP3 is thought to be a unique marker of Tregs [14]. T effector cells may also express FoxP3, but only transiently after activation and at significantly lower levels than those found in Tregs [15]. In the following the cells referred to as Tregs are the FoxP3 + T cells.

Tregs have been studied using different methods: mRNA expression levels in BALF [16], the relative number of cells in BALF [17,18], and the frequency of cells in peripheral blood [16,19], etc. However, the concordance between these results or the concordance with the actual frequency of Tregs in tissue has not been thoroughly addressed. This lack of data makes the comparison and summarization of results from different studies difficult.

Some studies have found a positive correlation between the frequency of Tregs in blood and prolonged allograft survival in animal models [20] and better lung function in humans receiving a lung transplant [20,21]. Several other studies suggest that a correlation exists between acute rejection and the number of Tregs present in BALF of lung transplanted patients and between acute rejection and the number of Tregs present in endomyocardial biopsies of heart transplant recipients. Notably, in these studies this correlation does not extend to the number of Tregs found in blood [11,16,22,23], indicating that Tregs accumulate in the graft. This accumulation might be important for the general outcome and BALF might be used to predict the presence of Tregs in lung tissue. It is therefore important to further investigate whether the number of Tregs present in BALF is comparable to those found in tissue sections.

2. Objective

We aimed to correlate the frequency of lymphocytes expressing FoxP3 in transbronchial biopsies with the levels of FoxP3 mRNA in BALF from lung transplant recipients. In addition, we attempted to correlate the frequency of Tregs in tissue to the degree of acute rejection of the transplanted lung.

3. Materials and methods

3.1. Material

A total of 40 samples were collected from 23 consecutive lung transplant patients enrolled in the surveillance follow-up of the Danish National Lung Transplant program at Copenhagen University Hospital, Rigshospitalet. The patients were enrolled in the study over a 6-month period (September 2008–March 2009). The samples consisted of corresponding BALF and lung biopsies obtained during the same bronchoscopy procedure for each patient. The biopsy specimens contained an average of 4.9 biopsies, and all of the biopsies were scored for acute rejection according to ISHLT criteria (A0–A4) by one observer (CBA) [24]. 23 samples showed no sign of acute rejection (A0), 9 samples showed minimal acute rejection (A1), 7 samples showed mild acute rejection (A2) and 1 sample showed moderate acute rejection (A3). The time after transplantation varied from 2 weeks to 2 years, with a median time of 6 weeks.

All of the patients received initial standard immunosuppression consisting of induction therapy with antithymocyte globulin (ATG, 1.5 mg/kg for three days), cyclosporine, azathioprine and prednisolone.

The demographic characteristics of the study population are shown in Table 1.

The study was approved by the Ethical Committee of Copenhagen.

3.2. PCR analysis of FoxP3 mRNA in BALF

PCR results were obtained for 40 samples. The results of the PCR analysis for FoxP3 mRNA in BALF have been previously published, and the details of the technique were described [25]. Briefly, samples were analyzed using qRT-PCR, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the primary reference mRNA using the Taqman assay. ΔCt (difference in threshold cycles) for the GAPDH-normalized FoxP3 levels was calculated.

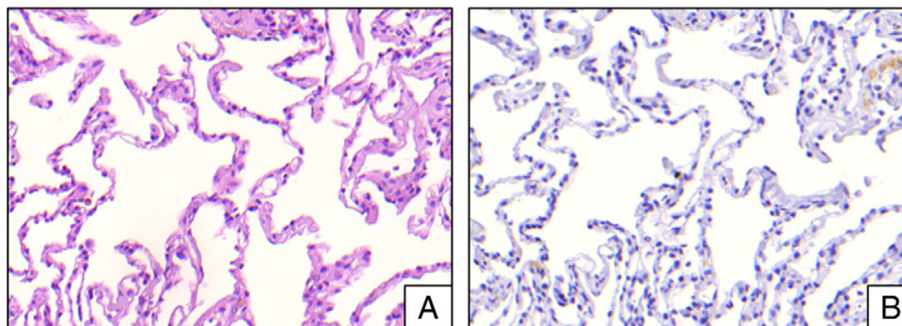

Fig. 1. Lung biopsy without acute cellular rejection (A0). H&E stain (A) showing no lymphocytic infiltration and immunohistochemical staining (B) showing only a single FoxP3 positive nucleus (×20).

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