Calcineurin inhibitors in bronchiolitis obliterans syndrome following stem cell transplantation

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ABSTRACT

Background: Bronchiolitis obliterans (BO) is a complication after allogeneic hematopoietic stem cell transplantation (HSCT). BO-management comprises intensive immunosuppression, but treatment response is poor. We investigated the effect of cyclosporine A (CsA), tacrolimus (FK506), methylprednisolone (mPRED), mycophenolate mofetil (MMF), and everolimus on the proliferation of primary lung myofibroblasts from HSCT-patients with bronchiolitis obliterans syndrome (BOS).

Methods: Cells were isolated from surgical lung biopsies of 8 HSCT-patients with BOS. Proliferation was assessed by $[^{3}H]$-thymidine-incorporation. Results: Biopsies revealed constrictive BO in 3 and lymphocytic bronchiolitis (LB) in 5 patients. CsA and FK506 significantly induced proliferation of myofibroblasts. mPRED and MMF caused a significant inhibition of proliferation, whereas everolimus had no effect. Co-stimulation with FK506, mPRED and MMF significantly inhibited proliferation. Serial pulmonary function tests over 12 months after lung biopsy and under the triple-therapy demonstrated that patients with LB had a significant improvement of their FEV1, whereas FEV1 of patients with BO was unchanged. Conclusion: Our data demonstrate a pro-poliferative effect of calcineurin inhibitors on primary human lung myofibroblasts obtained from patients with BOS after HSCT. In contrast, based on the observed anti-proliferative capacity of MMF in vitro, MMF-based calcineurin inhibitor-free treatment strategies should be further evaluated in patients with BO after HSCT.

Key words: bronchiolitis obliterans, chronic allograft rejection, immunosuppressive therapy, myofibroblasts
Abbreviations: α-SMA: α-smooth muscle actin; BAL: broncho-alveolar lavage; BO: bronchiolitis obliterans; BOS: bronchiolitis obliterans syndrome; CsA: cyclosporine A; CT: computed tomography; FCS: fetal calf serum; FEV1: forced expiratory volume in 1 second; FK506: tacrolimus; GvHD: graft-versus-host disease; HSCT: hematopoietic stem cell transplantation; LB: lymphocytic bronchiolitis; MMF: mycophenolate mofetil; mPRED: methylprednisolone; PFT: pulmonary function test; SEM: standard error of the mean, VATS: video-assisted thoracoscopic surgery.
INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established treatment option for several malignant and non-malignant disorders (1). However, the long-term survival is limited by recurrence of the underlying disease and development of chronic graft-versus-host disease (GvHD), the most common late complication after allogeneic HSCT (2). The manifestation of chronic GvHD in the lungs, bronchiolitis obliterans (BO) (3), has a reported incidence between 5 and 20% (4-5). BO is characterized by the new development of fixed airflow obstruction, manifesting with cough, dyspnea, and wheezing (3, 6). The term bronchiolitis obliterans stands for a histopathologically proven diagnosis, showing damage to the bronchiolar epithelium, obliteration of bronchiolar lumens, and inflammation between the epithelium and smooth muscle (7). In the absence of a histologic confirmation, the clinical diagnosis of bronchiolitis obliterans syndrome (BOS) requires decreased airflow in the pulmonary function tests and air trapping in computed tomography (CT) scans (3, 8).

Even though BO is strongly associated with chronic GvHD (9), the heterogeneous histopathologic findings and clinical course imply that formation of BO is a multifactorial process involving both allo-immune and non-allo-immune mechanisms (10). The pathogenesis of BO is believed to be initiated by repetitive lung tissue injuries. Those injuries may be caused by different factors, such as the interaction between donor T-lymphocytes and recipients epithelial cells (11), tissue damage caused by the conditioning regimen (12), or infection (13). Whether or not immunosuppressive agents play a role in BO development and/or progression after HSCT - beyond an increased susceptibility to infections - has not been studied yet.
Data from lung transplantation literature suggest that immunosuppressive agents such as calcineurin inhibitors and corticosteroids are likely beneficial by slowing down the progression of BOS (14-15). Therefore, treatment for BO after HSCT is based on reinstitution or augmentation of immunosuppressive therapy with high dose systemic corticosteroids and other agents, namely cyclosporine A (CsA) and tacrolimus (FK506) (16-17). However, despite different treatment approaches, prognosis of BO remains poor, with an overall 3-year mortality of up to 65% (18). Additionally, the potential benefit of augmented immunosuppression might be limited due to the occurrence of life threatening pulmonary infections. Currently, no data from large controlled trials are available, and BO treatment is based on expert opinion and small case series only.

The fibrogenic effects of CsA and FK506 have been studied extensively in renal allografts (19), and are predominantly mediated by elevated expression of transforming growth factor-β (20-21). Additionally, previous data have demonstrated that CsA may directly stimulate cell proliferation (22-23), raising the possibility that calcineurin inhibitors may contribute to the development of chronic GvHD in the lung. The aim of the present study was to investigate the in vitro effect of the immunosuppressive agents CsA, FK506, methylprednisolone (mPRED), mycophenolate mofetil (MMF), and everolimus on the proliferative capacity of primary human lung cells obtained from patients with BOS after HSCT.
MATERIALS AND METHODS

Patients
Primary human lung cell cultures were successfully established from lung tissue obtained from surgical lung biopsies of 8 patients with BOS after HSCT. All patients had an irreversible decline in their forced expiratory volume in 1 second (FEV1) of > 15% and symptoms suggestive of BO, and thus underwent surgical lung biopsy for final diagnosis. Before lung biopsy all patients had a high resolution CT scan and underwent flexible bronchoscopy with broncho-alveolar lavage (BAL) to exclude infection as a cause of lung function decline. CT scans were analyzed for pulmonary infiltrates, bronchial wall thickening, bronchiectasis, and air trapping. In the lavage fluid total and differential cell counts were performed. Lung biopsy was performed by video-assisted thoracoscopic surgery (VATS). Lung wedges were inflated and fixed with 4% buffered formalin. After paraffin embedding, specimens were cut into 4 µm sections and routinely stained with hematoxylin and eosin, elastica van Gieson, alcian blue-periodic acid Shiff, and Grocott’s methenamine sliver stain. Tissue sections were reviewed by two experienced lung pathologists. Patient characteristics are shown in table 1. All patients are part of a larger cohort the clinical characteristics of which will be described in more details in a separate paper (24).

Pulmonary function tests
Patients underwent a clinical assessment and a complete set of pulmonary function tests (PFT) before and after HSCT, before VATS, and 3, 6, and 12 months after VATS. PFTs were performed by using body plethysmography and the carbon monoxide diffusion capacity (Jaeger, Wuerzburg, Germany). All testing was performed according to the European Respiratory Society standards (25). Pulmonary function follow-up data
were available from seven patients; one patient died four months after diagnosis of BOS due to severe infection.

**Cell Culture**

Human lung tissue was obtained with approval of the Human Ethics Committee of the University of Basel, and written informed consent was obtained from all patients who underwent surgical lung biopsy. Primary human lung cell cultures were established as reported previously (26). Briefly, fresh peripheral lung tissue was obtained from the lung of patients undergoing diagnostic resection, and was cut into small pieces of 1 mm³ which were placed into pre-wetted 25 cm² cell culture flasks. Culture medium, consisting of RPMI (Gibco, Invitrogen; Lucerne, Switzerland) supplemented with 10% fetal calf serum (FCS) (Gibco), 20 U/L penicillin, 20 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco), was replaced every fourth day. Cells were grown under standard conditions (37°C, 21% O₂, 5% CO₂). At 90% confluence the cell layer was trypsinized with a solution of 0.5% trypsin in 1 mM Na₂EDTA. All experiments were performed using cells at passage 3 to 6.

**Immunofluorescence analysis**

For immunofluorescence analysis cells were plated in 6-well plates and grown in RPMI, 10% FCS. Cells were fixed with 4% formalin (10 minutes), and permeabilized with acetic acid (3:1, ice-cold, 10 minutes). Cells were then blocked with 5% BSA (1 hour), and incubated with antibodies against fibronectin (Santa Cruz, LabForce AG; Nunningen, Switzerland), α-smooth muscle actin (Epitomics, LabForce AG; Nunningen, Switzerland), and cofilin (Santa Cruz) for 1 hour. The primary antibodies were detected by addition of fluorescein-conjugated goat anti-rabbit (cofilin) (Bethyl Laboratories, Lubio Science; Lucerne, Switzerland), fluorescein-conjugated donkey anti-goat (fibronectin) (Southern Biotech, BioConcept; Allschwil, Switzerland) or cy3-conjugated goat anti-rabbit
antibodies (α-smooth muscle actin) (Invitrogen; Lucerne, Switzerland) for 1 hour. To visualise the nuclei, 4,6-diamido-2-phenylindole dihydrochloride (Sigma-Aldrich; Buchs, Switzerland) was added (5 minutes) and the well-plates were subsequently examined on a fluorescence microscope.

**Proliferation assay**

Primary human lung myofibroblasts were seeded onto a 96-well plate at a density of 1 x 10^5 cells/ml, and were grown until 60 to 70% confluent in growth medium. Cells were rinsed twice with phosphate buffered saline, and were then serum starved for 24 hours. Myofibroblasts were incubated in RPMI with or without drug (control). The effect of mPRED (methylprednisolone, Sigma-Aldrich), CsA (Sandimmune, Novartis; Basel, Switzerland), FK506 (Prograf, Fujisawa; Osaka, Japan), everolimus (Sigma-Aldrich), and MMF (in all experiments mycophenolic acid was used, which is the biologically active form of MMF) (mycophenolic acid, Sigma-Aldrich) were assessed. Myofibroblasts incubated with RPMI supplemented with 10% FCS were used as a control for proliferative response. After 19 hours 1 μCi [³H]-thymidine (PerkinElmer; Belgium) in 10 μl of RPMI was added to each well. After a further 5 hours incubation period, 100 μl of 1 M NaOH was added to each well, and cells were then harvested onto filter plates (PerkinElmer) using the Filtermate Unifilter-96 Harvester (PerkinElmer). The filter plates were then dried overnight at room temperature before addition of 20 μl of scintillation fluid (PerkinElmer) to each well. Counts per minute were obtained on a TopCount Microplate scintillation counter (PerkinElmer) for 1 minute per sample.

**Statistical analysis**

Statistical comparisons were made by using Student’s paired t-test for paired observations. p-values ≤ 0.05 were considered significant. Where applicable, data are
shown as mean ± standard error of the mean (SEM) from at least three independent experiments.
RESULTS
The characteristics of the 8 patients are shown in table 1. The mean decline of absolute FEV1 between pre-transplant (stable baseline) and lung biopsy was – 39% (range – 17.5% to – 65.5%) (table 1). Radiologic changes on high-resolution chest CT scans with focus on signs of air-trapping and bronchiectasis are summarized in table 1. Three patients had elevated numbers of neutrophils in their BAL fluid, and another 3 had a high proportion of lymphocytes. No patient had concomitant infection. Lung biopsy tissue revealed typical BO in 3 patients and lymphocytic bronchiolitis (LB) in 5 patients (table 1). Representative lung tissue sections of BO and LB are shown in figure 1. Post-biopsy BOS-treatment comprised a combination of steroids, FK506, and MMF in all patients (table 1).

As shown in figure 2, the cultured primary lung cells exhibited typical spindle-shape morphology of mesenchymal cells, and immunofluorescence studies demonstrated positive immunoreaction for both fibronectin (figure 2, panel B) and α-smooth muscle actin (α-SMA) (figure 2, panel D), identifying the cells as myofibroblasts. This was further confirmed by positive immunoreaction for the typical myofibroblast marker cofilin (figure 2, panel F). Cells kept these characteristics over several culture passages.

Proliferation of myofibroblasts after growth stimulation with 10% FCS was increased by 4 fold (406% ±115%) compared with cells grown in FCS-free medium (figure 3). We found no significant difference between baseline (FCS-free) and 10% FCS-stimulated proliferative capacity of myofibroblasts derived from patients with histologically confirmed BO compared with those obtained from patients with LB.

Low concentrations of CsA (0.01 mg/l, 0.1 mg/l) significantly induced cell proliferation compared to untreated cells (figure 4, panel A). CsA at 0.01 mg/l caused a
39.9%±11.9% induction of cell growth compared with untreated controls (n=8, p<0.05; figure 4, panel A), and 0.1 mg/l CsA had a similar stimulatory effect with 32.7%±12.6% induction of cell growth (n=8, p<0.05; figure 4, panel A). Only high dose CsA (50 mg/l) exerted a significant anti-proliferative effect of 56%±14.3% growth reduction (n=8, p<0.05; figure 4, panel A). However, this effect was accompanied by a decreased viability of cells (trypan blue exclusion), as we have reported earlier (27). Likewise, FK506 caused an induction of cell proliferation at low concentrations (figure 4, panel B): at 0.001 mg/l FK506 caused a 37.6%±15.1% induction of cell growth (n=8, p<0.05; figure 4, panel B), at 0.01 mg/l a 38.3%±15.5% induction of cell growth (n=8, p<0.05; figure 4, panel B), and at 0.1 mg/l FK506 had a 27.0%±12.2% stimulatory effect on cell proliferation (n=8, p<0.05; figure 4, panel B). A significant anti-proliferative effect of FK506 was observed only at a concentration of 5 mg/l (27%±15.4% inhibition; n=8, p<0.05; figure 4, panel B), but again combined with decreased cell viability. Methylprednisolone had no growth-stimulatory effect at any concentration, but caused a significant inhibition of proliferation at concentrations of 10 mg/l (14.8%±4.9% inhibition; n=8, p<0.05; figure 4, panel C), 50 mg/l (18.2%±7.5% inhibition; n=8, p<0.05; figure 4, panel C), and 100 mg/l (35.7%±7.5% inhibition, n=8, p<0.05; figure 4, panel C). Similarly, MMF caused a significant inhibition of proliferation at concentrations of 0.01 mg/l (17.9%±3.4% inhibition; p<0.05; figure 4, panel D) and 1 mg/l (25.6%±9.0% inhibition; p<0.05; figure 4, panel D). Everolimus had no significant effect on cell proliferation at any tested concentration (n=8; figure 4, panel E).
There was no difference in proliferative responses after drug treatment when cell lines were grouped according to histologic findings: cell lines derived from BO lungs did not differ in proliferative response from cell lines derived from LB lungs (data not shown). According to the administered BOS-treatment (table 1), myofibroblasts obtained from patients with BOS after HSCT were stimulated with the combination of FK506 (0.01 mg/l), mPRED (10 mg/l), and MMF (1 mg/l). As shown in figure 5, this triple-treatment caused a significant inhibition of cell proliferation (33%±6.9% inhibition, p<0.05; figure 5, black bar), which was more pronounced than with mPRED alone at the same concentration. To study MMF’s portion of the anti-proliferative effect, myofibroblasts were stimulated with MMF alone, and this caused a similar inhibitory effect as the triple stimulation (40.2%±5.0% inhibition, p<0.05; figure 5, second bar).

Follow-up pulmonary function tests were performed at 3, 6, and 12 months after lung biopsy. Figure 6A depicts the course of FEV1 (% predicted) of all patients from the time point of VATS, and 3, 6, and 12 months after lung biopsy (continuous lines = patients with BO; dotted lines = patients with LB). Figure 6B shows the development of mean FEV1 (% predicted) for the two groups with the dotted line representing the patients with LB and the continuous line depicting values for patients with histologically confirmed BO. Compared to pre-lung-biopsy values, patients with LB had a statistically significant mean improvement of their FEV1 (% predicted) after 3 months (+ 17.3%, n=5, p≤0.05; figure 6, panel B), 6 months (+ 30.9%, n=4, p≤0.05; figure 6, panel B), and after 12 months (+ 29.2%, n=4, p≤0.05; figure 6, panel B), whereas patients with histological BO showed no significant change of their FEV1 (% predicted).
**DISCUSSION**

In this study we investigated the *in vitro* effect of immunosuppressive agents on the proliferative capacity of primary human lung myofibroblasts obtained from patients with BOS after allogeneic HSCT. We found that the calcineurin inhibitors CsA and FK506 in clinically relevant concentrations (CsA: 0.01 mg/l, 0.1 mg/l; FK506 0.001 mg/l, 0.01 mg/l, 0.1 mg/l) enhanced proliferation of myofibroblasts, whereas MMF and mPRED caused a significant inhibition of cell proliferation. Finally, combination therapy of FK506, MMF, and mPRED – according to the administered BOS-treatment in our study group – caused a significant inhibition of myofibroblast proliferation.

Surgical lung biopsy remains the gold standard for diagnosis of BO (3). However, surgical lung biopsy is often avoided, due to potential high morbidity and mortality in this patient group. In a study by Santo Tomas et al. only 13% of patients with suspicion of BO underwent surgical lung biopsy to attempt histologic confirmation (28), and thus, only limited data on the histologic correlate of BOS is available. Among our 8 patients with BOS, only 3 (38%) showed a typical constrictive BO pattern in the lung biopsy. The other 5 patients showed the histologic pattern of LB with a prominent lymphocytic infiltration of the bronchiolar wall with variable epithelial inflammation and damage. The finding that approximately a third of patients with BOS exhibit the classic histologic pattern of constrictive BO is supported by the data of a larger series of HSCT patients with BOS who underwent surgical lung biopsy (24). Lymphocytic bronchiolitis has been categorized as a variant of lung GvHD (16, 29). However, the current standard of knowledge does not allow the decision on whether LB and BO are two independent processes, or whether LB and BO represent an early (LB) and a later (BO) stage of the
same disease process, i.e. chronic lung GvHD. The hypothesis that the fibrotic process observed in BO is preceded by an inflammatory phase (LB) is supported by the observations in a rat model of BO following lung transplantation where lymphocytic infiltration was a precursor of fibrous obliteration (30). Furthermore, on the basis of a patient case with slowly progressive BOS, it has been demonstrated recently that lymphocyte infiltration in the bronchial walls without fibrosis preceded the typical pathological findings of BO (31).

Irrespective of the histologic pattern, augmented immunosuppressive therapy – including calcineurin inhibitors - is widely used for the treatment of BOS after HSCT. However, to date a beneficial effect of the calcineurin inhibitor treatment for the course of BOS/BO after HSCT has not been demonstrated. CsA and FK506 both inhibit calcineurin, which is responsible for calcium-dependent signal transduction, thereby preventing the transcription of lymphokine genes, such as interleukin-2, and inhibiting T-cell activation and proliferation (32). However, a fibrogenic effect of CsA and FK506 has been described earlier in renal allografts and in animal and in vitro models (19-21). The fibrogenic effects are predominantly mediated by elevated expression of transforming growth factor-β, suppressed matrix metalloproteinase activity and augmented fibroblast collagen synthesis (20-21). To our knowledge, we are the first to culture primary human lung cells derived from HSCT patients with BOS, and by using these cells we were able to demonstrate that CsA and FK506 at clinically relevant immunosuppressive concentrations have a significant pro-proliferative effect on primary human lung myofibroblasts. The concentrations at which this effect was observed lied between 0.001 mg/l and 1 mg/l for CsA and between 0.001 and 0.01 mg/l for FK506, and thus
correspond to target whole-blood concentrations of 0.15 mg/l to 0.25 mg/l for CsA and 0.01 mg/l to 0.02 mg/l for FK506 (33). Our in vitro data showing a pro-proliferative effect of calcineurin inhibitors in primary human lung cells are in line with data obtained in other cell types (22-23), and correspond to the observed pro-fibrotic effect in vivo (34). Only at very high concentrations both CsA (50 mg/l) and FK506 (5 mg/l) exerted a significant anti-proliferative effect on primary human lung myofibroblasts in our in vitro model. But as this effect was accompanied by decreased cell viability (27), these concentrations were not further pursued. Even if tissue concentrations of CsA and FK506 are considerably higher than blood concentrations, it is very unlikely that these high tissue concentrations would be reached in the lung, since accumulation of these lipophilic drugs mainly occurs in lipid-rich organs (35). It is important to note that lung tissue concentrations are expected to increase by additional inhalation of CsA, and this new form of administration provided an advantage of survival over conventional therapy alone in lung transplant recipients with BO (36). Although we observed a similar pro-proliferative effect of CsA and FK506 when used in clinically relevant concentrations in our in-vitro model, clinical data demonstrated FK506 to be superior to CsA with regard to freedom from BOS after lung transplantation (37), and course of lung function in lung-transplanted patients with BOS (15). However, there is no comparable data for HSCT patients.

In contrast to the tested calcineurin inhibitors, mPRED exerted a significant growth-inhibitory effect at concentrations of 10, 50 and 100 mg/l. These concentrations might be reached by steroid pulses of 500 to 1000 mg per day (38), as might be administered initially after BO-diagnosis. The observed anti-proliferative effect of mPRED in our...
vitro model is in line with data presented previously (39-40). Our in-vitro data reflect the clinical experience that high-dose intravenous methylprednisolone may cause a favorable response in BO after bone marrow transplantation (41). In contrast, patients with isolated LB after lung transplantation did not show any improvement of their FEV1 after pulsed methylprednisolone treatment (42).

Similar to mPRED, MMF caused a significant inhibition of cell proliferation, and this is in line with previously published in vitro data (39, 43-44). The concentration of 1 mg/l MMF corresponds to the target whole-blood level for transplant patients (45). MMF has been demonstrated to be safe and effective when used as first line immunosuppression after lung transplantation (46), and conversion from calcineurin inhibitors to MMF in patients with BOS after lung transplantation led to a stabilization of graft function in some patients (47). Furthermore, in a prospective trial comparing MMF with azathioprine in lung transplant recipients it could be demonstrated that MMF significantly reduced graft loss due to BOS and tended to improve overall survival (48).

Everolimus, an inhibitor of the mammalian target of rapamycin, had no significant effect on cell proliferation in our in vitro model. An anti-proliferative effect of everolimus has been described earlier in hematopoietic and non-hematopoietic cells (49-50). However, in these studies everolimus only inhibited growth factor-triggered proliferation (49-50), whereas we looked at the effect of the drug in a FCS-/growth-factor-free cell culture medium. Even though data from lung transplantation demonstrated a significant reduction in the rate of the decline in lung function under everolimus treatment (51), no such data exist for BOS after HSCT. Finally, everolimus should be used with caution due to potential significant lung and liver toxicities (52-55).
After diagnosis of BO/LB, all 8 patients of this study group were treated with a triple-therapy comprising mPRED, FK506, and MMF. Therefore, we also tested the effect of this combination therapy in our in-vitro model, and the combination of mPRED and MMF was able to fully antagonize the pro-proliferative potential of FK506. Importantly, this effect might be attributed mainly to MMF, as the mono-stimulation with MMF exhibited a similar anti-proliferative effect as compared to the triple-stimulation. The observed in-vitro growth-inhibitory effect of MMF further substantiates the potential power of the drug to limit the progression of fibroproliferative diseases, and underlines the need for further clinical studies with this drug. We are aware that our in vitro model does not fully represent the multiple effects between different cell types and soluble factors resulting in BO in vivo. However, considering our in-vitro data, the role of calcineurin inhibitors in the treatment of BO after HSCT should at least be questioned, and MMF-based (calcineurin inhibitor-free) regimens may be further evaluated, including a combination of MMF with azithromycin, as a prophylaxis with the latter has been shown to be able to reduce BOS after lung transplantation (56). To date, only one randomized trial studied the effect of MMF in the initial treatment of chronic GvHD (57). However, since patients with BOS were excluded from this trial and numbers of newly diagnosed BOS were low, no conclusion concerning MMF’s effect on BOS could be drawn.

Finally, analysis of serial follow-up pulmonary function tests after lung biopsy and under the above mentioned triple-immunosuppressive therapy revealed that patients with histological LB experienced a statistically relevant improvement of their FEV1 (% predicted) whereas in patients with histologically confirmed BO FEV1 (% predicted) remained unchanged, and this is in line with the findings from a larger series (24). This
clinical observation supports our in-vitro data showing an anti-proliferative effect of the combination therapy with mPRED, MMF, and FK506. However, this beneficial effect seems to occur predominantly in patients with LB, but not in those with BO, and thus indicates that only in conditions with considerable inflammatory cell infiltrate the progression of disease can be delayed, whereas with only scant inflammation but pronounced fibrosis the potential benefit might be limited. Importantly, neutrophilia of > 15% in the BAL fluid has been demonstrated to be predictive of FEV1 response to azithromycin in patients with BOS after lung transplantation (58), whereas patients without neutrophilia in the BAL benefited from the addition of the leukotriene receptor antagonist montelukast (59). Transferring these findings to our study group, one would add azithromycin to the immunosuppressive regimen in those patients with histological LB and relevant BAL neutrophilia, and additional montelukast for those patients with low neutrophilic LB. Recent data obtained from HSCT-patients with BOS demonstrated that the combination of azithromycin and montelukast permitted a reduction of corticosteroid exposure (60), but lung function values did not improve under azithromycin treatment in a randomized placebo-controlled study (61). In contrast, combining standard immunosuppressive therapy with montelukast caused an improvement in PFT values in patients with chronic GvHD (62).

We acknowledge that the present study has some limitations. First, we used drug concentrations which are in the range of target whole-blood levels, but may not reflect lung tissue concentrations. However, no representative data exist on tissue concentrations of the studied drugs. Furthermore, our in vitro model rather resembles a constant infusion of the drug, in contrast to the peak and trough levels of the in vivo
situation. Second, the mechanism by which the calcineurin inhibitors exert their pro-proliferative effect was not addressed, but this would have exceeded the scope of this manuscript. Third, the number of patients studied is small. Since BO is a rare disease and only few patients undergo lung biopsy, it will be a big challenge to get larger patient numbers. However, given the uniformity of the results, we are confident that our data are representative for this type of experiments. Finally, we are aware that the transition of findings obtained by in-vitro models to clinical practice has to be done with caution, and controlled clinical trials are needed to test basic research data.

Conclusion

Our data showed that CsA and FK506 have a pro-proliferative effect on primary human lung myofibroblasts obtained from patients with BOS after allogeneic HSCT, and their role in the treatment of BO after HSCT might be questioned. However, combination of FK506, mPRED, and MMF significantly inhibited cell proliferation in vitro, which was mainly due to the growth-inhibitory effect of MMF. Our findings suggest that in patients with BO after HSCT MMF-containing, calcineurin inhibitor-free regimens should be further evaluated.
Table 1: Baseline demographics, pulmonary function tests, radiologic and histologic patterns, cytological analysis of broncho-alveolar lavage fluid, and treatment of patients with BOS after HSCT. Numbering of patients according to the date of surgical lung biopsy. BO: bronchiolitis obliterans; CT: computed tomography; FEV1: forced expiratory volume in 1 second; FK506: tacrolimus; LB: lymphocytic bronchiolitis; mPRED: methylprednisolone; MMF: mycophenolate mofetil.

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BO: bronchiolitis obliterans; CT: computed tomography; FEV1: forced expiratory volume in 1 second; FK506: tacrolimus; LB: lymphocytic bronchiolitis; mPRED: methylprednisolone; MMF: mycophenolate mofetil.
FIGURE LEGEND

Figure 1. A. Representative histologic section of a lung with bronchiolitis obliterans showing excentric, subepithelial connective tissue depositions (arrow) in the absence of inflammation. Elastica van Gieson staining, scale bar = 100 µm.

B. Representative histologic section of a lung with lymphocytic bronchiolitis demonstrating an intra-epithelial mononuclear inflammatory infiltrate, and lack of luminal obliteration. Hematoxylin and Eosin staining, scale bar = 100 µm.

C. Representative lung section of lymphocytic bronchiolitis that highlights the presence of intraepithelial CD8 lymphocytes. CD8 immunohistologic staining, scale bar = 100 µm.
Figure 2. Primary human lung cells immunostained for fibronectin (B), α-smooth muscle actin (D), and cofillin (F). Corresponding phase contrast pictures are shown in panels A, C, and E. Cells were grown to confluence in normal growth medium, were then fixed, and permeabilized. Primary antibodies were detected by addition of fluorescein-labelled (green) or Cy3-labelled (red) secondary antibodies. Visualisation by fluorescence microscopy, scale bar = 200 µm.
Figure 3. Effect of 10% fetal calf serum (FCS) growth medium on myofibroblast proliferation compared with serum-free medium. Primary human lung myofibroblasts (n=8) were incubated for 24 hours with serum-free RPMI medium or with RPMI containing 10% FCS. Cell growth was assessed by [³H]-thymidine incorporation. (A) Absolute values for each tested cell line. (B) Mean values ± SEM of eight independent experiments, expressed as relative proliferation compared with control (serum-free RPMI). Each experiment was performed in triplicates.
Primary human lung myofibroblasts (n=8) were incubated for 24 hours with increasing concentrations of cyclosporine A (A), tacrolimus (B), methylprednisolone (C), mycophenolate mofetil (D), or everolimus (E). Serum-free RPMI served as control. Cell
growth was assessed by \[^3\text{H}\]-thymidine incorporation, and values are presented as mean ± SEM of independent experiments performed in 8 different cell lines, expressed as relative proliferation compared with the control (serum-free RPMI). * p<0.05.
Figure 5. Effect of combination treatment with tacrolimus (FK506), mycophenolate mofetil (MMF), and methylprednisolone (mPRED) (third bar), and of MMF alone (second bar) on myofibroblast proliferation compared with serum-free RPMI (first bar). Primary human lung myofibroblasts were incubated for 24 hours with FK506 (0.1 mg/l), MMF (1 mg/l) and mPRED (10 mg/l) or with MMF (1 mg/l) alone. Serum-free RPMI served as control. Cell growth was assessed by [3H]-thymidine incorporation and values are presented as mean ± SEM of independent experiments, expressed as relative proliferation compared with the control (serum-free RPMI).

Figure 5

<table>
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<th>Treatment</th>
<th>mPRED 10 mg/l</th>
<th>FK506 0.01 mg/l</th>
<th>MMF 1 mg/l</th>
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<td>p = 0.007</td>
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Diagram shows relative [3H]-thymidine incorporation (%).
Figure 6. Forced expiratory volume in 1 second (FEV1) (% predicted) of all eight patients (panel A), and mean FEV1 (% predicted) values of the patient group with histologic bronchiolitis obliterans (n=3, continuous line; panel B) and of the patient group with lymphocytic bronchiolitis (n=5, dotted line; panel B). The time points shown are at the time of video-assisted thoracoscopic surgery, 3 months, 6 months, and 12 months after surgery. * p≤0.05.
Figure 6

A

![Graph A](image1)

B

![Graph B](image2)

- **Bronchiolitis obliterans (n=3)**
- **Lymphocytic bronchiolitis (n=5)**
REFERENCES


