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Synergism of imatinib mesylate and everolimus in attenuation of bronchiolitis obliterans after rat LTX

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Summary. Bronchiolitis obliterans (BO) is a progressive and fatal disease after lung transplantation (LTX). Dysregulated growth factor-induced proliferation of myofibroblasts seems to be responsible for the development of BO. The aim was to confirm the efficacy of both inhibitors of receptor tyrosine kinases (RTKI) and of mammalian target of rapamycin (mTORI) after rat LTX. We used a rat model of left lung allo-transplantation (F344-to-WKY) to evaluate the effect of imatinib (RTKI; 20 mg/kg/day; postoperative day (POD) 0-100) alone or in combination with everolimus (mTORI; 2.5 mg/kg/day; POD 14-100). Non-treated animals were the reference.

In non-treated rats, acute rejection (AR) peaked between POD 20 and 30 (19/19) and ended in chronic rejection (CR) on POD 60/100 (12/12). Imatinib alone did not prevent AR (6/6), but attenuated the degree of degenerated bronchioles on POD 30 (non-treated, 57%; imatinib, 4%), and increased the allografts free of CR on POD 60/100 (3/12). A combination of imatinib and everolimus significantly reduced AR, attenuated fibrotic degenerated bronchioles (5%) and vessels (non-treated, 24%; combination therapy, 11%) on POD 30, and reduced fibrotic degenerated vessels (non-treated, 97%; combination therapy, 43%) and bronchioles (non-treated, 88%; combination therapy, 34%) on POD 60/100. Fifty percent of the animals were completely free of BO and vasculopathy. In conclusion, co-application of RTKI and mTORI attenuated the development of BO and vasculopathy. Thus, imatinib might be an interesting therapeutic approach after LTX.

Key words: Lung transplantation, Rat, mTOR-inhibitor, RTK-inhibitor, Chronic rejection

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Introduction

Although lung transplantation (LTX) has evolved into established therapy for the majority of end-stage lung disease, long-term success rates are often limited by chronic allograft rejection (Verleden, 2001; Neuringer et al., 2005; Belperio et al., 2009). Its pathological manifestation mainly includes bronchiolitis obliterans (BO) (Verleden et al., 2005) as well as chronic vascular rejection, chronic inflammation of small and large airways, chronic interstitial fibrosis and chronic pleuritis (Snell and Westall, 2010). The pathogenesis of BO is multifactorial and can be divided into different phases (Neuringer et al., 2005): An early inflammatory phase initiates a strong immune activation followed by epithelial cell injury, necrosis, and ulcerations of the mucosa. While these cells failed to regenerate and reepithelialise the damaged organ, mesenchymal cells, in particular myofibroblasts persisted in the same tissue microenvironment and were involved in fibro-proliferative responses. These processes were controlled by growth factors that were secreted by airway macrophages and myofibroblasts (Jaramillo et al., 2003). In particular, platelet-derived growth factors (PDGF) have been implicated in the development of the fibroproliferative phase (Neuringer et al., 2005). Experimental and clinical data suggest that during the fibroproliferative phase of BO development, the growth of the fibroproliferative lesion is no longer responsive to augmented immunosuppression. New therapeutic options, including anti-proliferative and anti-fibrotic agents, are warranted to prevent BO (Belperio et al., 2009). In order to achieve therapeutic success, we hypothesized that there is a need of both an anti-proliferative drug - everolimus in combination with an anti-fibrotic drug - imatinib.

The majority of immunosuppressive regimens after LTX in clinical use exert their effect by blocking the

pathways involved in the clonal expansion of alloreactive T cells or by reducing lymphocyte subpopulations. Current drugs used as immunosuppressants (such as Cyclosporine A) are toxic, and relatively unspecific. It was suggested that the progressive fibrotic lesions after LTX are analogous to chronic vasculopathy in kidney and heart transplants (McDyer, 2007). Mammalian target of rapamycin (mTOR) inhibitors such as everolimus were successfully used to improve long-term outcomes after heart transplantation (Schaffer et al., 2010). This new class of drugs acts by interfering with T cell proliferation by blocking a kinase, causing cell cycle arrest (Kuo et al., 1992; Price et al., 1992). Therefore, mTOR inhibitors were also used to treat lung transplant patients. However, long-term results have been unsatisfactory so far.

Another therapeutic strategy - the use of receptor protein tyrosine kinase (RTK) inhibitors - was already described in 1999 by Kallio et al. (1999). They used a heterotopic rat tracheal allograft model to demonstrate an increased mRNA expression of PDGF and its receptors (PDGFR) that correlated with a progressive loss of respiratory epithelium and airway occlusion in non-treated allografts compared with syngrafts. The inhibition of PDGF receptors prevented/attenuated the development of obliterative airway disease (OAD) (Kallio et al., 1999; Tikkanen et al., 2006). However, the subcutaneous placement of tracheal rings has major drawbacks, which limit their comparability to clinical reality. Problems with this model are initial ischemia, diffusion restriction, missing physiological ventilation, no difference between large and small airways, no adequate vascularisation to allow optimal drug supply, and finally, a short span of time to develop OAD.

Therefore, we used an established orthotopic lung transplant model in the F344-to-WKY rat strain combination (von Suesskind et al., 2012; Hirt et al., 1999) to evaluate the efficacy of an RTK inhibitor (imatinib) alone and in combination with an mTOR inhibitor (everolimus) to prevent the development of BO. Our data indicate that dual therapy with RTK and mTOR inhibitors reduces the extent of chronic rejection after rat LTX.

Materials and methods

Lung transplantation

Specific pathogen-free inbred male Fisher F344 (RT1lvl) and Wystar Kyoto WKY (RT1l) rats (Harlan-Winkelmann, Borchen, Germany; 200±30 g) were used. Syngeneic left lungs were transplanted orthotopically from F344 donors to F344 recipients and allografts from F344 donors to WKY recipients as described earlier (Matsumura et al., 1995; Hirt et al., 1999). For histological analysis rats were killed on postoperative day (POD) 20, 30 (early, inflammatory phase), 60 and 100 (late, fibroproliferative phase). All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the European Union Guide for the Care and Use of Laboratory Animals (publication No. 86/609/EWG). Approval was granted by the institutional ethical committee at the University of Regensburg.

Drug regimens and study groups

Imatinib (Glivec®, Novartis, Basel, Switzerland) inhibits PDGFR protein tyrosine kinase activity (Buchdunger et al., 2002), whereas everolimus (Certican®, Novartis) blocks growth factor-driven proliferation of hematopoietic and non-hematopoietic cells (Nashan, 2002). Imatinib (20 mg/kg body weight (bw), per gavage) and everolimus (2.5 mg/kg bw, per gavage) were dissolved in polyethylene glycol (molecular weight 300) and administered daily. The dosages used were chosen on the basis of pharmacologic data given to us by the manufacturer (Shaker et al., 2011), and our own experience (von Suesskind et al., 2013). An increase in the dosage of imatinib (50-100 mg/kg bw) and its intraperitoneally application failed due to strong side effects (e.g. cardiotoxicity) (data not shown).

No additional immunosuppression was applied. The following study groups were performed: control-group (n=37), without treatment, no vehicle; imatinib-group (n=30), imatinib alone (POD 0-100), and combination-group (n=25), combination of imatinib (POD 0-100) and everolimus (POD 14-100).

Histology

To analyze histological symptoms of chronic rejection (CR) in transplanted lungs, these lungs and the nontransplanted right lungs were removed at the time of death (see above), fixed in formalin and embedded in paraffin. The embedded lungs were cut into 5 μm sections and stained with hematoxylin-eosin (HE) and Masson Goldner Trichrome (MGT) for visualization of connective tissue. Acute allograft rejection was graded according to the actual working formulation of The International Society for Heart and Lung Transplantation (ISHLT) (Stewart et al., 2007; von Suesskind et al., 2012) (Table 1). Briefly, acute vascular rejection was graded into A0-A4 depending on the extent of perivascular and interstitial mononuclear cell infiltrates. The degree of acute airway inflammation was scored from B0-B2R according to the extent and intensity of lymphocytic bronchiolitis. Chronic airway and chronic vascular rejection was quantified by the assessment of chronic altered bronchioles and small/medium-sized vessels (Table 2). The degradation of the bronchioles was classified as none (no chronic alterations), mild (first signs of granulation tissue into the small bronchioles), and severe (pronounced fibrotic degeneration). In this context, severe chronic airway rejection (BO) was defined as an excessive proliferation

of granulation tissue either within the airway wall (constructive bronchiolitis) or within its lumen (granulation tissue obliterates the lumen), or both (Mueller et al.,1987; King, 1998; Jonigk et al.,2011). The same scoring system was used for small and medium-sized vessels (no chronic alterations, mild degenerations (obstruction of vessels) and severe degenerations (vasculopathy). The percentage of affected bronchioles or vessels relative to the total amount of structures per tissue section was used to quantify the degree of chronic airway and chronic vascular rejection, respectively.

Collagen measurement

Lung collagen (hydroxyproline) content was detected by a multiplex-enzyme-linked immunosorbent assay (ELISA) system (BlueGene; Rat Hydroxyproline Elisa kit). Briefly, frozen lung tissue was homogenized using Tissue Lyser LT (Quiagen, Valencia, CA), and hydrolysed with 6 M hydrochloric acid. Hydroxyproline content was determined according to the manufacturer's instructions. The total amount of collagen in each sample was calculated, assuming that lung collagen contains 12.2% w/w hydroxyproline (Laurent et al., 1981), and expressed as μg collagen per mg lung tissue (Mutsaers et al., 1998).

Data requisition and analysis

Histological scoring was performed by three independent investigators in a blinded fashion.

Data were expressed as means ± standard deviation (SD). For statistical analysis of chronic vascular and chronic bronchiolar rejection, data from allografts on POD 60 and 100 were summarised. A Wilcoxon-Mann-Whitney-U-test was used as a non-parametric statistical hypothesis test for assessing whether one study group tends to have improved during therapy compared to the control-group or the imatinib-group. To compare all study groups on POD 20, 30 and 60/100 we used the Kruskal-Wallis test. Statistical package SPSS 18.0 (SPSS, Chicago,IL,USA) was used for statistical analysis. A P value ≤0.05 was considered statistically significant.

Results

Imatinib attenuated the development of BO after rat LTX

In the early inflammatory phase (POD 20), all allografts of imatinib-group 2 exhibited a severe acute vascular and airway rejection (ISHLT-A4/B2R). Diffuse perivascular, interstitial and air-space infiltrates of

Table 1. Brief summary of the Histological classification of acute rejection according to Stewart et al. 2007.

Acute rejection Vascular degeneration (A)	Grade	Characteristics
None	0	no rejection
Minimal	1	scatterned MNC
Mild	2	MNCs, perivascular cuffing
Moderate	3	MNCs, perivascular cuffing and extension into alveolar septa and air spaces
Severe	4	MNCs, perivascular and diffus infiltrations, endotheliasis
Acute rejection		
Lymphocytic degeneration (B)		
None	0	no rejection
Low	1R	MNCs in bronchiolar submucosa, infrequent
High grade	2R	MNCs , dense infiltraton of bronchiolar submucosa, percolation of basement mambrane

MNC: mononuclear cells.

Table 2. Brief summary of the Histological evaluation of chronic affected vessels and bronchioles according to Stewart et al. 2007, modified by von Suessind et al. (2012a).

Chronic degeneration of terminal bronchioles	Characteristics
free of rejection	no rejection
mild	first signs of granulation tissue and peribronchiolar fibrosis
severe	(BO), distinct peribronchiolar (concentric/eccentric) fibrosis
Chronic degeneration of small and medium sized vessels	
free of rejection	no rejection
mild	first signs of fibrointimal thickening and obstruction of small vessels
severe	distinct fibrointimal thickening and obstruction of small and medium sized vessels; (Vasculopathy).

mononuclear cells with prominent alveolar pneumocyte damage and endothelialitis dominated the tissue sections. Furthermore, there was evidence of bronchiolar epithelial damage in the form of necrosis and metaplasia and marked intra-epithelial lymphocytic infiltration. Airway inflammation was associated with fibro-purulent exudate, cellular debris and granulocytes. The degree of AR was comparable between the control-group and imatinib-group. In addition, allografts on POD 20 exhibited pronounced alveolar pneumocyte damage (APD) in varying degrees of organization.

On POD 30, all lung allografts showed moderate to high grade AR (control-group, ISHLT-A4/B2R; imatinib-group, ISHLT-A3-4/B2R) (Figs. 1, 2a,b). More than 75% of bronchioles of each section exhibited extensive infiltration of mononuclear cells in the submucosa. Moreover, there was evidence of epithelial damage in form of necrosis, metaplasia and marked intra-epithelial lymphocytic infiltration. Cell debris and fibro-purulent exudates were accumulated in the lumina of the bronchioles (ISHLT-B2R) (Fig. 2b).

Besides excessive inflammatory infiltrations, each allograft from the control and imatinib-group presented the first signs of chronic airway and vascular alterations. Figure 2c shows an example of a preliminary stage of BO (mild chronic airway alteration) with protrusion of granulation tissue into the lumen of a bronchiolus, attenuation of the epithelium and fibrotic thickening of subepithelial structures. In addition, a multitude of small vessels was characterised by incipient perivascular scarring accompanied by persisting vasculitis (Figure 2d). Subdivision of vessels and bronchioles into different grades of degeneration (Table 2) is shown in Figure 3. More than 50% of the vessels from allografts of the control and imatinib-group showed fibrotic alterations. However, there was no significant difference in the percentage and extent of chronic altered vessels between the control and imatinib-group (Fig. 3a). In contrast, 87±42% and 66±38% of bronchioles in allografts from the control-group and the imatinib-group, respectively, were degenerated (not significant). However, the percentage of severe degenerated bronchioles was significantly higher in the control-group (Fig. 3b, p=0.03). Independent of treatment strategy, the collagen content increased significantly after allogeneic LTX on POD 30 ($p \leq 0.001$) (compared to native right lungs). However, there was no difference between the allografts of the control and imatinib-group (Fig. 4).

In the long term follow-up (POD 60/100), progressed fibrotic degenerations of lung tissue inhibited a detailed scoring of the degeneration stage of small vessels and bronchioles. All allografts from the control-group exhibited vasculopathy (Figs. 5a, 6a) and BO (Figs. 5b, 6a). Only a small percentage of small vessels and bronchioles in the sections of these allografts were free of chronic alterations (Fig. 5a,b). Inflammatory infiltrations receded from fibrotic lung tissue. The collagen content of allografts from the control-group increased significantly compared to the native lungs ($p<0.001$) and compared to allografts from POD 30 ($p=0.01$) (Fig. 4). Long-term application of imatinib alone prevented the development of BO and vasculopathy in 3/12 animals (Fig. 7b). Two of these allografts showed a patchy distribution of inflammatory infiltrations (ISHLT-A3-4/B1R-B2R) and one allograft was more or less free of inflammatory infiltrates. The remaining allografts presented severe chronic degradations associated with reduced inflammatory infiltrations. The number of affected vessels in all tissue sections of the imatinib-group was significantly decreased compared to the control-group (Fig. 5a). In addition, especially on POD 60, a scattered distribution of metaplastic-regenerative type II pneumocytes were present in about 30% of the allograft sections of control-roup and imatinib-group (data not shown). The appearance of alveolar macrophages especially in the allografts was conspicuous in imatinib-group (Fig. 7d). The collagen content increased with prolongation of imatinib treatment ($p=0.037$). However, the concentration of collagen on POD 60/100 was about two thirds ($p=0.06$) of the concentration in the control-group (Fig. 4).

Imatinib and everolimus decreased the number of rats with BO after LTX

On POD 20, the application of imatinib and everolimus (combination-group) significantly reduced

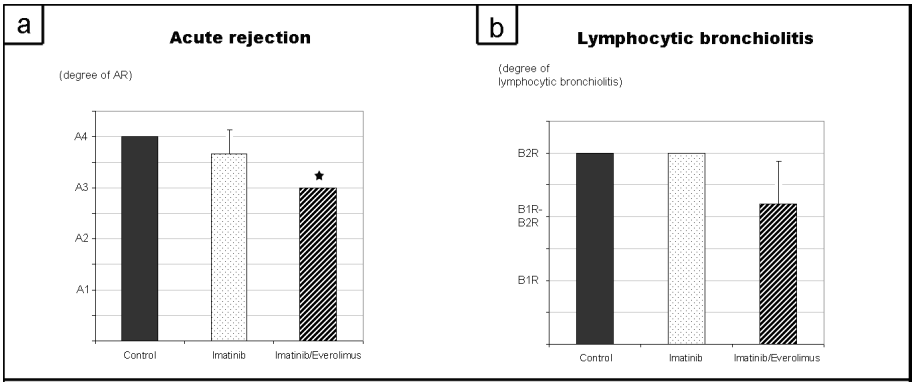

Fig. 1. Effect of imatinib alone and a combination therapy (imatinib/everolimus) on the development of acute vascular rejection **(a)** and lymphocytic bronchiolitis **(b)** after rat LTX (POD 30). Histological evaluation of allograft sections from the control-group (n=9), imatinib-group (n= 6), and combination-group (n= 5) according to the actual working formulation of The International Society for Heart and Lung Transplantation (ISHLT) (Stewart et al., 2007). There is a significant decrease of acute rejection (asterisk) after therapy with imatinib and everolimus compared to the control-group (p=0.03).

AR (ISHLT-A3, p=0.03) and lymphocytic bronchiolitis (ISHLT-B1R-B2R, p=0.03) compared to the control-group. Forty percent of the allografts exhibited severe acute vascular and high grade small airway inflammation. The remaining lungs developed mild to moderate acute vascular rejection and low grade airway inflammation.

On the next 10 days, the process of vascular infiltration and lymphocytic bronchiolitis persisted (Fig. 1a,b). The majority of vessels were cuffed by a dense mononuclear cell infiltrate spreading into the interstitium. All allografts presented APD in a different

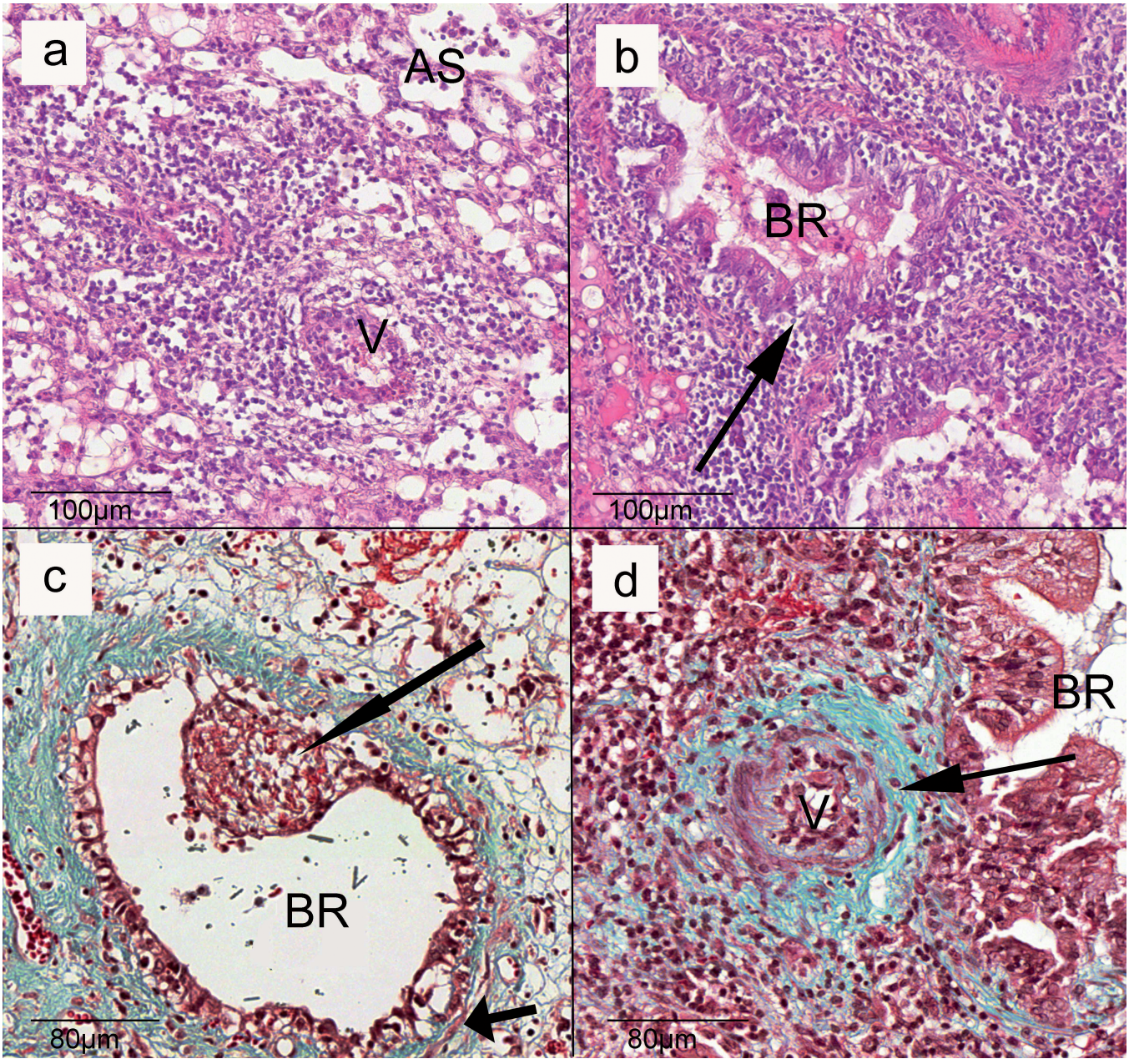

Fig. 2. Histological evaluation of lung allografts on POD 30. Representative lung allografts from imatinib-treated rats (HE) **(a, b)**, and from non-treated rats (control-group; MGT) **(c, d). a.** Moderate to severe acute vascular rejection (ISHLT-A3-4). A circumferential band of mononuclear cells surrounds the small vessels (V). Inflammatory cells spread into the perivascular interstitium and the alveolar septa (AS). Lumina of vessels are full of mononuclear cells and granulocytes (short arrow). **b.** High grade small airway inflammation (ISHLT-B2R). Intense peribroncholar (BR, bronchiole) and airspace infiltration with mononuclear cells; evidence of epithelial damage (arrow). **c.** First signs of fibrotic degeneration of a terminal bronchiolus. Granulation tissue protrudes into the lumen of the bronchiolus (long arrow). Residuals of smooth muscle cells are visible (short arrow). **d.** Persisting severe inflammation is associated with first signs of perivascular scarring (arrow). The adjacent bronchiolus (BR) exhibits no fibrotic degenerations.

expression (ISHLT-A3). Mononuclear cells formed a circumferential band around the bronchioles. They infiltrated the submucosa. In 2 of 6 animals (30%) there was evidence of epithelial damage and intra-epithelial lymphocytic infiltration (ISHLT-B2R). The remaining allografts showed only mild bronchiolar lymphocytic infiltration (ISHLT-B1R).

As shown in Fig. 3, allografts from combination-group developed significantly less severe chronic vascular and bronchiolar alterations ($p \leq 0.05$ compared to the control-group). Furthermore, the additional application of everolimus significantly decreased the percentage of vessels with severe fibrointimal thickening compared to the control-group and the imatinib-group (both $p \leq 0.05$, Fig. 3a). The content of collagen of the allografts remained unchanged compared to the control-group and the imatinb-group (Fig. 4).

While within the next 4 weeks the inflammatory process stagnated and the CR progressed in the transplanted lungs from control-group 1 and the imatinib-group , the inflammatory response persisted in all allografts of the combination-group. On POD 60, two allografts were classified with ISHLT-A4/B2R and three allografts with ISHLT-A2/B1R. On POD 100, one allograft showed moderate acute vascular rejection and lymphocytic bronchiolitis (ISHLT-A3/B2R), two allografts presented mild signs of acute vascular rejection and lymphocytic bronchiolitis (ISHLT-A2/B1R), and one allograft was completely free of acute vascular rejection and lymphocytic bronchiolitis. Co-

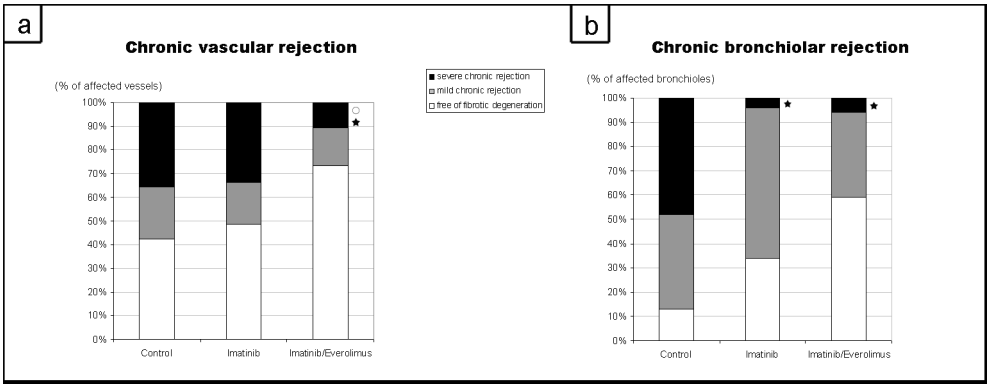

Fig. 3. Effect of imatinib alone and a combination therapy (imatinib/everolimus) on the development of chronic vascular degenerations **(a)** and chronic bronchiolar degenerations **(b)** after rat LTX (POD 30). Percentage of small and medium-sized vessels (**a)** and terminal bronchioles **(b)** with none (white bar), mild (grey bar) and severe (black bar) chronic degenerations of each group (control-group, n=9; imatinib-group, n=6; combination-group, n= 5). There was a significant reduction of severe chronic vascular degenerations in the combination-group compared to the control-group (asterisk; $p \leq 0.05$) and compared to the imatinib-group (circle; $p \leq 0.05$). Severe chronic bronchiolar degenerations were significantly reduced (asterisk; $p \leq 0.05$) in the imatinib and in the imatinib/everolimus group compared to the control-group.

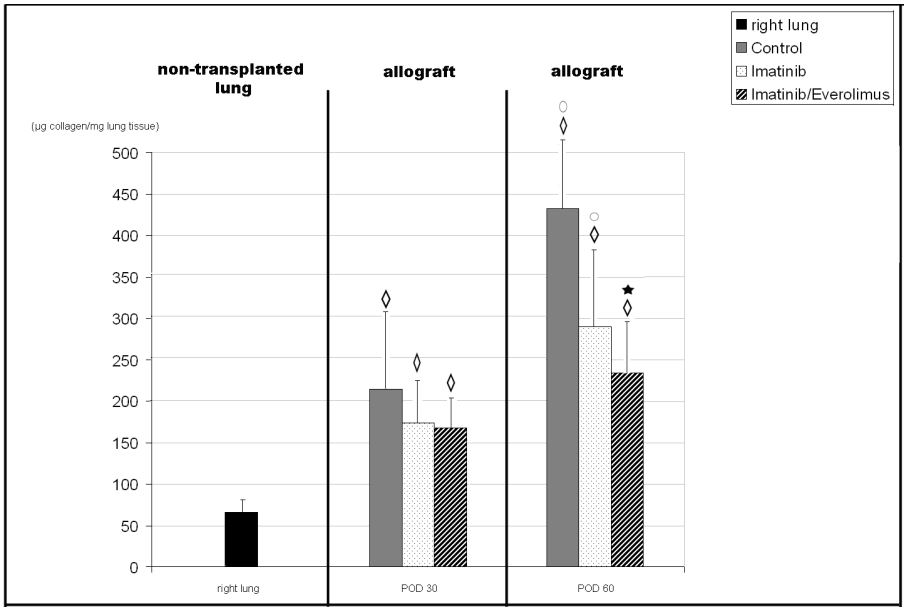

Fig. 4. Collagen content of native right lungs and lung allografts. Each bar represents the mean ± SD of collagen (μg) in one mg lung tissue of each group (control-group: POD 30, n=6; POD 60/100, n=6; imatinib-group: POD 30, n=6; POD 60/100, n=12; combination-group: POD 30, n=5; POD 60/100, n=10; right lungs: POD 30, 60 and 100; n=45). The collagen content in lung allografts on POD 30 and POD 60/100 of each group was significantly higher than in the native right lungs (lozenge shape, ($p \leq 0.001$). There was a significant increase in the collagen content from POD 30 to POD 60/100 regarding allografts from the control-group and the imatinib-group (circle, $p=0.03$). Lung allografts from the combination-group showed significantly lower content of collagen compared to the control group on POD 60/100 (asterisk, $p=0.04$).

application of imatinib and everolimus significantly reduced the percentage of severe chronic altered small vessels (p=0.01) and terminal bronchioles (p=0.03) (compared to the control-group) (Fig. 5a,b). Furthermore, the additional application of everolimus significantly reduced the number of chronic altered terminal bronchioles (compared to the imatinib-group) (p=0.04). According to the histological findings of the imatinib-group, accumulations of macrophages in the alveolar space were also observed, especially in the transplanted lungs of the combination-group.

Furthermore, there was a significant reduction of the lung collagen content in the combination-group compared to the control-group (p=0.04) (Fig. 4). Finally, the combination of everolimus and imatinib decreased the number of rats with BO and vasculopathy after LTX (5/10).

Discussion

The present study describes the effects of RTKI and mTORI on the development of CR after LTX in a rat model. We demonstrated that early application of imatinib in combination with everolimus significantly decreased the number of animals with BO and vasculopathy.

Kallio et al. (1999) reported previously that specific inhibition of PDGF receptors significantly reduced the proliferation of myofibroblasts as well as the development of OAD in rats. However, they used a heterotopic tracheal allograft model which has apparent drawbacks, including initial ischemia, limited diffusion, missing of small airway and ventilation. These restrictions limited its clinical relevance. Therefore, we applied imatinib in an orthotopic rat LTX model resembling the clinical situation. The combination of moderately histoincompatible rat strains (F344-to-WKY) without an additional immunosuppression allows the histological evaluation of (1) AR episodes in early time after transplantation, (2) the emergence of early chronic alterations, and (3) pathological vascular and airway alterations in the long-term follow-up (Hirt et al., 1999; Zweers et al., 2004; von Suesskind et al., 2012).

As expected, monotherapy with imatinib did not influence the extent of inflammatory response early after LTX. Both non-treated and imatinib-treated allografts developed severe acute rejection and lymphocytic bronchiolitis (ISHLT-A4/B2R) with diffuse perivascular, interstitial and airspace infiltrates of mononuclear cells accompanied by prominent APD and endothelialitis (Stewart et al. 2007). The study of Tikkanen et al. (2006) confirmed our data. Imatinib monotherapy did not reduce inflammation of tracheal allografts. The alloimmune activation could only be reduced by the application of an inhibitor of the vascular endothelial growth factor- receptor (PTK/ZK) alone or in combination with imatinib (Tikkanen et al., 2006). The additional application of a proliferation inhibitor - everolimus - at the time of maximum acute inflammation (POD 14 - 100) reduced the amount of animals with severe acute vascular rejection and high grade airway inflammation. The study design of delayed treatment of transplanted rats with everolimus resulted in several clinical and experimental observations. (1) Treatment of everolimus at the time of LTX increased the risk of disorders for wound healing (Røine et al., 2010) and reduced drug tolerance (Hausen et al., 1999; Schaffer and Ross, 2010). (2) Acute rat lung allograft rejection seems to be refractory to monotherapy with a high dose of everolimus (Hausen et al., 1999). Only the combination of early imatinib and delayed everolimus treatment initiated the anti-inflammatory effect in the allografts on POD 20. Monotherapy with everolimus was only successful in reducing AR in our rats when applied early after transplantation (von Suesskind et al., 2013). Similar data were shown by Reis et al. (2002). They induced an anti-inflammatory effect after rat corneal transplantation by using monotherapy with everolimus. Everolimus significantly attenuated the recruitment and activation of inflammatory cells in this

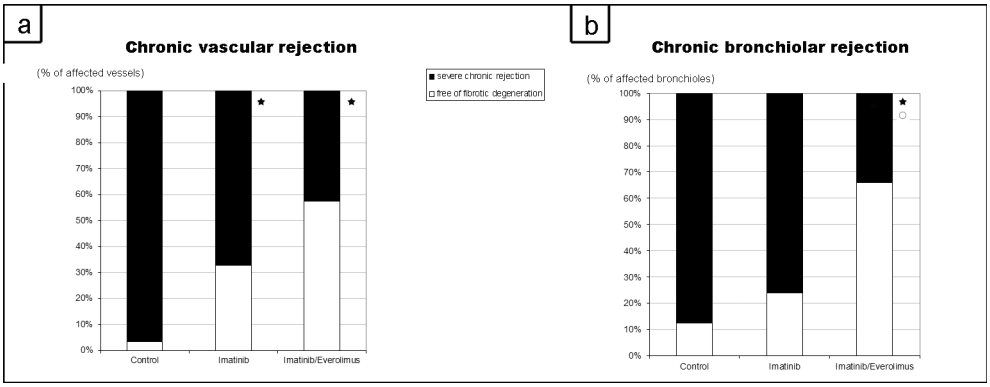

Fig. 5 Effect of imatinib alone and a combination therapy (imatinib/everolimus) on the development of chronic vascular degenerations **(a)** and chronic bronchiolar degenerations **(b)** in the long-term follow up (POD 60/100). Percentage of small and medium-sized vessels **(a)** and terminal bronchioles **(b)** with none (white bar) and severe (black bar) chronic degenerations of each group (control-group, n=12; imatinib-group, n=12; combination-group, n=10). There was a significant reduction in the percentage of affected vessels in the imatinib- and in the combination-group (asterisk, p≤0.05). Only the combination-group exhibited a significant attenuation of severe chronic bronchiolar degeneration compared to the control- group (asterisk, p=0.03) and compared to the imatinib-group (circle, p=0.04).

animal model. As a consequence, fewer immunocompetent cells in the graft prolonged graft survival (Reis et al., 2002). The inhibitory effect of AR was also shown after heart transplantation as reviewed by Schaffer and Ross (Schaffer and Ross, 2010).

On POD 30, our present non-treated model represents a "borderline form" that includes the coexistence of AR and chronic alterations (von Suesskind et al., 2013). While a delayed monotherapy with everolimus (POD 14-100) could not prevent the

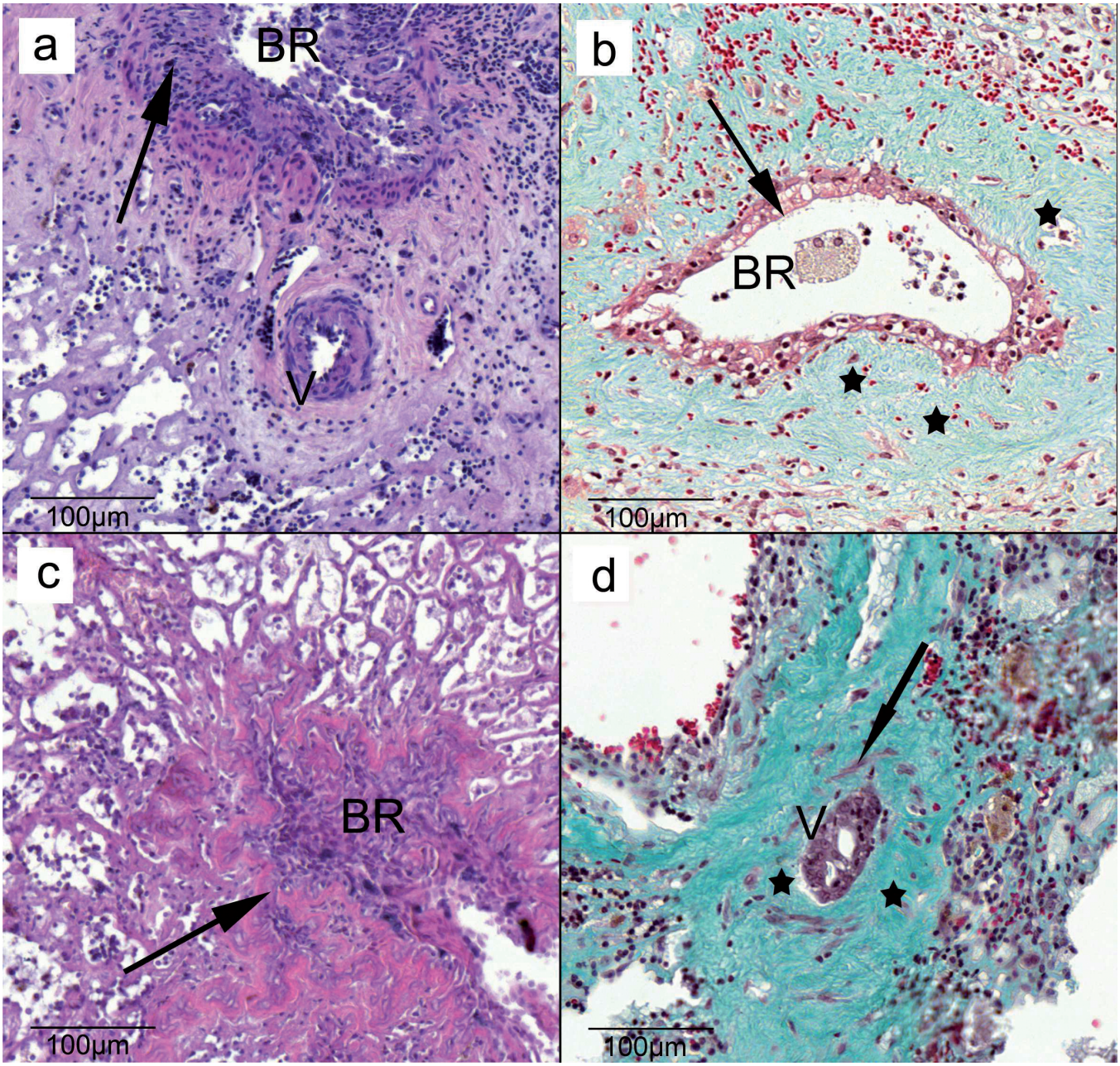

Fig. 6. Histological evaluation of lung allografts on POD 60/100. Lung allograft of the control-group (POD 60, HE) **(a)**; the imatinib-group (POD 100, MTG) **(b)**; the combination-group (POD 100, HE) **(c)**; and the imatinib-group (POD 100, MTG) **(d)**. **a.** Subepithelial thickening of the bronchiolus (arrow) dominates this section. The small vessel (V) shows a distinct perivascular fibrosis. **b.** Submucosal scarring (stars) of a terminal bronchiolus. The epithelium is attenuated and shows residuals of ciliated epithelial cells (arrow) (severe chronic bronchiolar degeneration). **c.** Eosinophilic fibrosis of the submucosa (arrow) surrounds the small airway. Parts of the airway lumen are already occluded. Occasional peribronchiolar mononuclear cell infiltrate exists. **d.** Fibrointimal thickening and perivascular fibrosis (stars) dominates this section. Residual structures of smooth muscle cells (arrow) are visible.

progression of AR and the development of the first signs of chronic alterations (von Suesskind et al. 2013), early application of imatinib alone attenuated the formation of severe bronchiolar fibrotic degenerations. We supposed that the suppression of the first signs of chronic airway alterations after treatment with imatinib delayed the long-term development of BO and vasculopathy after rat LTX. Even though imatinib protected airway structures on POD 30 (but without inhibition of intimal thickening of small vessels), in the long-term follow-up the majority of terminal bronchioles showed severe chronic degenerations (whereas the number of small vessels free of vasculopathy decreased). We speculated that early occlusion of bronchiolus-associated small vessels might

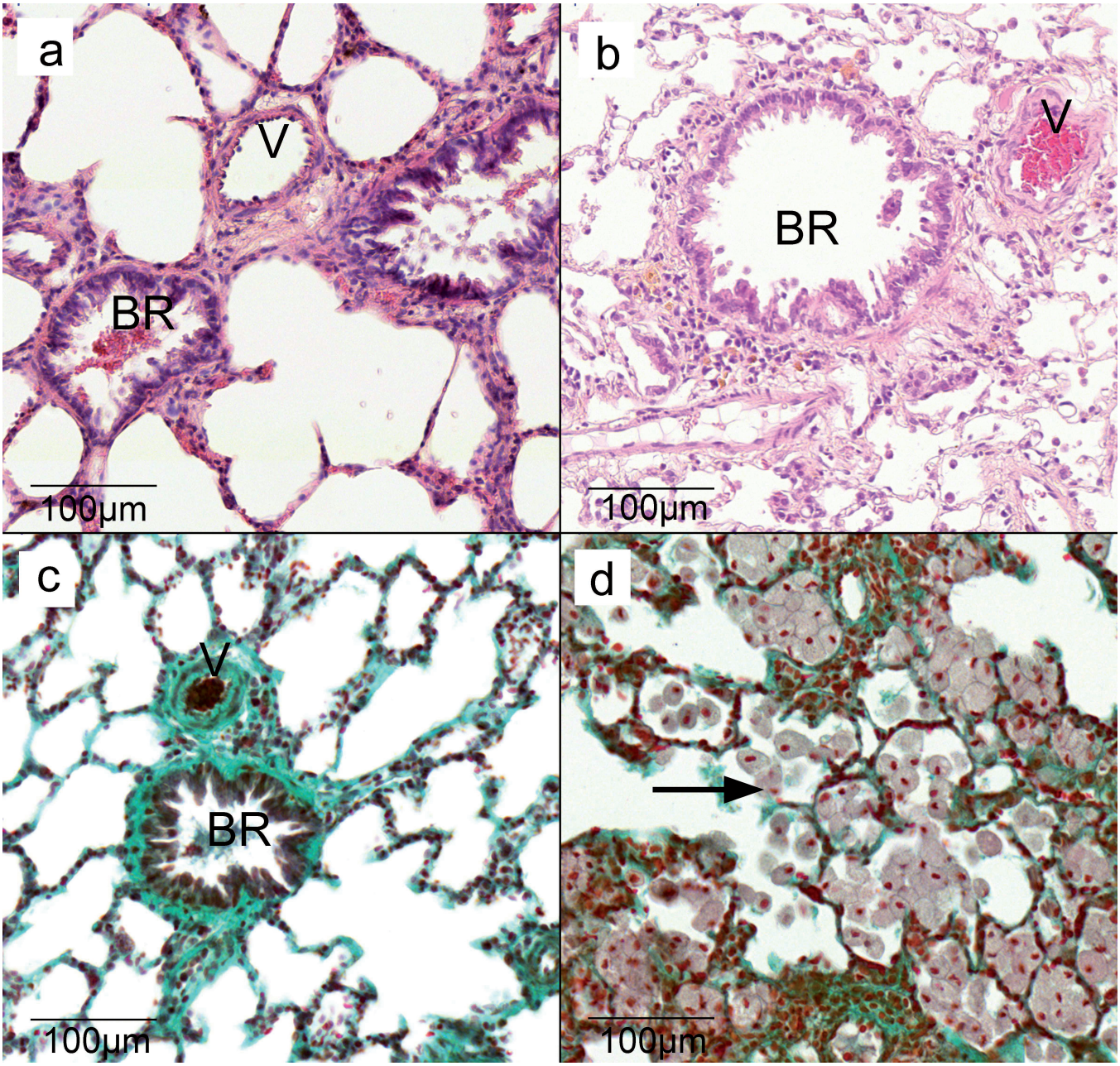

Fig. 7. Histological evaluation of lungs on POD 100. Representative sections of a non-affected right lung from the control-group (HE) **(a)**, and a lung allograft from the imatinib-group (HE) without chronic degenerations **(b)**. Subepithelial areas and adjacent interstitium of the allograft (b) was infiltrated with scattered mononuclear cells. **c.** Representative section of a non-affected lung allograft from the combination-group (MTG). **d.** Accumulation of macrophages (arrow) in the alveolar spaces of a lung allograft from the imatinib-group (MTG). BR, bronchiolus; V, vessel.

cause local ischemia that contributes to loss of epithelia integrity and the up-regulation of proinflammatory cytokines/chemokines that recruit injurious inflammatory cells. Furthermore, local ischemia will stimulate hypoxia inducible factors, which stimulate angiogenesis, a requirement to support chronic inflammation/fibroobliteration (Belperio et al., 2009). The reduction of chronic vascular rejection on POD 60/100 might reflect the presence of neoangiogenetic processes. Nevertheless, monotherapy with imatinib decreased the number of animals with BO and vasculopathy. In the long-term follow-up, two allografts on POD 60 and one allograft on POD 100 were free of BO and vasculopathy with almost normal lung structures. The protective mechanism of bronchiolar structures was also demonstrated by Kallio et al. (1999). They showed that selective inhibition of PDGFR significantly reduced the development of OAD after rat tracheal implantation (Kallio et al., 1999).

On POD 30, co-application of imatinib and everolimus showed synergistic anti-inflammatory, anti-proliferative and anti-fibrotic effect. While monotherapy with imatinib only reduced the percentage of chronic bronchiolar alterations, the addition of the mTOR inhibitor significantly reduced the percentage of chronic altered small vessels. The underlying mechanism might be an inhibition of smooth muscle cell proliferation and prevention of neointimal thickening and transplant arteriosclerosis (Neumayer, 2005). The described synergistic effect of the combination therapy maintained up to POD 100. The number of small vessels and terminal bronchioles with severe chronic alterations was reduced. Our data allowed the speculation that long-term application of the double therapy improved regeneration potential. Half of the allografts were free of BO and vasculopathy and another 25% of the transplanted lungs of this group only showed isolated structures with fibrotic degenerations. Nevertheless, it remains unclear why only some of the animals have responded to our therapy and the other part of the lung allografts developed distinct chronic airway and vascular rejection. We speculated that there were some mechanisms of resistances and pseudoresistances to imatinib in late fibrogenesis as they were already described for patients with chronic myeloid leukemia (CML) (Quintás-Cardama et al., 2002; Zhang et al., 2009). An increase in the dosage of imatinib (50 mg/kg/rat, 100 mg/kg/rat) could not improve our results. A dose-depended increase in cardiac side effects was observed. These dose-dependent side effects have already been described by Herman et al. (2011). In future studies, the neutralizing effect of alpha1-acid glycoprotein (AGP) should be analyzed. AGP is mainly expressed in alveolar type II cells in the lung (Crestani et al., 1998) and is a major drug binding protein that binds imatinib and mediates drug resistance (Azuma et al., 2007; Gambacorti-Passerini et al., 2000, 2003). The development of imatinib resistance was also described in patients with gastrointestinal stromal tumors (Pantaleo et al., 2010). Only the additional application of everolimus improved inhibition of tumor growth and metabolism in these patients. The underlying mechanism is still unclear. Mancini et al. (2010) showed that the combination therapy enhanced the effects of imatinib in CML by raising the nuclear expression of c-ABL protein. The underlying mechanism is still unclear.

Future studies regarding imatinib resistance, as well as the application of other RTKIs (e. g. Nilotinib, Dasatinib) in our rat LTX model might clarify the specific effect of RTKI and mTORI to prevent BO and vascular sclerosis.

The limitations of our rat model are the presence of persisting high grade inflammatory infiltrations that also affected lung parenchyma and extensive lung fibrosis on POD 100. Both limitations aggravate the clinical transferability. However, this model also allows the presentation of various developmental stages of chronic airway degenerations, as well as the evidence of BO-like lesions in the terminal bronchioles in the allografts after allogeneic LTX.

In conclusion, inhibition of RTK improved lung allograft regeneration after rat LTX. Everolimus synergistically increased the anti-fibrotic effect of imatinib. The introduction of RTKI and mTORI might be a promising therapeutic option in the immuno-suppressive regimen after LTX.

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