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A model of chronic lung allograft rejection in the rat

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Running Head: chronic rejection in rat lung allograft

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Bronchiolitis obliterans, the pathological hallmark of pulmonary chronic rejection, severely impacts long term survival following lung transplantation. However, experimental reproduction of this pathophysiological phenomenon has not been achieved with contemporary *in vivo* models. Here, we describe a model of chronic rejection with sensitized recipients receiving unilateral orthotopic rat lung transplants.

LEW-rats, sensitized with BN-skin seven days before receiving left lung transplants from $LBNF_1$-donors, were analyzed from day21 until day84. The development of chronic rejection was modulated by a treatment with rapamycin and cyclosporine and characterized by histology, immunohistochemistry and RT-PCR.

Characteristic histopathological changes leading to chronic rejection were induced over time by an initial treatment with cyclosporin in the presence of continuous rapamycin application. At day 84, fibrotic lesions replaced the respiratory epithelium within small bronchioles with strong expression of α-SMA and upregulated mRNA for Th1-cytokines, α-SMA, TGF-ß and CCL5 but decreased gene expression for FOXP3.

We describe a reproducible and clinically relevant experimental setup for progressive chronic rejection in rat pulmonary allografts. This model will allow for a better understanding of the pathological changes of small airways during the development of bronchiolitis obliterans, and may serve as an *in vivo* setup for testing the efficacy of novel therapeutic interventions.

Introduction

Surgical technique, anti-infective, and immunosuppressive therapy have increased the overall 1-year-survival following lung transplantation (Tx) to 85-90% [1], but long term prognosis has not improved similarly. The major obstacle on the way to unlimited graft acceptance remains the phenomenon of chronic rejection (CR). Several etiologic factors, both immunologic and non-immunologic, have been linked with CR. Donor-associated parameters, preservation and reperfusion injury, viral infection and acute rejection (AR) episodes influence the development of CR [2, 3]. In contrast to AR, where the histologic pattern reveals perivascular lymphocytic infiltrates and parenchymal damage, biopsies from pulmonary grafts undergoing CR show proliferative remodeling within small airways, leading to luminal obstruction and organ-specific lesions that are described as bronchiolitis obliterans (BO). BO may be found in 30-60% of recipients within the first three years after Tx and corresponds to fibro-obliterative changes of small bronchioles, the hallmark of CR [4].

Several experimental approaches have been developed to simulate CR *in vivo*, since a reproducible animal model for the disease is essential. A widespread setup employs the subcutaneous or intraperitoneal placement of tracheal rings [5, 6]. However, these models have major drawbacks which limit their comparability to the clinical reality since they are non-physiologic: first, tracheal segments undergo severe initial ischemia, which may alter the course of the subsequent immune response. Following this initial ischemic phase, viability of the tracheal rings rely on diffusion from the surrounding tissue alone. Second, physiological ventilation as a central functional aspect in lung transplantation does not occur. Third, this concept does not take the anatomic and physiologic differences between large and small airways into consideration, and fourth, the observed changes occur within a short span of time, in contrast to the slowly developing BO in human lung transplant recipients. Other approaches include end-to-end orthotopic tracheal Tx [6] or direct insertion (heterotopic) of an allograft trachea into the recipient's pulmonary parenchyma [7]. Yet, these models

continued to suffer disadvantages similar to the heterotopic tracheal Tx technique. Attempts to create CR in orthotopic lung Tx models in the rat also have been unrewarding [8, 9].

Recipient sensitization toward MHC-Ag due to multiple blood transfusions, previous engraftments and pregnancies, constitutes a significant problem in current transplantation medicine. These patients are at a greater risk for AR episodes, which, even if successfully treated, may predispose for a progressive decline in organ function over the years in comparison to unsensitized hosts. In 1994, Korom et al. developed a model of CR in heterotopically transplanted rat cardiac allografts [10]. Simulating recipient sensitization, they chose an established model of accelerated rejection in combination with rapamycin (RPM) therapy [10]. Employing this mTOR inhibitor [11] markedly diminished acute and accelerated rejection episodes, but did not prevent the progressive loss of specific organ function that occurred while the morphologic pattern of CR developed [12].

Drawing from the experience in this experimental set up, we here describe a novel approach of inducing CR in a rat orthotopic lung transplantation model in sensitized recipients.

Materials and Methods

Experimental model

Inbred male rat (Harlan, Netherlands), weighing 250 to 300 g, were used. Animals received adequate care according to *The Principles of Laboratory Animal Care* (National Institutes of Health, promulgated in 1985, most recently revised in 1996) and the study was approved by the local animal committee (Licence No. 217/2006). For allogeneic Tx (n = 5-8), left pulmonary grafts were recovered (Lewis [LEW] x Brown-Norway [BN] F_1 hybrids [$LBNF_1$]) and transplanted into LEW (*RT-1*) recipients applying the orthotopic left single lung Tx, using a cuff technique [13]. For syngeneic Tx (n = 4), pulmonary grafts from LEW rats were used. For pre-sensitization, a skin flap of 3×3 cm was orthotopically placed on the dorsal thorax of prospective recipients at day -7. In order to identify the optimal therapeutic regimen of RPM application, a series of pilot experiments, assessing RPM-doses of 0.25 mg/kg/d to 0.75 mg/kg/d and CsA-doses of 2.5 mg/kg/d of various time frames were performed (Fig. 1).

Histologic assessment

For morphometric examination of the sections, standard staining with hematoxylin and eosin was used. All grafts were evaluated according to the classification of the Working Formulation for Classification and Grading of Pulmonary Rejection (ISHLT-Grading).

Immunohistochemistry for CD68, CD3, and α-SMA

Immunohistochemistry was performed according to a previously established protocol [14]. The primary antibody for CD68 and CD3 (AbD Serotec, Düsseldorf, Germany) was applied in a dilution of 1:200 and for α-SMA (Sigma-Aldrich, St. Louis, MO, USA) in a dilution of 1:100, incubated for 2.5 hours, washed in Histo-Tris and incubated for 30 min with the biotinylated secondary link antibody (Vector Laboratories, Servion, Switzerland) before being washed twice in TBS again. They are then incubated for 30 min at room temperature with

ABC, the signal was detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB), producing a brown stain at the site of the reaction. Sections were lightly counterstained with Mayer's haematoxylin. Calculation of CD68 positively stained alveolar macrophages (MΦ) and CD3 positively stained T cells was determined as described previously [15]. H&E counterstaining of corresponding sections identified mononuclear cells as MΦ. Three different representative sections from each graft were analyzed. MΦ and T cells in 20 alveoli were counted in a blinded fashion, and the absolute number of positively stained cells was expressed as the mean number ± standard deviation.

Immunofluorescence staining for Th1, Th2 cytokines

Lung tissue was fixed with OCT-mounting medium, immediately frozen in liquid nitrogen, and stored at -80°C until use. The tissue was cut at -22°C into 5µm slices and put on positively charged glass slides and stored at -80°C until use. The slides were washed in 2/3 Methanol and 1/3 Acetone for 10 min, and air dried. After washing in Histo-Tris, sections were blocked for 1 hour in 1% BSA Histo-Tris, Tween+Goat in a dilution of 1:20. After incubation with the first mouse-anti-rat antibody (IL-2, IL-12 1:50; IL-4, IL-10 1:200), slides were washed 3×15 min and incubated with anti mouse FITC in 1%BSA/HTT (1:100) for 1h. After an additional rinsing, counterstaining of cell nucleus was performed with DAPI (1:1000 in PBS) for 5 min and slides were mounted with DAKO Fluorescent Mounting Medium (DAKO, Chromogen system, Carpinteria, CA, USA). Staining was analyzed with a Leica AF6000 microsystems fluorescent microscope (Leica, Microsystems, Wetzlar, Germany). Three different representative sections from each graft were analyzed. Positively stained cells in 20 alveoli were counted in a blinded fashion, and the absolute number of positively stained cells was expressed as the mean number ± standard deviation.

Quantitative RT-PCR for Th1, Th2 cytokines, TGF-ß, RANTES, α-SMA and FOXP3

The total RNA was extracted from 50 mg lung tissue using TRIzol reagent (Invitrogen, Paisley, UK). Five micrograms RNA were reverse-transcribed using the Thermoscript RT-PCR kit (Invitrogen, Basel, Switzerland), yielding the complementary DNA template. The quantitative real-time polymerase chain reaction (PCR) amplification and the data analysis were performed using an ABI-Prism 7000 Sequence Detector system. TaqMan gene expression assays (PE Applied Biosystems, Rotkreuz, Switzerland) for IL-2 (Rn99999181_m1), IL-12 (Rn00575112_m1), IFN-γ (Rn00594078_m1), IL-10 (Rn00563409_m1), IL-13 (Rn00587615_m1), TGF-β (Rn99999016_m1), α-SMA (Rn01759928_m1), RANTES (Rn00579590_m1), and FOXP3 (Rn01525085_m1) were used to quantify the messenger RNA (mRNA) expression of the respective genes. The mRNA expression levels for each sample were normalized to 18*S* RNA. The results gained represent fold induction versus baseline levels in control syngeneic transplanted rats.

Statistical Analysis

Data analysis was performed using SPSS for Windows 15.0 (SPSS, Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). For categorical values Pearson's chi-square test was used. The Mann-Whitney test was performed to compare the differences between the two groups. A P value < .05 was considered statistically significant.

Results

Histological changes in sensitized allografts during CR

Recipients were treated with different RPM doses at various time points (Fig. 1). 42 days after Tx, allografts were analyzed under the lowest dose of RPM (0.25 mg/kg/d) and showed near-complete necrosis due to AR. When increasing the dosis of RPM to 0.5 mg/kg/d, we observed a destruction and detachment of the respiratory epithelium in small bronchioles (Fig. 1A), with partial arterial occlusion *(ISHLT-Grade A2-A3*). When introducing 0.75 mg RPM per day, a severe lymphocytic bronchiolitis and a detachment of the respiratory epithelium were induced (Fig. 1B), but even this high dose of RPM did not succeed in controlling the acute rejection and vascular rejection patterns prevailed *(ISHLT-Grade A2-A4*), Cyclosporin A (CsA) was now introduced in order to target the acute vascular rejection phase. Introduction of low-dose CsA (2.5 mg/kg/d) during the first three weeks after Tx, together with RPM (0.5 mg/kg/d) effectively suppressed the acute vascular rejection phase and resulted in striking graft preservation up to 70 days after Tx, yet, peri- and transbronchial lymphocytic sequestration occurred more frequently and more severe (Fig. 1C). Reducing the CsA treatment phase to one week after engraftment and continuous application of 0.5 mg RPM per day, we observed a substantial destruction of respiratory bronchial epithelium with development of fibro-proliferative, partially replacing the respiratory epithelium 84 days after Tx (Fig. 1D). At this time point, there was strong positivity for α-SMA within fibrotic lesions (Fig. 2).

Histology in sensitized syngrafts

In order to solely investigate the effect of skin sensitization, we transplanted syngeneic LEW rats that received BN-skin 7 days before Tx. Histology on day 42 after Tx revealed mild thickening of the alveolar wall with few numbers of leucocytes (Fig. 3A) without edema formation or inflammation of bronchioles (Fig. 3B). On day 60 after Tx, syngeneic grafts

displayed moderate alveolar wall thickening, accompanied by a mild sequestration of MΦ and neutrophiles into the alveolar wall (Fig. 3C). The peribronchial space and, to a lesser extent, the perivascular space, were discretely enlarged, and filled by MΦ while bronchioles were surrounded by a cuff of lymphocytes (Fig. 3D). There was no evidence of intraalveolar edema formation.

Macrophages and T cells in allografts and syngrafts

The activation of MΦ and T cells plays a critical role in the initiation of the pathways leading to CR. On day 21, MΦ, but also $CD3^+$ T cells were the most prominent cells within allografts (n = 6/group) (Fig. 4).

Th1 and Th2 cytokines in allografts

Th1-related cytokines are considered to be dominant over Th2 cytokines during acute rejection episodes. The Th1-cytokines IL-2 and IL-12 were detected with increased intensity at day 21 after Tx (Fig. 5, A, B), whereas Th2-cytokines IL-4 and IL-10 were expressed to a lesser extent within allografts (n = 6/group) (Fig. 5, C, D).

mRNA expression of Th1-, Th2-cytokines, TGF-ß, RANTES, α-SMA, and FOXP3 during CR

Quantitative real-time PCR analysis performed in allografts relative to syngrafts demonstrated a higher expression of mRNA at day 21 after Tx for IL-2, IL-12, IFN-γ (n = 4/group) and at day 84 for IL-2, IL-12, IFN-γ, IL-10, IL-13, TGF-ß, α-SMA and RANTES (n = 4/group), but at day 84 a decreased expression difference between sensitized allografts vs. syngrafts for FOXP3 (n = 4/group) (Fig. 6).

Discussion

Based on an established cardiac CR model, we here describe a clinically relevant experimental setup with stable reproducibility for the studying of CR in sensitized rat recipients. Rapamycin in combination with CsA in a defined treatment algorithm led to the progressive development of the characteristic fibro-proliferative changes associated with obliterating bronchiolitis in orthotopically transplanted pulmonary allografts.

The heterotopic tracheal transplantation model in mice has been extensively used for the study of BO [16]. Although this model is technically easy to perform, it is associated with major limitations. Alternative models have utilized orthotopic tracheal Tx [6] or intraparenchymal insertion of allogeneic tracheal segments into the host lung [7]. Yet, these approaches are hampered by similar pitfalls. Up to date, experimental *in vivo* models investigating small airway changes associated with BO in perfused organ Tx yield heterogeneous results and lack reproducibility: Tx models of moderately histo-incompatible inbred rat strain combinations (between F344 and WKY animals) revealed interstitial and peribronchiolar fibrosis but no pathological changes within small airways [8]. Late airway changes were detected 6 months after Tx by Lee et al., but the lesions were expressed infrequently [9]. We chose a first generation rat model between LEW and BN, thus intending to attenuate acute rejection episodes by the fully MHC-class II mismatched strains while maintaining a sufficient immunogeneic background. Yet, in this strain combination, acute rejection episodes occluded arteries and rendered allografts non-functional. This was particularly surprising as RPM therapy was shown to reverse acute rejection [17]. In contrast to various engrafted solid organs where interaction between host and recipient are confined to the vasculature of the transplant, the lung has a more complex anatomy. Bronchial and pulmonary circulation plus the alveolar exchange surface constitute a vast area for interaction between the recipient and

the *milieu exterieure*, which could in part explain the marked rejection early during the course of CR.

RPM therapy markedly diminishes acute and accelerated rejection episodes, but it does not prevent the progressive loss of specific organ function. Interestingly, RPM does not effectively abrogate the late expression of pro-inflammatory macrophage-associated gene transcripts like regulated on activation, normal T cell expressed and secreted (RANTES), MCP-1, and IL-12 [12]. When looking for the presence of activated MΦ, we found a significant accumulation of MΦ in allografts early during CR. But also RANTES, which plays a pivotal role in recruiting mononuclear phagocytes during the pathogenesis of BO [18], was found to be elevated both, at early and late time points within the time frame of analysis. Furthermore, TGF-β mRNA levels were described to be higher in RPM-treated animals during the chronic rejection process compared to untreated recipients [10]. Confirming increased levels of TGF-β at day 84 rendered this mediator to be relevant during the development of CR.

Implementing low dose CsA to the treatment regime allowed for a shift toward bronchial immune interaction during the course of rejection. We then could observe the characteristic stages of destruction of small airways, which were all in accordance to the description of the working formulation in the diagnosis of lung rejection [19]. The recruitment of myofibroblasts, that partially replaced the respiratory epithelium at day 84, was shown to be an inevitable condition for fibrous obliteration in heterotopic tracheal allografts [20]. The significance of this histological feature is increasingly recognized by others [21] and current literature provides evidence that the myofibroblast/α-SMA phenotype can be a result of the upregulation of TGF-β trough activation of various pathways such as the MAPK, c-Jun pathway [22].

Transplanted individuals which were exposed to multiple blood transfusions, previous engraftments and pregnancies are thought to be sensitized toward MHC-Ag and are therefore at a greater risk for acute rejection episodes, which, if even successfully treated, predispose for a chronic decline in organ function over the years in comparison to unsensitized hosts. In the heterotopic heart model, pre-sensitization in combination with RPM resulted in characteristic signs of CR with the morphologic picture of accelerated graft arteriosclerosis [23]. However, in this model of CR, pre-sensitization did not have a severe impact on chronic rejection changes. This could be due to the different immunologic environment of the pulmonary tissue, or alternatively, RPM is inducing an attenuation of the allo-reactive antibody response.

While Ramirez et al. did not show an effector function of allo-reactive cells, of which they identified only few in the BO lesions [20], we may assume that airway wound repair is dependent on a delicate balance between pro- and anti-inflammatory cytokines. Changes in this balance can influence allograft airway remodeling. Low levels of IL-12 were demonstrated to predict the development of BO-syndrome in humans [24], whereas the domination of the Th2 response was favoured in promoting CR [25]. In a rodent heterotopic tracheal Tx model, Neuringer et al. suggested both pathways of Th to play a role during the development of CR [26], while others provided dichotomous findings on the role of Th1/Th2 [9, 27]. The marked infiltration of MΦ and perivascular CD3 positive T cells most likely lead to a strong Th1 response in allografts in our model. As it has been shown in the cardiac heterotopic Tx model [12], the presence of the Th1 cytokine IL 12 in long term allografts after RPM therapy suggests a late activation of MΦ that may also contribute to the chronic rejection of pulmonary transplants.

Current literature supports a protective effect conveyed by regulatory T cells in preventing rejection [28]. We therefore were not surprised to observe reduced levels of FOXP3 gene

expression in fibrotic allografts, although the phenomenon of BO did not affect the full circumference of bronchioles at the end of our investigation period.

Although we could provide clear evidence for chronic rejection in small airways in physiologic orthotopic allografts, some limitations should be considered in this experimental set-up. BO did not occlude the lumen completely, possibly due to the early and pronounced effect of CsA. Choosing an even longer period of experimental time frame could potentially induce a more pronounced histologic picture of BO. Second, the role of allo-antibodies, particularly antibodies against recipient HLA which are capable to damage allograft airway epithelium, needs to be investigated since sensitization is known to induce the synthesis of donor antibodies that have been associated with BO. Third, rat models lack the breadth of reagents and the genetic manipulations available to study both innate and adaptive immunity during chronic rejection. We were previously able to show ventilated and perfused syngeneic grafts to be functional up to day 90 post-Tx in a mouse model of orthotopic Tx [29]. This model could potentially serve as a suitable future tool for the investigation of chronic rejection.

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Figure legends

Figure 1, A-D: Histology (H&E) on paraffin sections in grafts on day 21 post-Tx (*A*), day 42 (*B*), day 70 (*C*) and day 84 (*D*) after allogeneic Tx from $LBNF_1$ → sensitized LEW. Fibroproliferative changes displayed in Fig. D occurred in 80% of animals. Within section *D*, fibroproliferative changes were found in 80% of all bronchioles in whole lung specimens in this group (n = 5-8/group), (Original magnification *A, B:* ×200; *C, D*: ×100). RPM, rapamycin; CsA, cyclosporine.

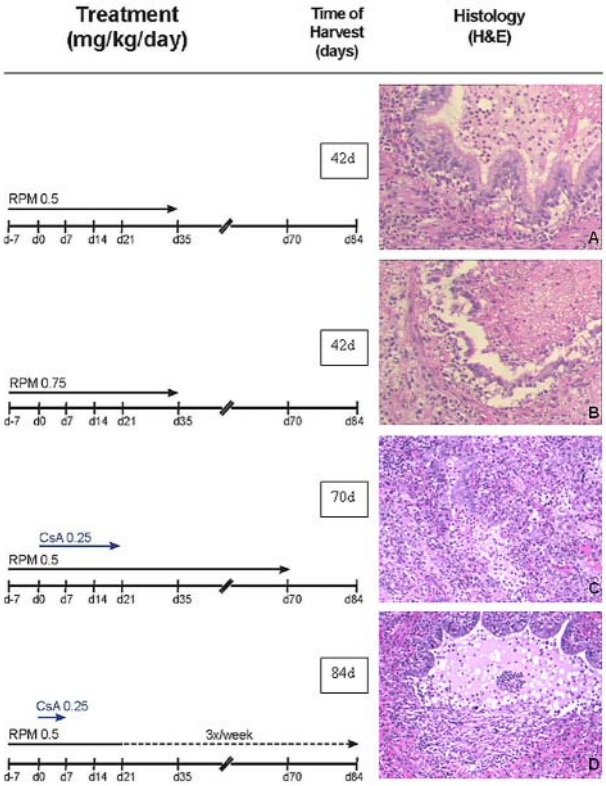


Figure 1

Figure 2, A-H: H&E-staining (*A, C, E, G*) and immunohistochemistry for α-SMA (*B, D, F, H*) in $LBNF_1$ lung allografts, transplanted into sensitized LEW recipients, 84 days after Tx (n = 8). Images are randomly chosen representative sections from the corresponding location. The primary antibody of α-SMA is detected using a horse-redish-peroxidase coupled antibody and DAB, the staining is identified as brown staining of myofibroblasts within bronchioles.

(Original magnification: ×100). Arrows indicate the site of loss of respiratory epithelium and the development of fibro-proliferative changes. SMA, smooth muscle actin.

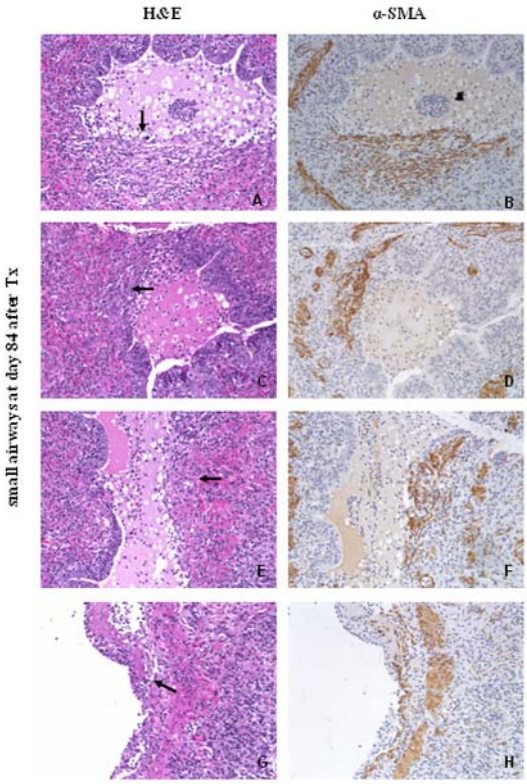


Figure 2

Figure 3, A-D: H&E-staining, alveolar and bronchial histology from syngeneic grafts (LEW → LEW; n = 4) in previously sensitized recipients, 42 days (*A, B*) and 60 days post-Tx (*C, D*), respectively. The ultrastructure of syngrafts was preserved at day 42 whereas syngeneic grafts at day 60 showed minimal signs of alveolar and peribronchial inflammation. (Original magnification: ×100).

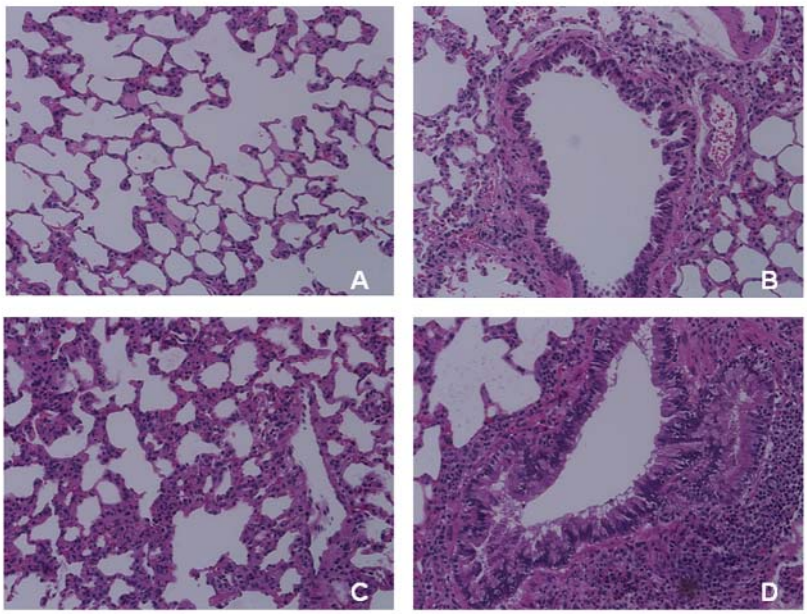


Figure 3

Figure 4, A-C: $CD68^{+}$ activated alveolar macrophages, 21 days after allograft Tx $LBNF_1$ → senzitised LEW (*A*) vs. sparse CD68-positivity in a syngeneic Tx (LEW → LEW) (*B*), 21 days post-Tx. The number of positively stained cells per alveolus were increased in sensitized allografts vs. syngrafts (allografts: 7.2±1.91 vs. syngrafts: 2.25±1.37; **P* = .0001, n = 6/group; **P* = .0001). Nuclei are counterstained with hematoxylin (Original magnification: ×200). Perivascular lymphocytic cuff of CD3-positive T-cells, 21 days after allograft Tx $LBNF_1$ → senzitised LEW *(C)*; the number of cells per alveolus did not differ significantly between sensitized allografts and syngrafts (allografts: 4.75±1.48 vs. syngrafts: 2.65±1.39; n = 6/group; **P* = .01 vs. control). Nuclei are counterstained with hematoxylin (Original magnification: ×200). Bars represent the mean ± SD for each group

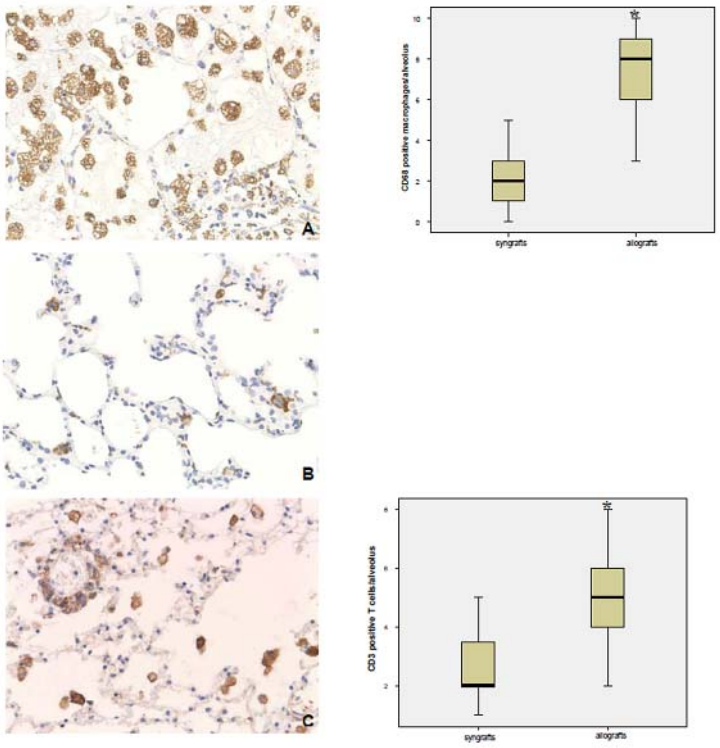


Figure 4

Figure 5, A-D: Immunofluorescence of Th 1- and Th 2-cytokines. The cytoplasm of cytokine secreting cells are stained. They are located within the alveolar wall, partially accumulating to groups of cells around vessels and small bronchi. The alveolar structure of the pulmonary tissue appears self-fluorescent in the background. There is significant more expression for IL-2 (*A*) and IL-12 (*B*), 21 days after allogeneic Tx, $LBNF_1$ → sensitized LEW vs. syngrafts (green: FITC-labeled; (Original magnification: ×400) The number of positively stained cells per alveolus was increased in sensitized allografts vs. syngrafts (IL-2, allografts: 7.65±1.73 vs. syngrafts: 3.65±1.53, **P* = .0001; and for IL-12, allografts: 5.3±1.72 vs. syngrafts 1.9±1.17, **P* = .0001; n = 6/group). Weak expression for immunofluorescent IL-4 (*C*) and IL-10 (*D*), 21 days after allogeneic Tx, $LBNF_1$ → sensitized LEW vs. syngrafts green: FITC-labeled; (Original magnification: ×400). The number of positively stained cells per alveolus was increased in sensitized allografts vs. syngrafts (IL-4, allografts: 2.8±1.47 vs. syngrafts:

1.3±0.98, *P = .001; and for IL-10, allografts: 3.8±1.47 vs. syngrafts: 3.2±1.44; NS, *P < .2, n = 6/group).

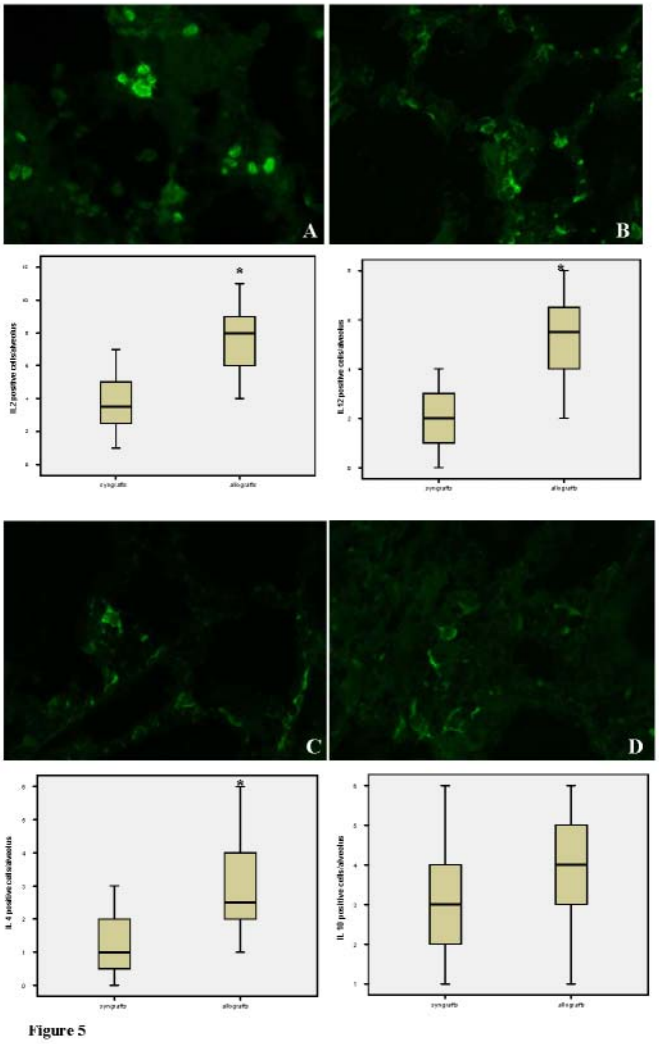
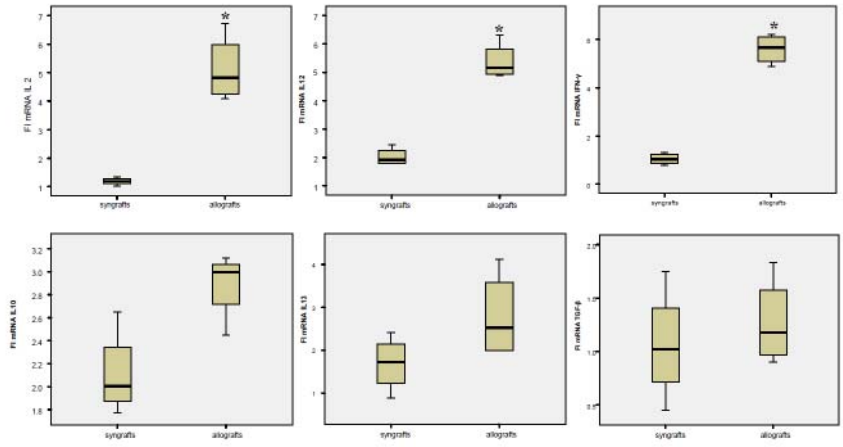


Figure 5

Figure 6: Real time PCR analysis of mRNA expression in pulmonary allografts on day 21 (A) and day 84 (B) after transplantation. Relative gene expression (mRNA) in sensitized allografts are increased in comparison to control syngrafts. Bars represent the mean ± SD for each group (d 21: *P = .003 vs. control syngrafts; d 84: *P = .02 vs. control syngrafts; n = 4/group). mRNA expression is decreased for FOXP3 (d 84: *P = .008 vs. control syngrafts; n = 4/group). Tx, transplantation, FI, fold induction.

A



B