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ORIGINAL ARTICLE

Adaptive and innate immune responses in a rat orthotopic lung transplant model of chronic lung allograft dysfunction

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Conflicts of interest
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Summary

Acute rejection and respiratory infections are major risk factors for chronic lung allograft dysfunction (CLAD) after lung transplantation. To shed light on the enigmatic etiology of CLAD, we test the following hypotheses using a new experimental model: (i) Alloimmune-independent pulmonary inflammation reactivates alloimmunity. (ii) Alloimmunity enhances the susceptibility of the graft toward pathogen-associated molecular patterns. Pulmonary Fischer 344 to Lewis rat allografts were treated with lipopolysaccharide (LPS), which consistently results in lesions typical for CLAD. Grafts, local lymph nodes, and spleens were harvested before (day 28) and after LPS application (days 29, 33, and 40) for real-time RT-PCR and immunohistochemistry. Mixed lymphocyte reactions were performed on day 33. Four weeks after transplantation, lung allografts displayed mononuclear infiltrates compatible with acute rejection and overexpressed most components of the toll-like receptor system. Allografts but not secondary lymphoid organs expressed increased levels of Th1-type transcription factors and cytokines. LPS induced macrophage infiltration as well as mRNA expression of pro-inflammatory cytokines and effector molecules of innate immunity. Unexpectedly, T-cell reactivity was not enhanced by LPS. We conclude that prevention of CLAD might be accomplished by local suppression of Th1 cells in stable grafts and by controlling innate immunity during alloimmune-independent pulmonary inflammation.

Introduction

Lung transplantation is the only therapeutic option for numerous patients suffering from end-stage lung disease. Patients, which survived the first year after surgery, are mainly menaced by a progressive decline in lung function as measured by a decrease in the forced expiratory volume in one second (FEV1), named chronic lung allograft dysfunction (CLAD) or bronchiolitis obliterans syndrome (BOS). Major risk factors for the development of CLAD are acute rejection episodes, respiratory infections, and acid aspiration due to gastro-esophageal reflux [1–3]. CLAD is assumed to be chronic rejection enhanced or triggered by a "second hit", which can be any kind of nonalloimmune inflammation. Accordingly, some success in the prevention of CLAD was achieved by application of azithromycin, an anti-inflammatory macrolide broad-spectrum antibiotic [4] and by antireflux surgery [5] in selected patients.

The cellular and molecular basis for the high susceptibility of lung allografts to alloimmune-independent inflammation and the mechanisms how inflammation translates into CLAD are still elusive. Nonalloimmune pulmonary inflammation increases the expression of MHC antigens, costimulatory molecules, adhesion molecules as well as a plethora of pro-inflammatory mediators, which together could enhance or reactivate T-cell alloimmunity. Reactivation of alloimmunity by lipopolysaccharide (LPS) or bacterial infection can indeed break operational tolerance to allografts and induce pulmonary injury after allogeneic bone marrow transplantation [6–9].

Infectious and sterile inflammatory stimuli, so-called danger- or pathogen-associated patterns (DAMP or PAMP), are sensed by numerous receptors such as toll-like receptors (TLR), which are expressed by leukocytes and epithelial cells [10–12]. Polymorphisms of TLR2, TLR4, TLR9, and CD14, a coreceptor for TLR4, seem to influence the risk of CLAD development [13]. As shown for small bowel grafts, alloimmune reactions can enhance TLR expression [14] and hence might increase the sensitivity of the graft toward PAMPS and DAMPS.

Recently, we established an experimental model, which mimics central aspects of human CLAD [15]. This model involves orthotopic left lung transplantation in the Fischer 344 (F344) to Lewis rat strain combination, a short course of immunosuppression, and intratracheal application of LPS to induce alloimmune-independent inflammation. Four weeks after transplantation, graft histopathology is compatible with moderate acute rejection [15]. At this time point, LPS application induces strong neutrophil influx into allografts, whereas right native lungs and isografts are only mildly infiltrated [15]. Twelve days after LPS instillation, first signs of vascular and bronchiolar remodeling as well as graft fibrosis are seen. Three months post-transplantation, pulmonary allografts are severely damaged including airway remodeling, vasculopathies, and lung fibrosis, closely resembling human end-stage CLAD/BOS [15]. Experimental CLAD develops consistently, when the alloimmune stimulus is combined with an alloimmune-independent stimulus but neither in LPS-treated isografts nor in control-treated allografts. Hence, CLAD seems to depend on the synergism of both stimuli.

The purpose of this study is to gain insights into the pathogenesis of CLAD and to further characterize our new experimental model. We dissect alloimmune-dependent and alloimmune-independent factors contributing to experimental CLAD and target two hypotheses: (i) Stimulation of innate immunity activates or re-activates T-cell alloreactivity, and (ii) preceding alloimmune reactions lead to overexpression of the TLR system, which predisposes allografts to exaggerated innate responses to TLR ligands.

Materials and methods

Animal experiments

Male Lewis (RT1 ^{l}) and Dark Agouti (DA, RT1^{av1}) rats were obtained from Janvier Labs (ST Berthevin, France), F344 (RT1^{lv1}) rats from Charles River (Sulzfeld, Germany). All animals were raised under specified pathogen-free conditions. The model for CLAD was recently described in detail [15]. Briefly, allogeneic left lung transplantation was performed in the F344 to Lewis rat strain combination, and isogeneic transplantations were performed in Lewis rats. Recipients were treated with ciclosporin for 10 days after transplantation. On day 28, rats were sacrificed or treated intratracheally with vehicle or LPS. Recipient rats were killed at different time points after transplantation, and lungs, spleens, and regional mediastinal lymph nodes (MLN) were snap-frozen. Lungs were fixed in buffered 4% paraformaldehyde and embedded in paraffin [16]. Animal care and experiments were approved by the Regierungspräsidium Giessen (V54-19c2015(1)GI20/10Nr.49/2007) and performed in accordance with German animal protection laws as well as the NIH principles of laboratory animal care.

Immunohistochemistry

Staining with monoclonal antibody (mAb) R73 to the α/β T-cell receptor, CD-68-like antigen (ED1), and CD163 (ED2) (all from Serotec, Düsseldorf, Germany) was performed on pulmonary paraffin sections as described [16]. Only for detection of R73, the CSA II biotin-free tyramide signal amplification system was used (Dako, Hamburg, Germany). Controls omitting the primary antibody were included in each experiment, which essentially remained unstained. In double-staining experiments, slides were stained with mAb R73 followed by incubation in 0.01 M sodium citrate buffer pH 6.0 for 15 min at 120 °C and 1.1 bar, application of mAb PC10 (Serotec) directed to proliferating cell nuclear antigen (PCNA) and detection with rabbit anti-mouse Ig labeled with alkaline phosphatase (Dako) and Fast Blue (Sigma-Aldrich, Taufkirchen, Germany).

Cryostat sections of native spleens were fixed in 4% buffered paraformaldehyde followed by 2 min in isopropanol. PC10 was detected with rabbit anti-mouse Ig labeled with HRP (Dako) and DAB. Thereafter, R73 was stained with rabbit anti-mouse Ig (Dako), mouse alkaline phosphatase anti-alkaline phosphatase (Dako) and Fast Blue. Three controls were included: omission of both primary antibodies followed by the complete detection system as well as two controls, where only one of them was omitted. These control revealed no unspecific signals. Sections were evaluated blinded for the experimental groups using an Olympus BX51 microscope and the analySIS software (Olympus, Hamburg, Germany). PCNA-positive T cells were counted manually. To evaluate leukocyte infiltration, a scoring system was used: grade 0, no obvious pathological changes; grade 1, a slight increase in the amount of immunopositive cells; grade 2, obvious increase in immunopositive cells; and grade 3, strong increase in immunopositive cells.

Real-time RT-PCR

RNA isolation and real-time RT-PCR were performed as described previously [16]. Primer pairs are indicated in Table 1 or have been published [16]. No product was

obtained in negative controls where cDNA was replaced by water. The specificity of the PCR was confirmed by separation in agarose gels and by product sequencing (SeqLab, Göttingen, Germany). The expression of the gene of interest was normalized with porphobilinogen deaminase (PBGD) and analyzed using the $2^{-\Delta Ct}$ method.

Mixed lymphocyte reactions

To obtain responder cells, blood was taken from LPS-treated (n = 5) and vehicle-treated (n = 4) allograft recipients on day 33 post-transplantation and from healthy Lewis rats. Stimulator cells were obtained from untreated F344 or DA

Table 1. Primers used for real-time RT-PCR.

Gene	Oligonucleotide Sequence (5′-3′)	Genbank-ID	Product lenght (bp)
CD14	F: CAGAATCTACCGACCATGAAGC	NM_021744	143
	R: GGATCTGAGAAGTTGCAGTAGC		
Foxp3	F: GCCCTCCAGTACAGCCGGACA	NM_001108250.1	104
	R: ACGGCAGAGGAGCTGCCGAA		
GATA-3	F: GCCTGCGGACTCTACCATAA	NM_133293.1	110
	R: GTCTGACAGTTCGCACAGGA		
GrB	F: CTCCTCTTGCTCCTGCTGAG	NM_138517.3	100
	R: CCATGTAGGGTCGAGAGTGG		
IL12p35	F: TCAGAGCCACAATCATCAGC	NM_053390.1	111
	R: GGAGCTTTCTGGTGCAGAGT		
MD2	F: GAGGCTGTCAACACAGCAATA	NM_001024279	94
	R: TATCCCCAGCAATGGCTTCT		
MyD88	F: GCCTTGTTAGACCGTGAGGA	NM_198130	145
	R: TTGTCTGTGGGACACTGCTC		
PCNA	F: TCCCAGACAAGCAATGTTGA	NM_022381.3	117
	R: CAGTGGAGTGGCTTTTGTGAA		
T-bet	F: CAACCAGCACCAGACAGAGA	NM_001107043.1	108
	R: AACATCCTGTAATGGCTCGTG		
TGF-β	F: GAAGTCACCCGCGTGCTAAT	NM_021578.2	114
	R: CACTGCTTCCCGAATGTCTG		
TLR1	F: CCTAGAGAAAGATGACATTCGGG	NM_001172120	253
	R: ATTGGTAGGGATGGAGTACTGTG		
TLR2	F: AGATGGCCACAGGACTCAAG	NM_198769	100
	R: TCACAGCCATCAAGATCCAG		
TLR3	F: AAGCAACCCTTTCAAAAACCA	NM_198791	111
	R: AGCTCTTGGAGGTTCTCCAGTT		
TLR4	F: CATGGCATTGTTCCTTTCCT	NM_019178	116
	R: TGTCATGAGGGATTTTGCTG		
TLR5	F: CCACTGGGCTACCTCACCAG	NM_001145828	111
	R: CTTCTTGTTGGCGGACTTGG		
TLR6	F: CTGGCGTCCGAGATATCTTG	NM_207604	149
	R: CTTCCGACTATTAAGGCCAGG		
TLR7	F: GCTCTGTTCTCCTCCACCAAA	NM_001097582	141
	R: CCATCGAAACCCAAGGACTC		
TLR8	F: TTGCCAAAATCTGCTCTCTGC	NM_001101009	133
	R: AATCCACGACTGAGGGGACA		
TLR9	F: GCTGCCCAGTTTGTCAGAGG	NM_198131	150
	R: GTAGGAAGGCAGGCAGGGTA		
TLR10	F: ACTGACCTCCCTGGGTGTGA	NM_001146035	105
	R: CTCCTGGCAGCTCTGGAAAA		
TRAM1	F: AGTTGGCGTATTGGCTCCAT	NM_001007701	113
	R: CCCAGCAATGTGGAAGAGGT		
TIRAP	F: GCCTCCTCCACTCAGTCCAA	XM_001055833	140
	R: CCATCCTGTGTGGCTGTCTG		
TRIF	F: GGTAGCTGCAGATGCTGTTCA	NM_053588	109
	R: TTGGAATGACAGAAGAGACACCA		

F, forward; GrB, granzyme B; R, reverse.

rats. Mononuclear leukocytes were purified by Percoll gradient centrifugation as described previously [17]. Stimulator cells were pretreated with mitomycin C (25 μg/ml, Roche Diagnostics, Mannheim, Germany) for 30 min, thoroughly washed and co-incubated in quadruplicates with an identical number of responder cells for 4 days in RPMI, 10% fetal calf serum (FCS Gold), 2 mM L-glutamine, penicillin/streptomycin (all from PAA, Cölbe, Germany). Thereafter, BrdU was applied for 24 h and incorporation was measured by Cell Proliferation ELISA (Roche Diagnostics).

Negative controls (medium alone, responder cells, stimulator cells) as well as a positive control [responder cells cultured with 5 μg/ml phytohemagglutinin-L (Roche Diagnostics)] were included. The relative specific incorporation of BrdU was calculated by the following formula: [OD of the experiment−OD of stimulator cells alone−OD of responder cells alone] divided by [OD of positive control−OD of responder cells alone].

Statistical analysis

Data were analyzed by Kruskal–Wallis test followed by Mann–Whitney rank sum test using SPSS software (SPSS, Munich, Germany). A $P \leq 0.05$ is considered as statistically significant.

Results

Leukocytic graft infiltration

Pulmonary graft infiltration by macrophages expressing a CD68-like antigen (mAb ED1) or CD163 (mAb ED2), and α/β T-cell receptor-positive T cells (mAb R73) was investigated by immunohistochemistry (Fig. 1a). Before application of LPS (day 28), peribronchiolar and perivascular areas of allografts contained more ED1-positive macrophages and T cells compared with isografts, which is in line with mild to moderate acute rejection described previously [15] (Fig. 1a and b). Intra-alveolar infiltration was low in isografts and allografts.

Application of LPS induced a strong influx of neutrophil granulocytes into allografts and to a lesser extend to isografts and right native lungs [15]. In addition, LPS induced a strong increase in ED1-positive macrophages in isografts, which returned to basal levels until day 40. On days 33 and 40, LPS-treated allografts displayed stronger macrophage infiltrates compared with isografts or PBS-treated allografts. These changes were most prominent in the alveolar region (Fig. 1a and b). ED2-positive macrophages did not differ between allografts and isografts. In contrast to macrophages, LPS application did not change perivascular and peribronchiolar T-cell infiltrates. Only in the alveolar region of LPS-treated allografts on day 40, T-cell infiltrates significantly increased (Fig. 1a and b).

T-cell polarization

We analyzed the mRNA expression of transcription factors T-bet, GATA-3, and Foxp3, characteristic for Th1, Th2, and Treg by real-time RT-PCR (Fig. 2). All factors were expressed at comparatively high levels in MLN, but no significant differences were seen between experimental groups (data not shown). On day 28, splenic T-bet expression was higher in isograft compared with allograft recipients. Application of LPS abolished this difference and reduced splenic Foxp3 mRNA expression in all recipients. Contrary to the spleen, day 28 allografts expressed more T-bet and Foxp3 mRNA compared with isografts. Foxp3, however, was detected at low levels. GATA-3 was abundantly expressed in all lungs, and mRNA levels were reduced by LPS and seemed to increase again on day 40. Of note, LPS did not change pulmonary T-bet and Foxp3 mRNA expression. In contrast, the mRNA of granzyme B, an effector molecule of cytotoxic cells, was induced by LPS exclusively in allografts (Fig. 2).

As T-cell transcription factor expression suggested that alloimmune reactions predominantly take place in the graft but not in secondary lymphoid organs, we focused on lungs and investigated mRNA expression of a set of T-cell cytokines. IFN-γ, IL-2, IL-10, and IL-21 mRNA was increased on day 28 in all allografts but not in isografts or native right lungs. IFN-γ, IL-2, and IL-10 expression was further enhanced on day 33 in response to LPS (Fig. 3). TGF-β1 and IL-4 mRNA was only increased in day 40 allografts compared with isografts (Fig. 3). No changes were observed in the mRNA expression of IL-23p19, and IL-17F mRNA levels remained near the threshold of detection throughout (data not shown).

T-cell proliferation

Next, we analyzed mRNA expression of proliferating cell nuclear antigen (PCNA) in MLN and spleens of pulmonary isograft and allograft recipients, which did not differ (Fig. 4a). LPS resulted in a stronger PCNA mRNA expression in lung allografts on day 33 compared with PBS-treated allografts (Fig. 4a).

To estimate T-cell proliferation, immunohistochemical double-staining of histological sections of spleens and lungs was performed with mAb R73 recognizing the α/β T-cell receptor and with mAb PC10 directed to proliferating cell nuclear antigen (PCNA) (Figs 4b and c, 5). Compared with spleens from healthy Lewis rats, the proportion of PCNA-positive T cells in periarteriolar lymphoid sheaths was increased on day 28 after isogeneic transplantation

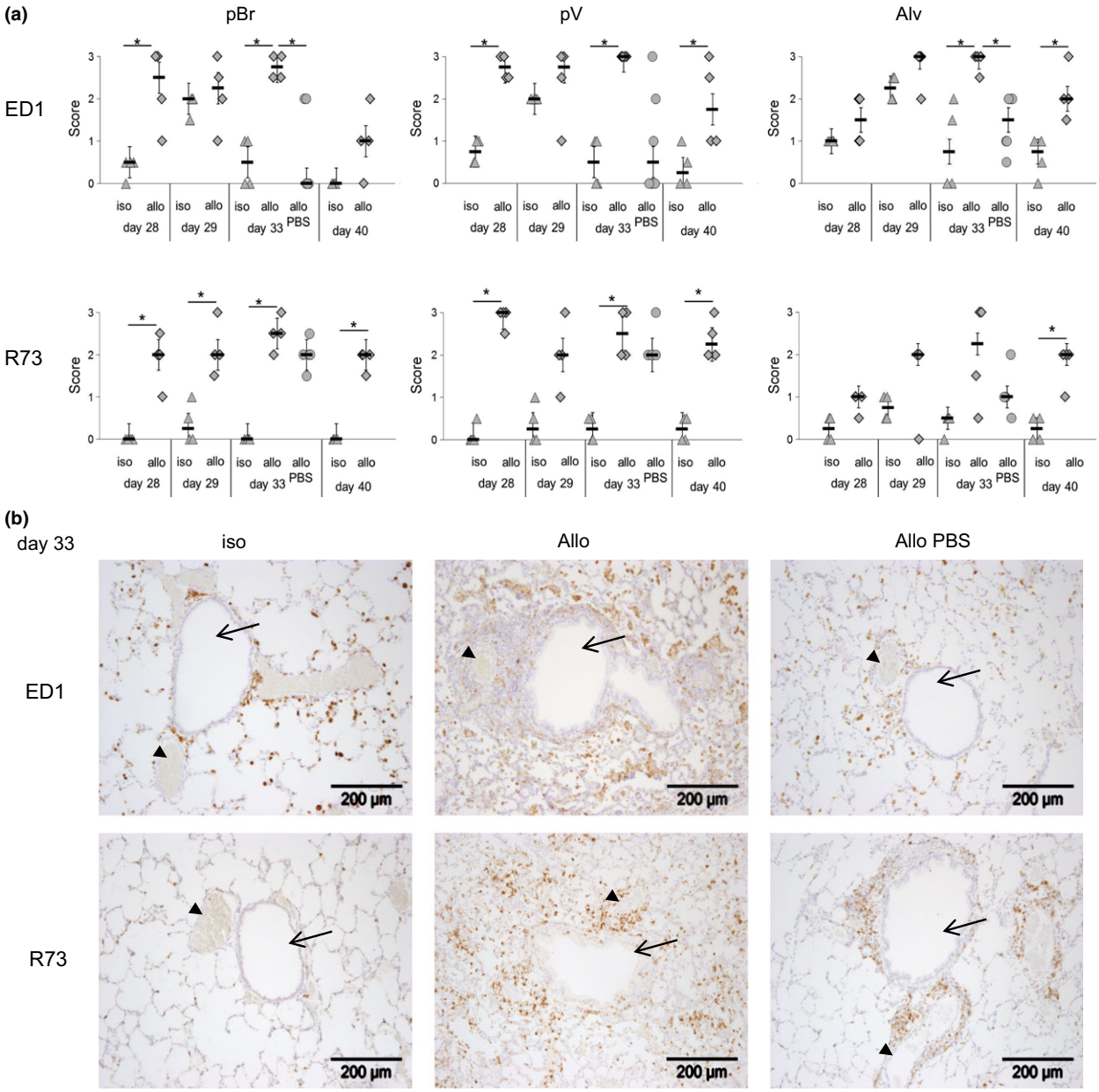

Figure 1 Leukocytic graft infiltration. Infiltration of pulmonary isografts (iso) and allografts (allo) by monocytes/macrophages and T cells was detected on paraffin sections by monoclonal antibody ED1 directed to a CD68-like antigen and by monoclonal antibody R73 directed to the α/β T-cell receptor. Graft recipients were investigated on days 28, 29, 33, and 40 post-transplantation. On day 28, experimental animals were sacrificed before application of lipopolysaccharide (LPS). Allografts and right native lungs, treated with vehicle (PBS) instead of LPS, were studied on day 33. (a) Graft infiltration was evaluated in peribronchiolar (pBr), perivascular (pV), and alveolar (Alv) regions separately using a scoring system; $n = 4$ per group, $^*P \leq 0.05$ Kruskal–Wallis test followed by Mann–Whitney *U*-test. (b) Micrographs depict examples of day 33 isografts and allografts, which were treated with LPS on day 28 as well as allografts treated with PBS instead of LPS. Immunoreactive cells are stained in brown, and slides were lightly counterstained with hemalum. Arrows are pointing to the lumina of bronchioles, arrowheads to pulmonary arteries.

($P \leq 0.05$) (Fig. 4b). In line with the mRNA data, isograft and allograft recipients did not differ irrespective of LPS treatment (Fig. 4b and c).

In mixed lymphocyte reactions (MLR), blood leukocytes from pulmonary allograft recipients treated with LPS or PBS did not proliferate in response to F344 cells, similar to blood leukocytes from naïve Lewis (Fig. 4d). In contrast, stimulation with phytohemagglutinin-L (data not shown) or with fully allogeneic DA cells resulted in strong proliferative responses (Fig. 4d).

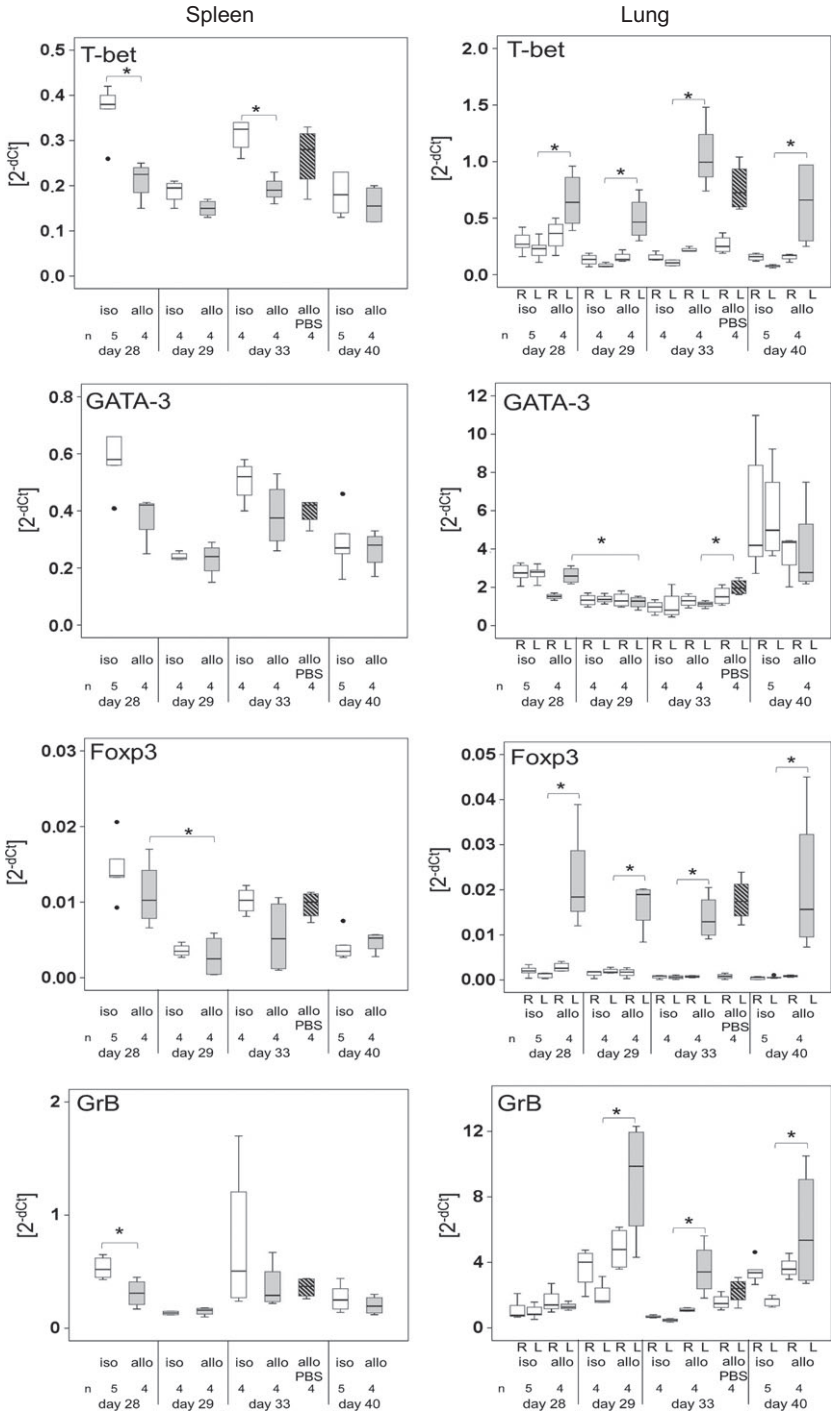

Figure 2 Real-time RT-PCR analysis of T-cell transcription factors and granzyme B. Expression of T-bet (Th1 cells), GATA-3 (Th2 cells, but also expressed by lung tissue), and Foxp3 (Treg cells) was studied by real-time RT-PCR on RNA extracted from spleen, right (R) native lungs and left (L) transplants of isograft (iso) and allograft (allo) recipients on days 28, 29, 33, and 40 post-transplantation. On day 28, experimental animals were sacrificed before application of lipopolysaccharide (LPS). Allografts and right native lungs, treated with vehicle (allo, PBS) instead of LPS, were studied on day 33. All other graft recipients were treated intratracheally with LPS on day 28. *$P \leq 0.05$, Kruskal–Wallis test followed by Mann–Whitney *U*-test.

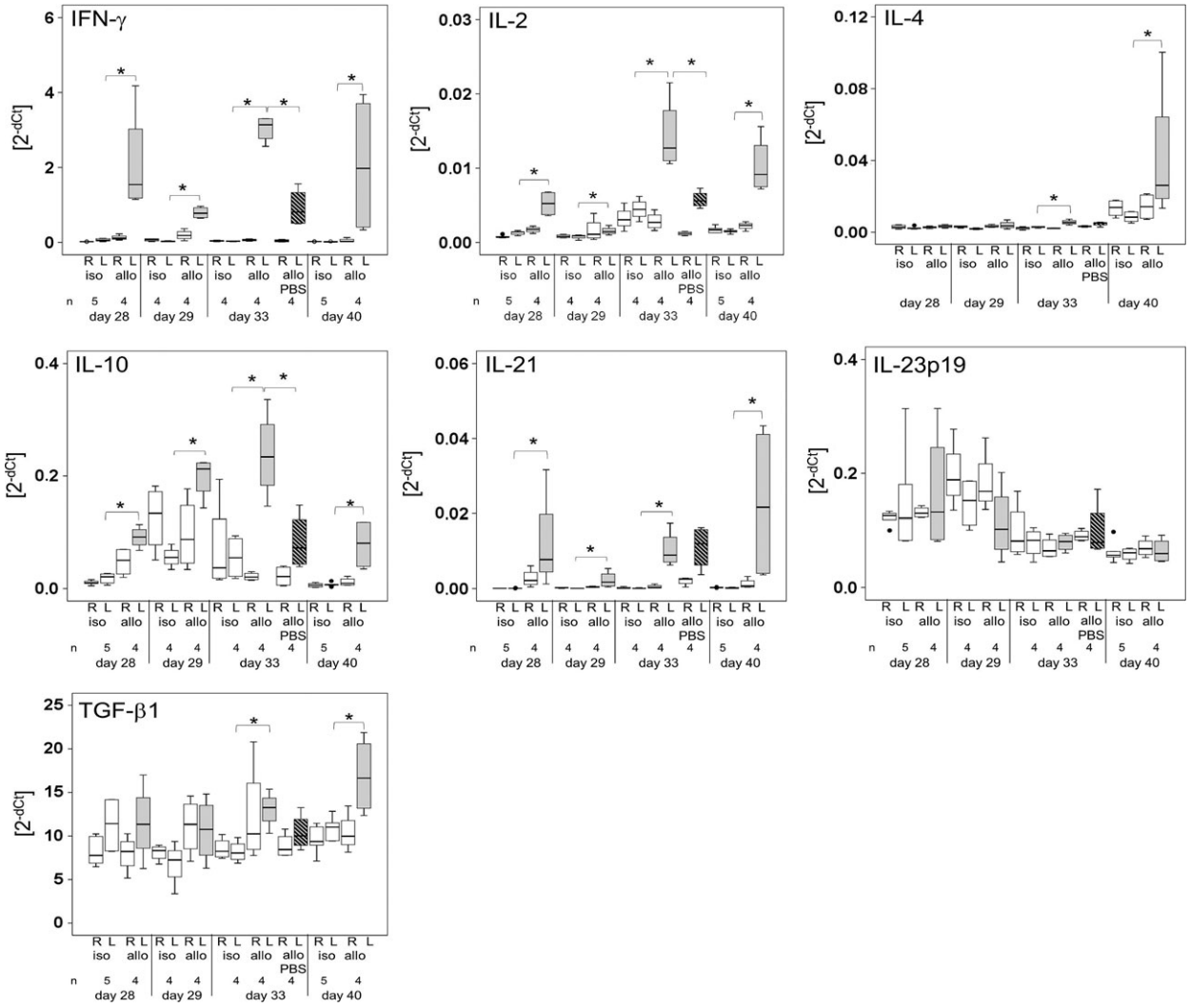

Figure 3 Pulmonary mRNA expression of selected cytokines typically produced by T cells. Real-time RT-PCR was performed on RNA extracted from right (R) native lungs and left (L) transplants of isograft (iso) and allograft (allo) recipients on days 28, 29, 33, and 40 post-transplantation. On day 28, experimental animals were sacrificed before application of LPS. Allografts and right native lungs, treated with vehicle (allo PBS) instead of LPS, were studied on day 33; $*P \leq 0.05$, Kruskal–Wallis test followed by Mann–Whitney *U*-test. The expression of IL-17F was very low and remained under the threshold of detection in most samples (data not depicted).

Innate immunity

Next, we tested the hypothesis that allogeneic lung transplantation results in increased mRNA expression of the TLR system on day 28 before application of LPS, which might increase the sensitivity of allografts toward LPS. In comparison with healthy Lewis lungs, isogeneic transplantation only resulted in subtle changes in the mRNA expression of the TLR system (data not shown). The mRNA expression of TLR1, TLR2, TLR3, TLR5, TLR7, TLR9, TLR10, CD14, and MD2 was significantly increased in allografts compared with isografts, whereas no significant difference was seen for TLR4, TLR8, Myd88, TRAM, and TIRAP (Fig. 6). Expression levels were relatively high for TRAM, TLR9, TLR7, and TLR4, and low for TLR1, TLR2, and TLR10 (Fig. 6). TLR6 remained below the threshold of detection in most samples (data not shown).

Finally, we investigated the mRNA expression of iNOS and of cytokines typically produced by monocytes/macrophages (Fig. 7). The expression of pro-inflammatory monokines pro-IL-1β and IL-12p40 was increased in allografts compared with isografts at day 28 post-transplantation. Within 24 h, treatment with LPS led to an increase of pro-IL-1β, IL-6, and iNOS in all lungs, whereas IL-12p35 was transiently induced in allografts. On day 33, pro-IL-1β and iNOS were overexpressed in LPS-treated allografts

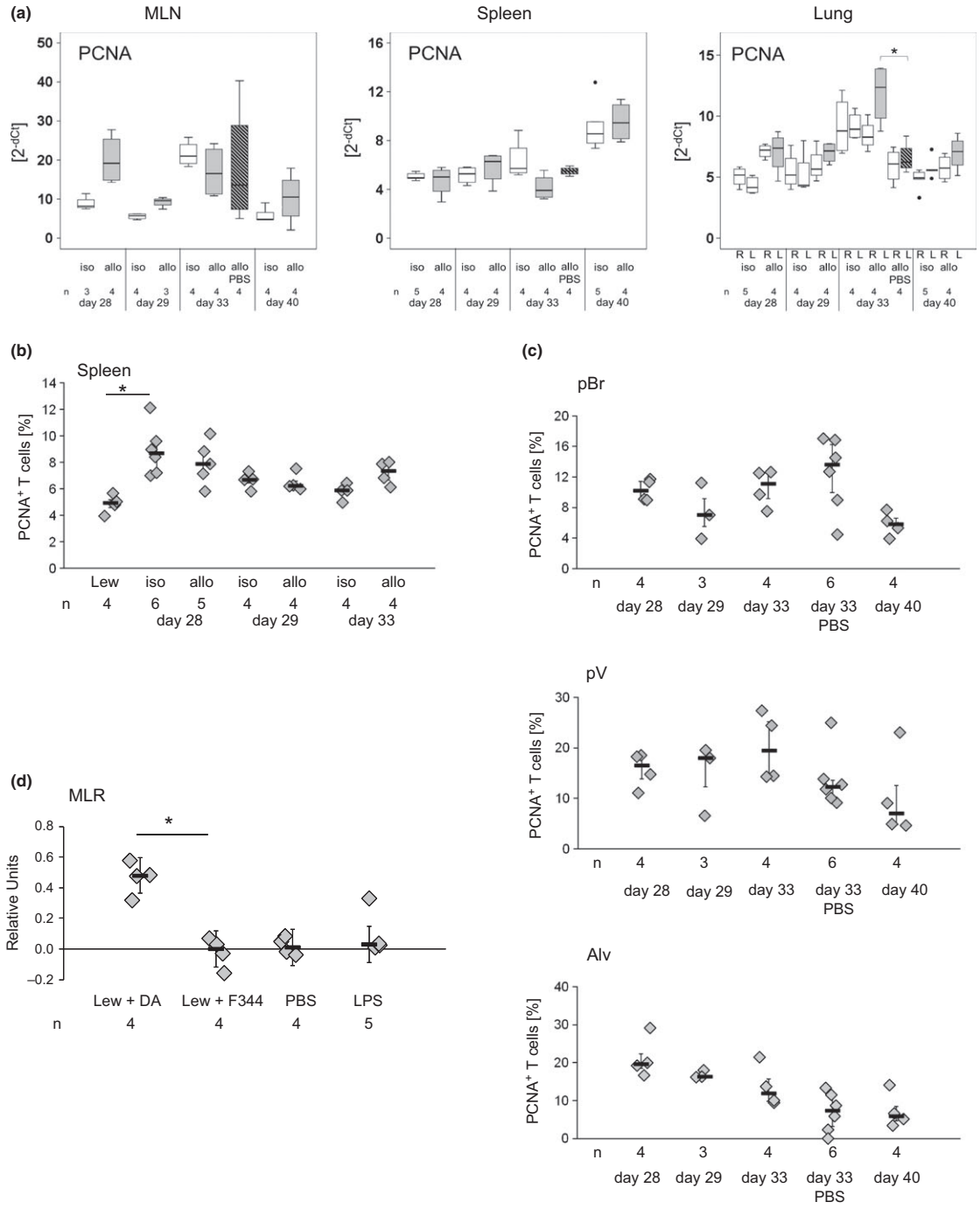

Figure 4 Cell proliferation. Mediastinal lymph nodes (MLN), spleens, right native lungs (R), and left transplants (L) of isograft (iso) and allograft (allo) recipients were investigated on days 28, 29, 33, and 40 post-transplantation. On day 28, experimental animals were sacrificed before application of lipopolysaccharide (LPS), and all other allograft recipients were treated intratracheally with LPS or vehicle (PBS) on day 28. (a) Proliferating cell nuclear antigen (PCNA) mRNA expression was analyzed by real-time RT-PCR; circles are indicating values beyond $\pm$ 3 $\times$ standard deviation. (b, c) Immunohistochemical double-staining for PCNA and α/β T-cell receptor was performed on histological sections of spleens from untreated Lewis (LEW) rats and graft recipients as well as on sections of lung allografts. (b) The percentage of double-positive cells was determined in T-cell areas of the spleen. (c) In lungs, areas surrounding the main bronchus (pBr), small blood vessels (pV), and the alveolar region were evaluated separately. (d) Mixed lymphocyte reactions (MLR) were performed on day 33 post-transplantation with mononuclear blood leukocytes from allograft recipients treated with PBS or LPS. In addition, naïve Lewis (LEW) leukocytes were incubated stimulator cells from fully allogeneic Dark Agouti (DA) rats or Fischer 344 (F344) rats; *$P \leq 0.05$, Kruskal–Wallis test followed by Mann–Whitney *U*-test.

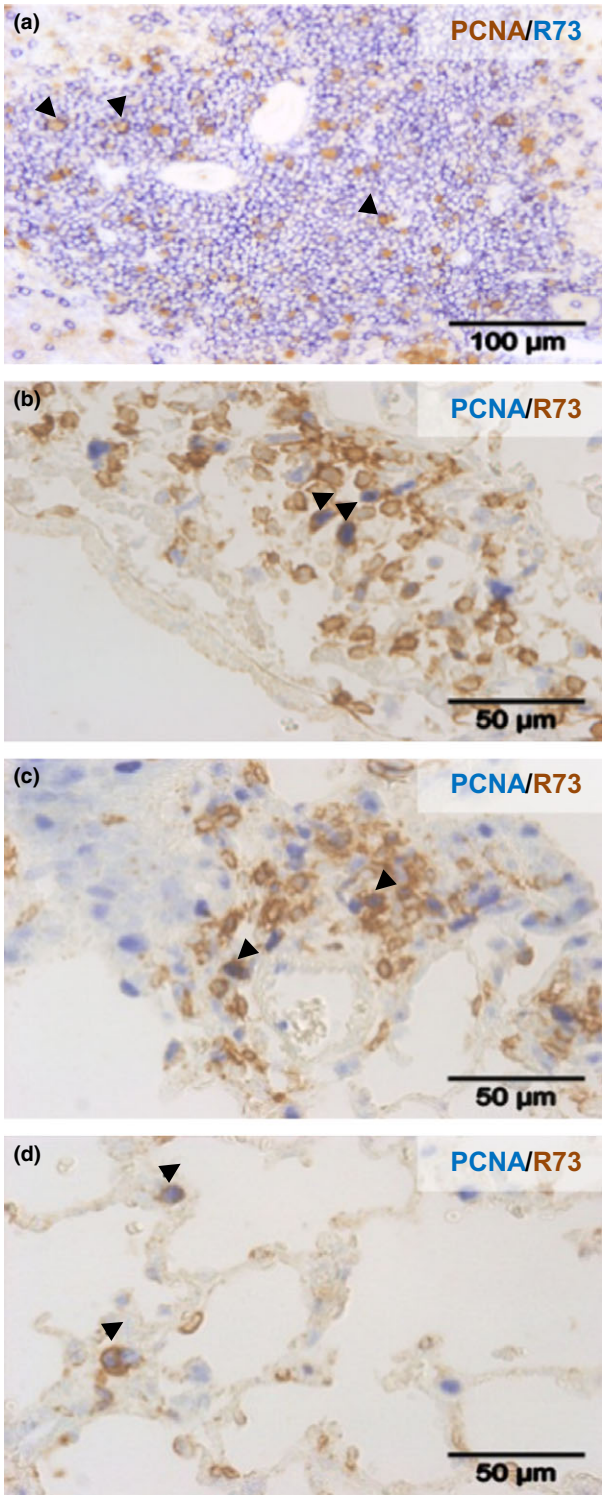

Figure 5 Immunohistochemical double-staining for PCNA and α/β T-cell receptor. Staining was performed on histological sections of (a) spleens from graft recipients as well as (b–d) on sections of lung allografts (n = 4, each). As an example, sections from allograft recipients sacrificed on day 28 before application of lipopolysaccharide are depicted. Arrows are pointing to PCNA-positive T cells. (b) Main bronchus, (c) perivascular region, (d) alveolar region. Arrows are pointing to PCNA-positive T cells.

compared with isografts and vehicle-treated allografts. IL-12p40 and TNF-α levels were increased in LPS-treated allografts on day 40 post-transplantation (Fig. 7).

Discussion

The interplay between alloimmune-dependent and alloimmune-independent inflammation in the pathogenesis of CLAD is poorly understood. We demonstrate that Th1-like alloimmune reactions dominate locally in experimental lung allografts as well as an increased pulmonary expression of major components of the TLR system. Alloimmune-independent inflammation leads to overshooting responses of innate immunity but surprisingly, neither to local nor to systemic augmentation of alloreactivity.

Allograft infiltration 28 days post-transplantation was consistent with mild to moderate acute rejection, a prototypical Th1-type of immune reaction. Accordingly, allografts overexpressed the transcription factor T-bet and cytokines typical for Th1 cells and, as recently shown, IFN-γ-dependent chemokines [15]. In response to LPS, the T-cell infiltrate did not change dramatically and apart from granzyme B, an effector molecule of cytotoxic T cells, NK cells and macrophages [18], Th1-type immunity was not enhanced. In the same line, clinical studies demonstrated that Th1-type immune reactions early after transplantation and certain genetic variants of IFN-γ predispose to CLAD [13,19,20]. Of note, T-cell polarization toward Th1 cells is seen in allografts but not in spleens and local lymph nodes.

The contribution of Th17 cells to the pathogenesis of CLAD is disputed [2,21–23]. In the rat, the Th17 cell-specific transcription factor RORγt is not identified as an isoform of RORγ (NCBI Gene ID: 368158). Therefore, we could not investigate RORγt expression. In pulmonary allografts, however, IL17F mRNA remained below the threshold of detection and IL-23p19 was not differentially expressed. Hence, Th17 cells do not seem to play a major role during the time points investigated.

Our data on Th2-type of immune reactions are also not fully conclusive as pulmonary expression of GATA-3, the transcription factor determining Th2 cells, is expressed by lung tissue [24]. Changes in pulmonary GATA-3 expression might rather reflect lung damage and regeneration than T-cell polarization. IL-4, however, was expressed at very low levels and only slightly increased in day 40 allografts.

Some authors claimed that a reduced frequency of Treg promotes CLAD in humans [23,25]. This assumption bases on reduced Treg counts in BAL fluid. Foxp3 was, however,

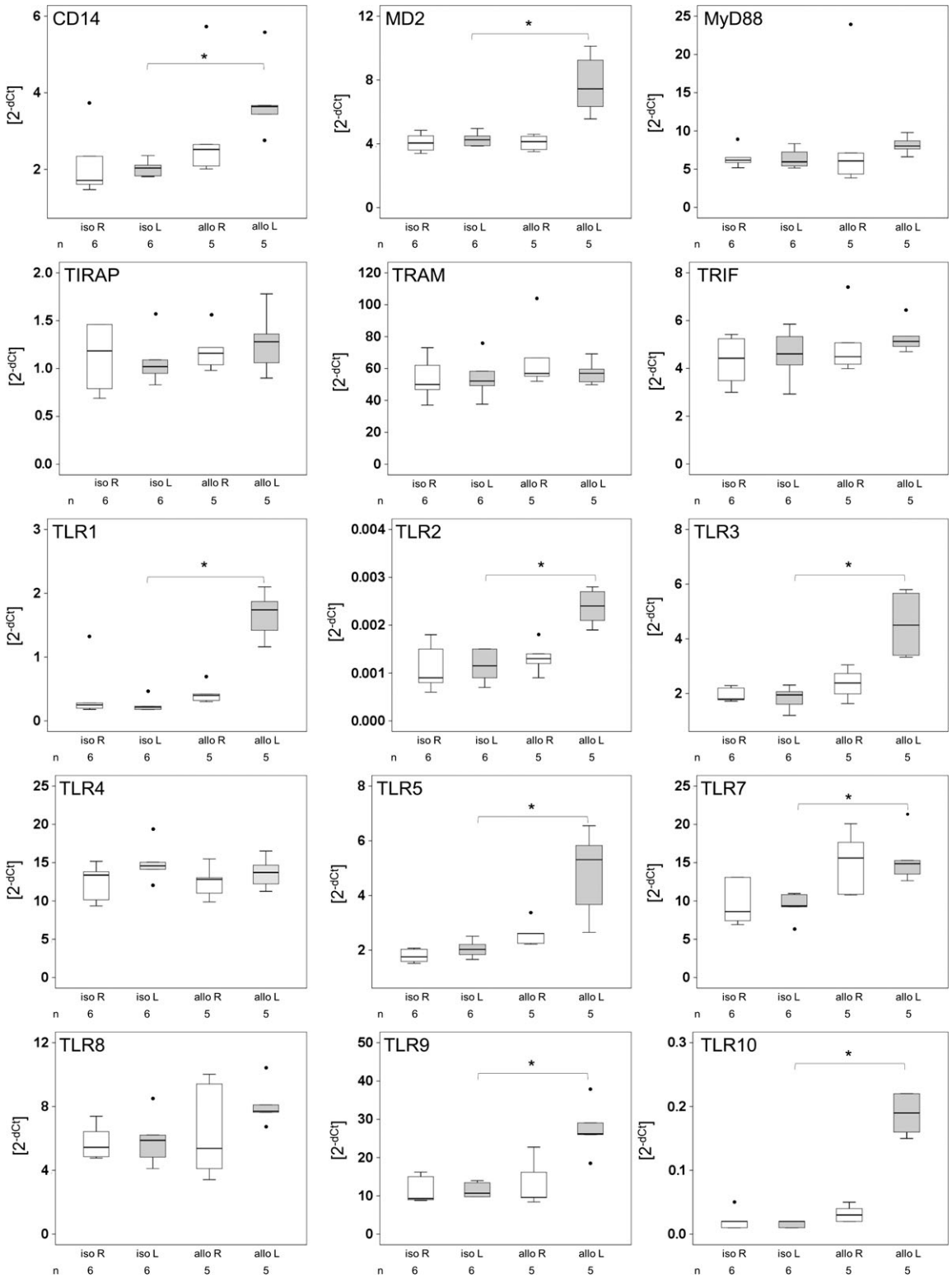

Figure 6 Comparison of mRNA expression of components of the toll-like receptor (TLR) system by isografts and allografts. Real-time RT-PCR was performed on RNA extracted from right (R) native lungs and left (L) transplants of isograft (iso) and allograft (allo) recipients on day 28 post-transplantation before application of LPS. Circles are indicating values beyond $\pm$ 3 $\times$ standard deviation.*$P \leq 0.05$ Kruskal–Wallis test followed by Mann–Whitney *U*-test.

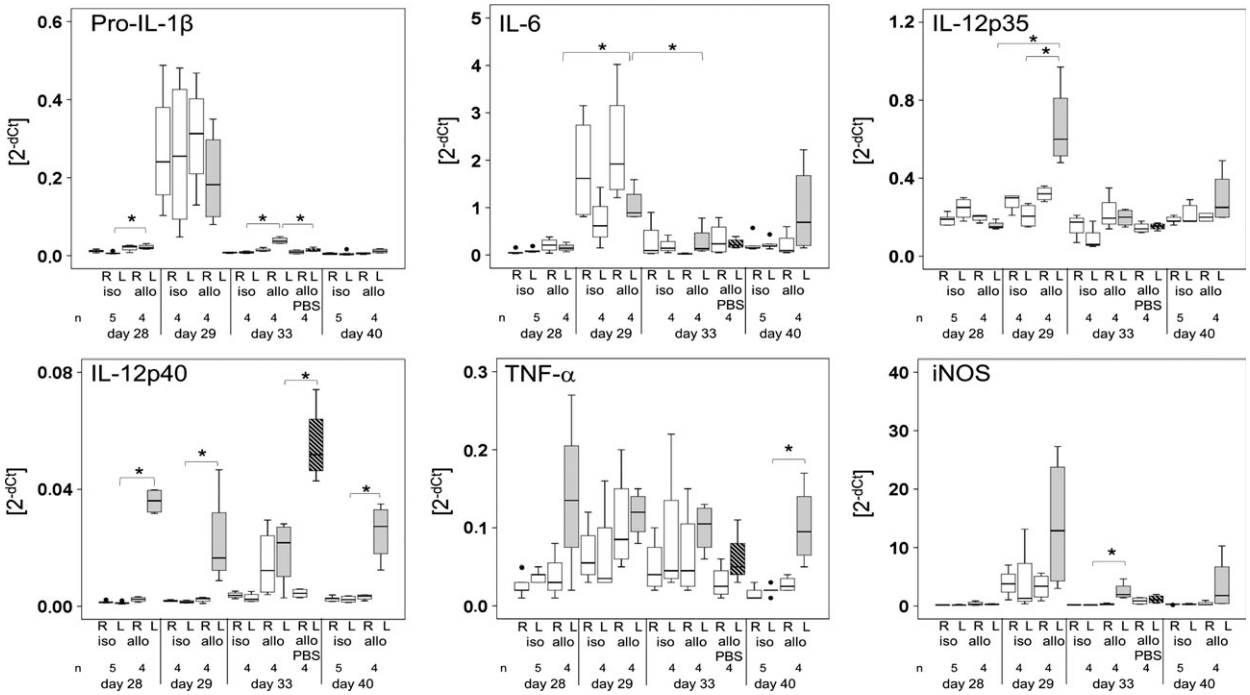

Figure 7 Pulmonary mRNA expression of selected cytokines and iNOS, typically produced by monocytes and macrophages. Real-time RT-PCR was performed on RNA extracted from right (R) native lungs and left (L) transplants of isograft (iso) and allograft (allo) recipients on days 28, 29, 33, and 40 post-transplantation. On day 28, experimental animals were sacrificed before application of LPS. Allografts and right native lungs, treated with vehicle (allo PBS) instead of LPS, were studied on day 33; $*P \leq 0.05$ Mann–Whitney *U*-test. Circles are indicating values beyond $\pm 3 \times$ standard deviation.

more strongly expressed in experimental allografts compared with isografts, and LPS did not change its expression. As graft outcome probably depends on the ratio of Th1 cells and Tregs, the slight induction of Foxp3 is probably not sufficient to prevent CLAD. In line with the moderate induction of Foxp3, however, slightly more IL-10 mRNA was expressed in allografts. Also TGF-β1 mRNA, which possibly contributes to human CLAD [13,22], was induced by LPS.

In the allograft itself but not in lymphatic organs of allograft recipients, T cells are strongly proliferating but neither local nor systemic T-cell alloreactivity is markedly enhanced by LPS. In the same line, blood leukocytes from vehicle- and LPS-treated allograft recipients are irresponsive toward donor antigen in MLR experiments. It is known, however, that Lewis rats sensitized by F344 skin allografts proliferate in MLR [26,27]. Technical problems can be excluded because blood leukocytes from naïve Lewis rats stimulated with PHA-L or with fully allogeneic Dark Agouti cells led to strong MLR responses. Data from other groups indicate that the lung itself can function as a secondary lymphatic organ and that pulmonary allograft rejection may be independent of spleen and lymph nodes [28,29]. These data would favor local immunosuppression of pulmonary transplant patients, which indeed seems to be efficient [30].

The hypothesis that alloimmune responses result in an increased expression of components of the TLR signaling system was supported and our data are compatible with the assumption that experimental allografts are particularly susceptible to ligands for TLR4 and TLR9, because TLR4 and TLR9 are strongly expressed, and co-receptors of TLR4, MD2, and CD14, as well as TLR9 are overexpressed in allografts. Infections leading to human BOS are caused by *Pseudomonas aeruginosa*, respiratory viruses, and cytomegalovirus [31]. Pseudomonadaceae and the F protein of respiratory syncytial virus are detected by TLR4, and TLR9 senses viral DNA from adenoviruses and cytomegaloviruses [32,33]. Together with published genetic evidence from human transplant recipients [13], our data suggest that overexpression of components of the TLR system contributes to the pathogenesis of CLAD.

Next, we investigated infiltration by monocytes/macrophages and mRNA expression of monokines and effector molecules. Allograft infiltration by macrophages resembled infiltration by T cells, but we observed a persistent increase in alveolar macrophages upon LPS instillation. Isografts were only mildly infiltrated on day 28 post-transplantation

but, in sharp contrast to T cells, LPS induced an obvious transient infiltration of macrophages. Clinical data and the allograft-specific overexpression of pro-inflammatory monokines (pro-IL-1β, IL-6, IL-12, TNF-α) and iNOS suggest that destructive M1-type immune responses play a central role in CLAD [22,23,34].

The most important limitations of this study are inherent to the experimental approach: species-specific differences between rat and man, discontinuation of immunosuppression 10 days post-transplantation in the rat versus life-long treatment in patients, application of LPS versus multiple infections and noninfectious noxes in patients—to give just a few examples. Furthermore, the degree of MHC mismatch in our model, that is a localized class I mismatch in the presence of a full class II match, does not reflect the clinical situation, where mismatches generally are more extensive. Nevertheless, the experimental model for CLAD used in this study is among the most telling available [15]. A broad but not exhaustive set of cytokines, transcription factors, effector molecules, and pattern recognition receptors were analyzed on the mRNA level, and no protein and no gene methylation data are given. In addition, we ignore which cell types are involved in their production as well as antibody mediated mechanisms. T-cell reactivation was investigated by several methods in different organs and at different time points, but we are aware that any negative result can be questioned. Finally, future interventional studies are needed to confirm the relevance of our findings.

In despite of all the limitations, our data allow for the following conclusions. Lung transplantation in the F344 to Lewis rat strain combination results in persistent local but not systemic activation of Th1-like immune reactions and in an increased expression of major components of the TLR signaling system. Th1-driven immune reactions seem to set the stage for an exaggerated M1-like innate response to consecutive alloantigen-independent irritants, which seems to contribute to CLAD. Therapeutic strategies aiming at a prevention of CLAD should focus on an early local down-modulation of Th1 cells and of the TLR system. During nonalloimmune pulmonary inflammation, destructive innate immune responses should be targeted rather than augmenting classical T-cell-focused immunosuppression.

Authorship

AA and SA: performed experiments, analyzed data, and wrote the manuscript. GF-M, KP and AZ: performed experiments. MH: planned and performed experiments. WP: planned experiments and wrote the manuscript. VG: planned and performed experiments and wrote the manuscript. All authors read and approved the final manuscript.

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