The Role of Chemokine Receptors in Acute Lung Allograft Rejection

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Abstract

Introduction: Recruitment of inflammatory cells to vascularised allografts is a hallmark of rejection, and paves the way for chronic allograft injury. Chemokines play pivotal roles in the directed movement of leukocytes. Here we define the distribution of chemokine receptors for the most common cell types during human lung allograft rejection as a prerequisite for therapeutic interventions.

Methods: Immunohistochemistry was performed on lung allograft biopsies from 54 patients for the chemokine receptors CCR5, CXCR3, CXCR1 and the Duffy antigen/receptor for chemokines (DARC).

Results: Perivascular infiltrates in acute lung rejection are composed of subsets of mononuclear cells expressing the chemokine receptors CXCR1, CXCR3, CCR5. The number of DARC positive small-sized and capillary vessels was associated with sites of inflammation and increased during episodes of acute lung rejection. DARC expression correlated with an increase in interstitial CCR5 positive T-cells and CXCR1 positive leukocytes. Leukocytic infiltrates in bronchial/bronchiolar rejection express CXCR1 and CXCR3.

Conclusion: This is the first study that demonstrates an induction of the chemokine binding protein DARC at sites of acute human lung allograft rejection. Colocalization with the chemokine receptors CXCR1 and CCR5 may indicate a role for DARC expression during leukocyte adhesion and interstitial infiltration.

Introduction

Lung transplantation has grown into a routine treatment option for terminal lung failure [1]. Lung allograft rejection is a life threatening complication, and bronchiolitis obliterans is thought to be the manifestation of chronic lung allograft rejection [2]. The rejection of a lung allograft is characterized by the recruitment of inflammatory cells, predominantly T cells, to particular compartments in the lung. Mechanisms involved in this recruitment are incompletely understood.

Chemokines are chemotactic cytokines, which share a motif of conserved cysteine residues in their primary structures [3]. Chemokines function via seven transmembrane spanning receptors, which signal upon binding of chemokines of one subgroup, and are therefore labeled accordingly (CCRs, CXCRs etc). Chemokines play pivotal roles in the recruitment of inflammatory cells to sites of injury including vascularized allografts [4, 5]. The cell type recruited depends in part on the pattern of chemokine receptors expressed. CXCR1 binds the ligand CXCL8/IL-8, which is a major chemoattractant for neutrophils [3]. CXCL8/IL-8 protein was increased in allograft biopsies after reperfusion and its level of expression correlated with lung function [6]. In a lung ischemia-reperfusion model the expression of CXC chemokines was associated with neutrophil recruitment [7]. Furthermore CXC chemokines were increased in lavages from allograft recipients with reperfusion injury [7]. CXCR3 and CCR5 are two receptors which are predominantly expressed on activated, T helper type 1 cells. In a rat lung transplantation model of acute

rejection the increased expression of CXCL9/Mig and CXCL10/IP-10 (both ligands for CXCR3) was associated with the recruitment of CXCR3 positive cells to the allograft [8]. Inhibition of CXCL9/Mig reduced the recruitment of CXCR3 positive cells, resulting in lower rejection scores [8]. The chemokines CXCL9/Mig and CCL5/RANTES (ligand of CCR5) were found in alveolar lavages of lung allograft recipients with acute rejection [9, 10].

DARC, the Duffy antigen/receptor for chemokines, is a member of a growing family of chemokine binding proteins which do not signal [11]. These proteins can modify chemokine functions via scavenging, up-take, degradation or transcellular transport [12]. DARC binds proinflammatory CC and CXC chemokines, and shares ligands with CCR5 and CXCR1. This protein has first been described as a blood group antigen, but it is also expressed on endothelial cells [13, 14]. DARC was found to be up-regulated on small vessels during pneumonia and in various forms of renal allograft rejection [15]. Therefore DARC might modulate the chemokine milieu in lung allografts during rejection, but also during infection.

Here we describe the expression of the chemokine receptors CCR5, CXCR3, CXCR1 and the chemokine binding protein DARC on a large series of lung allograft biopsies with acute allograft rejection.

Material and Methods

Included were transbronchial lung allograft biopsies from 54 patients (one biopsy per patient) who underwent surveillance bronchoscopy for clinical suspicion of infection or acute rejection (early type) or in the setting of obliterative bronchiolitis to rule out acute rejection (late type). In the latter group cases without acute rejection were not included. Serial sections were used for histological diagnosis and immunohistochemical analysis. In addition to standard stainings, immunohistochemistry of consecutive sections were performed for the cytokeratin marker CAM5-2 and with an antibody against CD3. These two immunohistochemical stainings were used to semiquantitatively assess T cell infiltration and to grade acute pulmonary rejection according to the revised working formulation of the Lung Rejection Study Group on behalf of the International Society for Heart and Lung Transplantation [16]. Each biopsy was evaluated at low magnification for obvious mononuclear infiltrates. The minimal number of parenchymal venules/arterioles required to evaluate rejection was one with a median range of 1 to 6 vessels per transbronchial biopsy. Acute rejection is graded according to the nature and extent of perivascular leukocyte infiltration (adventitia of small/middle sized venoles and/or arterioles). In minimal acute rejection (A1) the infiltrate consists of CD3 positive predominantly small lymphocytes, in mild (A2) and moderate (A3) rejection more activated larger lymphocytes together with macrophages, eosinophils and neutrophils. Extension of the infiltrate into adjacent alveolar walls defines A3 rejection. Coexistent

airway inflammation manifesting as bronchiolar/ial lymphomononuclear infiltrates was graded B0: none; B1: minimal; B2: mild; B3 moderate; B4 severe; BX: ungradable (due to sampling). The cohort of the present study included cases of rejection grades A0, A2, A3, all of which are defined entities readily identifiable at low magnification in transbronchial biopsies. We excluded minimal rejection A1 and severe rejection A4.The first because it is usually not subject to mandatory therapeutic intervention in our center, the latter because of its rare appearance. The study was performed according to the institutional ethical standards and in accordance with the declaration of Helsinki.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded lung biopsies similar as described previously [17]. In brief, dewaxed, rehydrated tissue sections were incubated in 3% hydrogen peroxide (to neutralize endogenous peroxidases). Antigen retrieval was performed in an autoclave oven using Antigen Retrieval Solution (Vector, Burlingame, CA). The Avidin/Biotin blocking Kit (Vector) was used to block endogenous biotin. Incubation with the primary antibody was followed by incubation with biotinylated secondary antibodies (Vector), and the ABC reagent (Vector). 3'3'Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) was used as a detection system. As negative controls the primary antibody was replaced by isotype matched irrelevant mouse IgGs on tissue sections from human tonsils. The monoclonal antibodies used were: anti-CD3 antibody

(Novocastra, UK); anti-cytokeratins 7/8 (clone CAM 5-2, BD Biosciences Pharmingen); MC5 (gift from M. Mack, University of Regensburg) against CCR5; anti- CXCR3 (clone 1C6,BD Biosciences Pharmingen); anti-CXCR1 (R&D) and 2C3 against DARC (gift from Yves Collins, Paris).

Quantification

The number of positive cells in the perivascular area of small to middle sized arteries and veins (between 1 and 7 middle-sized blood vessels per biopsy) was expressed as percentage of the overall perivascular infiltrates. Each specimen was also scored for the presence or absence of DARC immunostaining on alveolar capillaries (0=absent, 1=focal, at least one area per biopsy, 2= more than 2 separate foci), on medium-sized vessels and semiquantitatively for the presence of interstitial CCR5 and CXCR1 positive cells as follows: no infiltrate, 0; mild 1; moderate 2, and severe 3. All scores are presented as a mean +/- standard error of the mean (SEM). Means were compared with the non-parametric Kruskal-Wallis Test. A $p<0,05$ was considered to be statistically significant.

Results

Demographics and Clinicopathological Data

The study group comprised 37 males and 17 females ranging in age from 15 to 68 years (median age at the time of lung transplantation, 49 years). The median time between transplantation and allograft biopsy was 309 days (range, 8 – 1520 days). All patients received basal immunosuppression consisting of cyclosporine, mycophenolate mofetil

(MMF) and steroids. In the study we included 24 specimens with mild acute rejection (A2), 7 with severe acute rejection (A3), and 23 biopsies showed no evidence of acute rejection (A0). To keep a stringent separation of entities we choose biopsies with no evidence of chronic bronchiolar and/or chronic vascular rejection. Biopsies with minimal rejection (A1) were also not included.

Lymphocytic bronchitis/bronchiolitis recognized as a possible harbinger of bronchiolitis obliterans [16] could be assessed in 28 out of 37 biopsies and graded B1-B3 (see table 1). The presence of concomitant acute and/or chronic infection was also noted in 24 out of 31 biopsies graded A2/A3 and 11 out of 23 graded A0. Hemosiderosis was seen in 6 out of 24 A2/A3 and 3 out of the A0 graded biopsies.

Upregulation of DARC expression on capillary vessels in allograft rejection and inflammation

The control group consisted of 23 patients without signs of acute allograft rejection according to the ISHLT criteria. In all examined samples a small number (3 to 7 per biopsy, median number 4) of middle to large sized pre/postcapillary blood vessels (mostly veins) of the alveolar parenchyma exhibited a distinct DARC signal on endothelial cells (Fig. 1A). DARC immunostaining was also readily detectable on vessels of the bronchiolar region. This corresponds to the constitutive pattern described in normal adult lung tissue [15].

Specimens with histological evidence of acute cellular rejection (perivascular lymphocytic infiltrates with/without spilling over into the interstitium) demonstrated an overall increase in the DARC staining of small sized blood vessels in particular of capillaries of the alveolar wall (Fig. 1C, Fig. 2A). This was reflected in the number of foci (2 to 3 per biopsy). At the same time we noted a stronger expression of DARC on the endothelium of middle-sized blood vessels (see table 1 and Fig 2B).

Focally restricted expression of the Duffy antigen was also detected in smaller vessels and capillaries of the alveolar septa in 15 of 23 biopsies without evidence of acute allograft rejection. In the majority of these samples (11/15) concomitant parenchymal inflammation could be observed. The degree, the distribution and the composition of the inflammatory reaction ranged from acute bronchitis to pneumonia with/without signs of organization. This pattern of DARC expression in infection-mediated lung injury reflects the patchy alveolar staining described in suppurative pneumonia [15].

Out of fifteen samples with DARC expression with rejection grade A0, four did not display any signs of infection (histology confirmed by clinical data), but still exhibited foci of DARC antigen–expressing capillaries. A distinguishing feature of the morphology of lung parenchyma of three of them was focal or diffuse hemosiderosis (Fig 1B). Investigation into the case history of these three patients revealed a previous episode of acute rejection (3 weeks to 6 months prior to actual biopsy).

When comparing the mean scores of DARC-positivity of capillaries in specimens without (A0) and with rejection (A2/A3) and correlating them to the presence of signs of inflammation (most likely caused by infection) we noted that although the increase in the number of DARC positive capillaries in rejection is notable, it is more significant still when infection is added to rejection (Fig. 2A). Inflammation alone increased the DARC score in a more dramatic way than mild or moderate vascular rejection. This is even more evident when the DARC score of small to middle sized vessels is examined. Here the highest DARC scores were found in samples with both rejection and inflammation and the difference between rejection alone and rejection plus infection is even more striking (Fig. 2B). Therefore both types of injury to the allograft contribute to the increase in DARC positive vessels.

Interstitial CCR5 positive T cells are associated with an increased number of DARC-positive capillaries in acute lung rejection

CCR5 positive cells formed a significant part of the infiltrating cells in the majority of biopsies with histological evidence of acute rejection. Many of them (22 out of 24) were clustered in areas of the alveolar walls, where DARC-expressing capillaries could also be observed on consecutive sections (Fig. 1D). In contrast only 6 out of 23 biopsies without evidence of acute cellular rejection showed a correlation between DARC positive capillaries and interstitial CCR5 positive leukocytes (presence of interstitial CCR5 positive

cells in at least one area with prominent DARC-positive capillaries). In those samples either inflammation or hemosiderosis were detected.

Interstitial CXCR1 positive leukocytes are associated with increased numbers of DARC-positive capillaries in lung biopsies with evidence of inflammation

Twenty-four out of 31 specimens with grade A2 and A3 rejection and 11 out of 23 biopsies without histological evidence of rejection presented signs of infection. CXCR1 positive granulocytes, the hallmark of acute inflammation where present in all specimens with acute bronchitis. Distinct focal infiltration of alveolar parenchyma by CXCR1 positive granulocytes could be seen in different stages of infection-triggered lung injury, as well as in incipient/resolving pneumonia (n=11/54). In these biopsies (6 with rejection grade A2+A3, 5 with rejection grade A0) the presence of interstitial CXCR1 expressing leukocytes was observed in at least one area with prominent DARC-positive capillaries per biopsy.

Perivascular mononuclear cells express CXCR3, CCR5 and CXCR1 in acute lung rejection

Cuffing of blood vessels by activated, CD3 positive T-cells is the hallmark of acute pulmonary rejection. We routinely stain biopsies with antibodies against CD3 in order to assess (semiquantitatively) the density of lymphocytes in the infiltrate and grade acute

rejection. This is particularly helpful in biopsies containing only few blood vessels. In higher degrees of rejection the presence of macrophages, neutrophils and eosinophils within the perivascular infiltrate is more pronounced (A3>A2>A1).

CD3 staining correlated with the amount of perivascular lymphocytes seen by light microscopy: 54,7% and 38,3% of all perivascular cells (median percentage) expressed CD3 in A2 and A3 acute rejection respectively. A0 rejection is defined by lack of perivascular lymphocytes; in these areas no CD3 expressing cells can be observed. In the studied biopsies T-cells in the infiltrate strongly expressed CXCR3: 44,2 % of all perivascular cells were CXCR3 positive in A3 and 50,2 % were positive in A2 acute rejection (median percentage).

The number of CCR5 expressing mononuclear cells in the neighborhood of blood vessels was considerably smaller: 21,4 % of CCR5 positive cells were observed in infiltrates of A3 acute rejection, 27,9% in A2 acute rejection (median percentages) (see also Fig. 3E). CXCR1 expressing perivascular neutrophils demonstrated the highest variability in numbers. Their mean percentage in perivascular infiltrates in A3 and A2 rejections varied between 0 and 40% with a mean of 8,1% in A2 and 7,1% in A3.

Bronchial/bronchiolar infiltrates contain CXCR3 and CXCR1 positive leukocytes

Bronchial/iolar infiltrates contained a mixed population of lymphocytes and macrophages in varying density representing the airway part of acute rejection. The density of these

infiltrates is reflected in the grading. The immunostaining profile seen in B1 and B2 acute bronchial/iolar rejection shows cuffing of bronchioles and/or bronchi with intact epithelium by CXCR3 positive leukocytes. Higher grades of bronchial/iolar rejection (in our collective B2 –B3) had a varying number of infiltrating CXCR3 positive activated lymphocytes in the epithelium. Additional presence of CXCR1 positive neutrophils in the denser band-like infiltrate of the submucosa was also often observed. B2 and B3 grading was ascribed to rejection in cases where infection could be clinically and histomorphologically excluded. Prominence of CXCR1 positive neutrophils either in exsudate and/or in the airway epithelium was consistent with (bacterial) infection concomitant with acute rejection (Fig. 4A, B).

Discussion

This study includes the largest cohort of human lung allograft biopsies studied for the expression of three chemokine receptors and the chemokine binding protein DARC.

DARC is a seven transmembrane protein which binds a variety of proinflammatory CC and CXC chemokines [18, 19]. It has first been identified as a red blood group antigen involved in transfusion reactions and has later been shown to be a coreceptor for malaria parasites [13, 20-22]. Besides red blood cells DARC is expressed on endothelial cells, notably on high endothelial cells, the site of lymphocyte recirculation [23].

In normal lung tissue constitutive expression of DARC was described on the endothelium of middle-sized parenchymal and bronchiolar vessels [15]. In acute suppurative pneumonia DARC antigen expression is newly present on alveolar septa. These vascular beds with low shear stress are thought to be the sites of leukocyte extravasation. This is partially due to an environment favorable for interactions between chemokines and their receptors. An increased number DARC positive interstitial peritubular capillaries has been demonstrated in renal allograft rejection [24, 25]. In the kidney antibody and cell mediated rejection both resulted in an increased number of DARC positive peritubular capillaries [25]. Interestingly, the combination of both forms of allograft injury resulted in an additional effect on the number of DARC positive peritubular capillaries.

This study provides the first evidence that endothelial expression of DARC is upregulated in acute lung allograft rejection. Samples with no or minimal evidence of rejection presented with a focally restricted and infrequent expression of DARC in smaller blood vessels and capillaries of the alveolar septa. This is consistent with the pattern described in normal parenchyma. In acute mild and moderate lung allograft rejection (A2, A3) we could observe an increase in DARC expression on the endothelium of all sizes of blood vessels, most prominently at the small/capillary level. Additionally, inflammation (most likely related to infection) increased the number of DARC positive vessels. The combination of rejection and infection resulted in the highest numbers of DARC positive

capillaries, and only in combination resulted in an increased number of medium sized DARC positive vessels.

This pattern of DARC positive capillaries mirrored the one in the renal interstitium in renal allograft rejection except that it seemed to persist after acute rejection has resolved [24, 25]. In an attempt to keep the morphological entities as stringent as possible, all of the examined biopsies (one per patient) where performed at time points when no suspicion or clinical evidence of chronic airway rejection (obliterative bronchiolitis) was present. Prominent foci of DARC expressing capillaries could be found in A0 lung samples rich in hemosiderosin-carrying macrophages. Although hemosiderosis is considered a non-specific finding indicating past endothelial injury and blood extravasation, it is nevertheless a characteristic residual feature of rejection episodes in lung allografts. As endothelial DARC is involved both in transcytosis of chemokines into the bloodstream as well as induction of leukocyte emigration, persisting DARC induction in these samples could be linked to a role in restoration of chemokine homeostasis [12].

Upregulation of DARC in episodes of mild or moderate lung allograft rejection was associated with prominent recruitment of CCR5 positive cells at the site of rejection and in the interstitium. Previous studies have revealed an upregulation of CCL5/RANTES in solid organ allografts. A significant increase of CCL5/RANTES protein was demonstrated in bronchoalveolar lavages from patients with lung rejection with or without concomittant CMV infection [26-28]. These studies were also able to demonstrate that

pulmonary CMV associated increase of BALF CC-chemokines can predict the development of obliterative bronchiolitis. In rat lung allografts the expression of CCL5/RANTES was associated with recruitment of cells expressing CCR1 and CCR5, and treatment with antibodies against CCL5/RANTES significantly reduced inflammatory cell influx [10]. The functional role of DARC in vascularized allografts is still under intense investigation. A differential effect is postulated for DARC on red blood cells (most likely scavanging chemokines), whereas endothelial DARC might be involved in the transendothelial transport and apical presentation [12]. It can therefore be hypothesized that with the increased number of DARC positive vessels (in acute rejection with and without infection) the pro-inflammatory role of DARC might become more important. This would explain the accumulation of CCR5 positive cells in these areas.

CXCR3 the shared receptor of the chemoattractants CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC is a prominent mediator of CTL responses not only in allograft rejection, but also in other TH1 related disorders. In our population, episodes of grade A2 or greater lung allograft rejection were associated with striking expression of CXCR3 and lesser of CCR5 chemokine receptors by perivascular lymphocytes. These findings extend the results of a previous study where immunohistochemical analysis of selected cases of human lung allograft rejection demonstrated CXCR3 positive T-cell infiltrates in perivascular and interstitial areas [29].

In the present study we could observe a recruitment of CXCR1 positive neutrophils both in severe forms of lung allograft rejection and in acute (bacterial) infection. Activated neutrophils have been implicated in the pathogenesis of tissue injury in early and late events of lung transplantation. They are sequestered after reperfusion, or with episodes of infection and/or rejection and activated by CXCL8/IL-8 and its two receptors CXCR1 and CXCR2 [6, 30]. Several lines of evidence suggested that neutrophils could play a key role in the development of acute cellular graft rejection through direct or indirect mechanisms like TH1 specific polarization of T-lymphocytes [31]. In our study population CXCR1 positive neutrophils were seen at sites of higher grades of acute rejection (A2-3, A3), where these are components of the perivascular infiltrates. We could also observe a varying number of CXCR1 positive neutrophils in segments of the bronchial tree. Neutrophils infiltrating the mucosa and/or submucosa were either a feature of higher grades of acute bronchial/bronchiolar rejection (B2, B3) or an evidence of airway inflammation. A clear distinction of the two events on morphologic grounds alone was only possible when CXCR1 positive neutrophils were seen to cluster and form purulent exsudates. Either cause of bronchial/bronchiolar infiltration is known to create a proinflammatory environment that predisposes to obliterative bronchiolitis, the most important long-term complication after lung transplantation [32, 33].

In summary this is the first study that demonstrates a prominent induction of the chemokine binding protein DARC at the site of CCR5-positive T- cell infiltration in

human lung allografts. It was associated with a recruitment of CXCR1 positive neutrophils not only in acute infection but also in severe forms of lung allograft rejection. CXCR3 was the receptor most commonly expressed by lymphocytes during lung allograft rejection. The results of this study suggest that endothelial DARC and chemokine receptors on leukocytes together with their major ligands CCL5/RANTES and CXCL8/IL-8 may enhance recruitment to sites of rejection and promote cell-mediated lung injury. The studied chemokine receptors could be viewed as potential therapeutic targets and further studies need to address the potential of antagonists (single and in combination) in lung allograft models.

Table 1: Quantification of the immunohistochemical evaluation (column 2-4 illustrate the fraction of positive cells, column 5-8 illustrate scores, mean ±sem; *p<0,05; **p<0,01, ***p<0,001 versus A0, part 1)

Rej.	CXCR3 perivascular	CCR5 perivascular	CXCR1 perivascular	DARC capill.	DARC middle	CCR5 Int.	CXCR1 int.	Infection
A3B3	0,4	0,2	0,2	2	2	3	1	yes
A3B2	0,2	0,2	0,0	1	2	1	0	no
A3B2	0,6	0,3	0,1	2	3	1	1	yes
A3B2	0,4	0,0	0,0	1	2	0	2	yes
A3B2	0,5	0,3	0,0	1	2	1	3	yes
A3B2	0,4	0,1	0,2	1	1	1	1	yes
A3BX	0,6	0,4	0,1	3	2	2	1	yes
A3 n=7	*0,4 (±0,05)****	*0,2 (±0,05)***	*0,08 (±0,03)**	*1,6 (±0,3)*	*2,0 (±0,2)*	*1,3 (±0,36)**	*1,3 (±0,4)*	
A2B3	0,4	0,3	0,0	1	2	1	1	yes
A2B2	0,6	0,2	0,0	2	2	1	1	yes
A2B2	0,7	0,3	0,2	1	2	1	1	yes
A2B2	0,3	0,3	0,0	3	3	2	2	yes
A2B2	0,7	0,4	0,0	2	2	2	1	yes
A2B2	0,6	0,5	0,3	1	2	2	2	yes
A2B2	0,7	0,4	0,0	1	3	1	2	yes
A2B1	0,4	0,5	0,0	1	3	1	0	yes
A2B1	0,7	0,3	0,0	1	3	1	1	yes
A2B1	n.d.	0,0	0,0	0	1	1	1	no
A2B1	0,4	0,0	0,0	1	2	0	0	no
A2B1	0,6	0,5	0,2	3	3	2	0	no
A2B0	0,5	0,3	0,3	1	2	0	0	yes
A2BX	0,4	0,5	0,1	3	2	2	1	yes
A2BX	0,6	0,1	0,0	0	2	0	1	yes
A2BX	0,4	0,4	0,4	2	2	2	1	yes
A2BX	0,7	0,1	0,1	1	3	1	1	yes
A2BX	0,5	0,1	0,1	0	1	0	1	no
A2BX	0,5	0,4	0,0	1	1	2	0	yes
A2BX	0,5	0,1	0,2	0	1	1	2	no
A2BX	0,3	0,0	0,0	1	2	1	0	yes
A2BX	0,3	0,1	0,0	1	1	2	1	yes
A2BX	0,3	0,4	0,0	0	1	0	0	no
A2BX	0,7	0,5	0,1	3	3	2	1	yes
A2 n=24	*0,5 (±0,03)****	*0,3 (±0,03)****	*0,08 (±0,03)***	*1,3 (±0,2)*	*2,0 (±0,15)***	*1,2 (±0,16)***	*0,9 (±0,14)*	

Table 1: Quantification of the immunohistochemical evaluation (column 2-4 illustrate the fraction of positive cells, column 5-8 illustrate scores, mean ±sem; part 2)

Rej.	CXCR3 perivascular	CCR5 perivascular	CXCR1 perivascular	DARC capill.	DARC middle	CCR5 Int.	CXCR1 int.	Infection
A0B1	0	0	0	1	2	0	1	yes
A0B1	0	0	0	1	1	0	1	yes
A0B1	0	0	0	1	1	1	1	yes
A0B1	0	0	0	0	1	0	1	no
A0B1	0	0	0	0	1	0	0	no
A0B1	0	0	0	1	2	1	2	yes
A0B1	0	0	0	0	1	0	1	no
A0B1	0	0	0	1	2	1	2	yes
A0B1	0	0	0	1	1	0	1	no
A0B1	0	0	0	0	1	1	1	yes
A0B0	0	0	0	2	2	0	2	yes
A0B0	0	0	0	0	1	0	1	no
A0B0	0	0	0	2	2	1	0	no
A0B0	0	0	0	2	2	1	2	yes
A0B0	0	0	0	0	1	0	1	no
A0B0	0	0	0	0	1	0	1	no
A0B0	0	0	0	1	2	1	1	no
A0B0	0	0	0	1	2	0	1	no
A0BX	0	0	0	3	1	1	1	yes
A0BX	0	0	0	0	1	0	0	no
A0BX	0	0	0	1	1	0	1	no
A0BX	0	0	0	1	2	0	1	yes
A0BX	0	0	0	1	1	1	2	yes
A0 *n=23*	*0 (±0)*	*0 (±0)*	*0 (±0)*	*0,9 (±0,3)*	*1,4(±0,1)*	*0,4(±0,1)*	*1,1 (±0,12)*	

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Figure legends

Figure 1: Immunohistochemical detection of DARC in parenchymal capillary vessels and of interstitial CCR5 positive cells of lung allografts
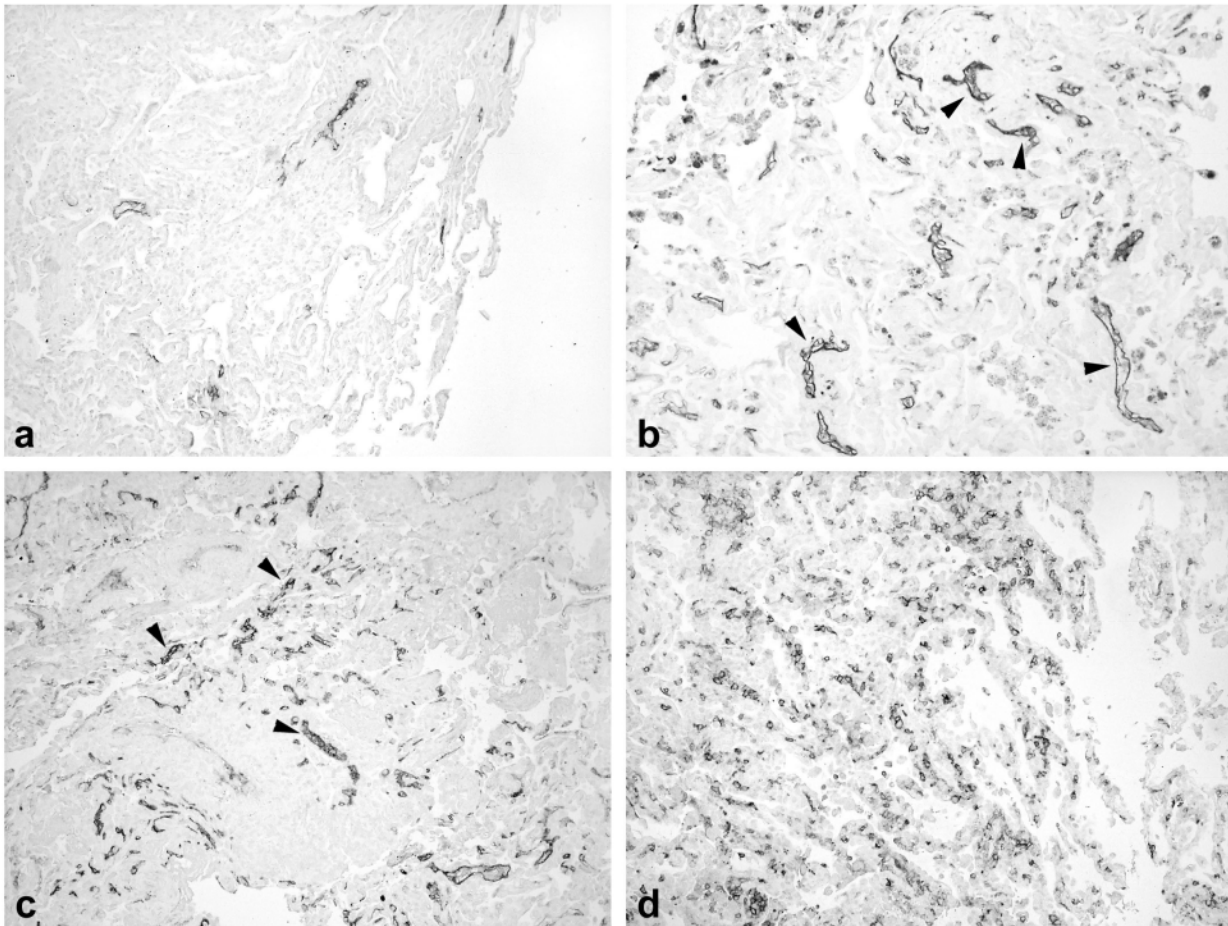
Immunohistochemistry of transbronchial lung biopsies stained with monoclonal antibodies against DARC (**A-C,** x 400) and CCR5 (**D**, x 400). **A**: normal parenchyma of transplanted lung with no evidence of rejection or infection shows moderate DARC labelling of endothelial cells of middle sized (pre/postcapillary) blood vessels, but absent staining of capillaries of the alveolar septa. **B**: transbronchial biopsy negative for acute

rejection with features of infection: Distinct DARC labelling of alveolar capillaries and blood vessels of different sizes can be observed (arrowheads); the alveolar spaces are filled with hemosiderin-ladden macrophages. **C**: Focus with many DARC positive capillaries and small-sized blood vessels (arrowheads) in a transbronchial biopsy from a patient with A3 acute rejection. **D**: In a parallel section of the same biopsy CCR5 positive interstitial leukocytes colocalized with DARC-positive capillaries.

Figure 2: Mean DARC scores of capillaries (A) and middle-sized vessels (B) according to the presence of acute allograft rejection (AO vs. A2/3) and the presence of inflammation (no inflamm vs inflamm). The **error bars illustrate SEM. (A **p<0,01 A2/3 inflamm. vs A0 no inflamm.; B ***p<0,001 A2/3 inflamm. vs A0 no inflamm.)**

A   ******
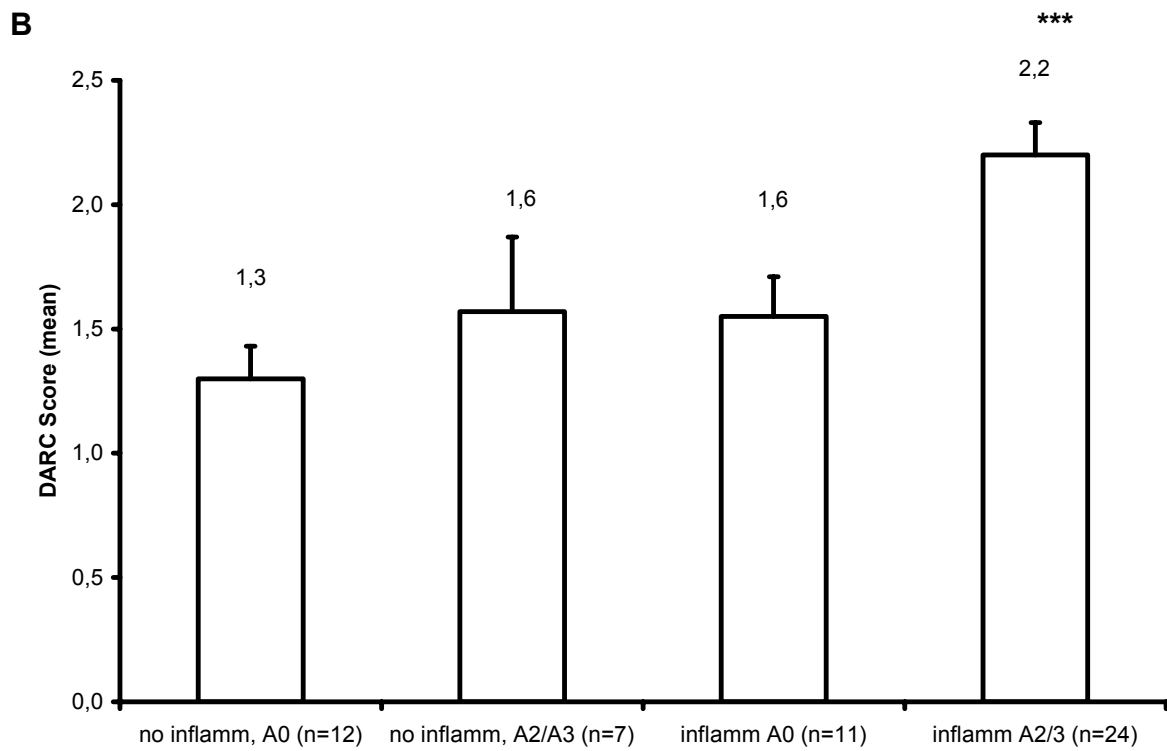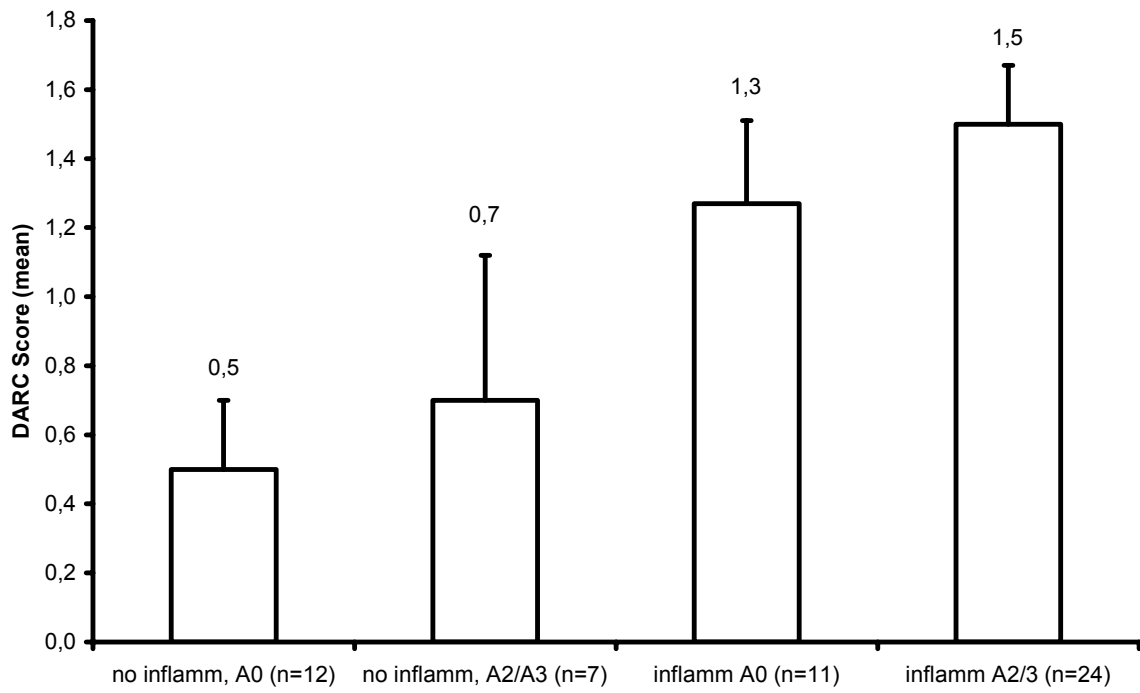
B

Figure 3: Distribution of CXCR3 and CCR5 positive cells in lung allografts

Transbronchial biopsies of lung allografts are illustrated in **A-C**: A central middle-sized blood vessel with no evidence of perivascular infiltrate corresponding to A0 rejection and in **D-F**: A dense perivascular infiltrate with expansion of the adventitial space, but no involvement of alveolar walls consistent with grade A2 rejection. **A** and **D**: HE staining, x 400. **B** and **E**: Immunohistochemistry for CCR5 positive mononuclear cells. In **B** no perivascular CCR5 positive cells are observed. The CCR5 positive fraction of the perivascular infiltrate (arrowhead) in **D** shows mainly large cells with a weak to moderated membrane staining x 400. **C** and **F**: Immunohistochemistry for CXCR3 expression by lung T cells. **F:** Strongly stained CXCR3 positive lymphocytes (arrowhead) can be demonstrated in the perivascular space in A2 rejection; **C**: they are absent in vessel walls with no infiltrate. Only scant CXCR3 positive small lymphocytes can be seen in the alveolar septa of biopsies negative for acute rejection x 400.
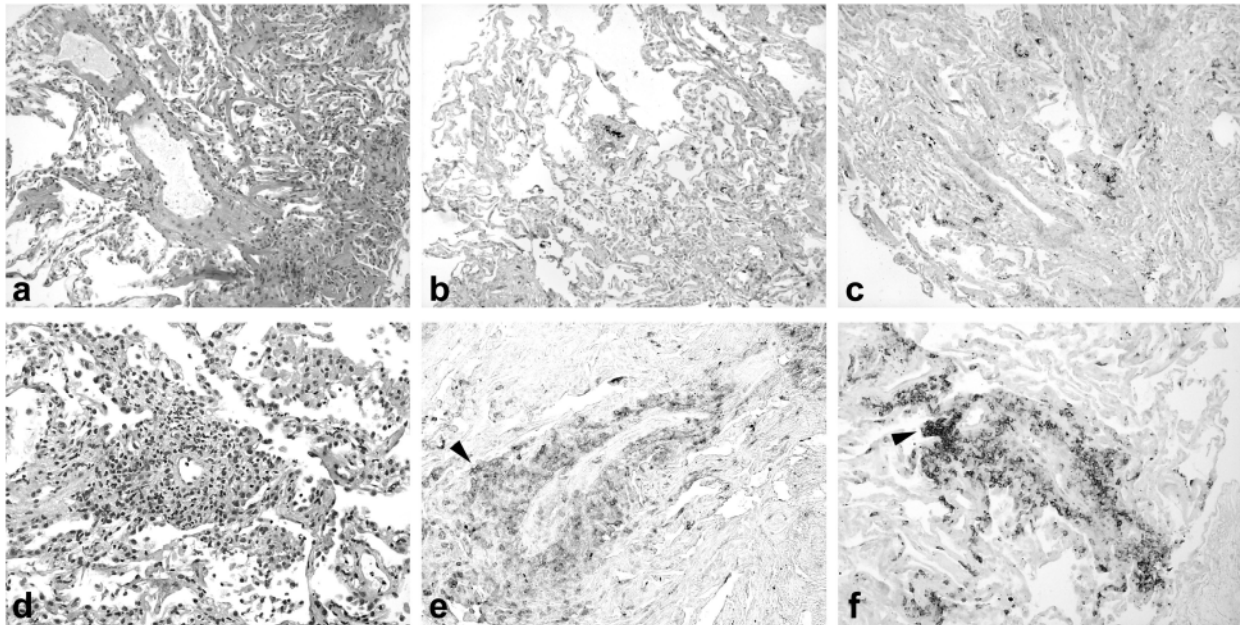
Figure 4: CXCR1-expressing leukocytes in lung allografts with evidence of infection. Immunohistochemistry for CXCR1. **A**: High power view of a bronchiolar wall with intact columnar epithelium infiltrated by polymorphs expressing CXCR1 (arrowhead), x 800. **B**: in fibrinopurulent exsudate containing many CXCR1 expressing polymorphs x 200.