The Killer Immunoglobulin-Like Receptor *(KIR)* Group A Haplotype is Associated With Bronchiolitis Obliterans Syndrome After Lung Transplantation

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Background: The development of bronchiolitis obliterans syndrome (BOS) after lung transplantation is associated with viral infections. Natural killer (NK) cells are involved in the lysis of viral infected cells, and their activation is largely controlled by activating and inhibitory killer immunoglobulin-like receptors (KIRs). We hypothesized that KIR ligand incompatibility and recipients' individual KIRs could influence the development of BOS and the incidence of cytomegalovirus reactivation after lung transplantation.

Methods: The *KIR* gene contents were determined in 48 patients who received a lung transplant, and human leukocyte antigen (HLA)-Cw and HLA-Bw4 typing was performed on their respective donors.

Results: BOS developed in 7 patients and cytomegalovirus reactivation occurred in 16. BOS developed in 5 of 19 patients homozygous for *KIR* haplotype A compared with 2 of 27 patients with *KIR* haplotype AB and B (homozygous; $p = 0.03$; log-rank test). In none of the patients with BOS was the activating *KIR2DS5* gene detected, whereas it was present in 35% of patients without BOS ($p = 0.04$; log-rank test). No correlation was found between *KIR* gene content and cytomegalovirus reactivation.

Conclusion: Our results suggest that the lack of activating KIRs may play an important role in the development of BOS but not in the control of cytomegalovirus reactivation after lung transplantation. J Heart Lung Transplant 2008;27:995–1001. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

Lung transplantation is an ultimate therapy in patients with end-stage lung diseases who have no other therapeutic options left. Survival rates have improved over the years but are still restricted by the development of bronchiolitis obliterans syndrome (BOS).[1] At present the exact mechanism of this chronic allograft rejection is not clear, but it has become evident that both innate and adaptive immune responses play an important role in the onset and pathogenesis of BOS.[2–5]

Natural killer (NK) cells are one of the main cellular components of the innate immune system and are important in the lysis of virally infected cells and tumors. The NK cells express a multitude of intracellular and extracellular proteins in order to recognize and eliminate target cells. Recognition of target cells occurs through integration of different signals derived from cell-surface receptors, including major histocompatibility complex (MHC) class-I receptors (killer immunoglobulin-like receptors [KIRs], CD94/NKG2A, leukocyte immunoglobulin-like receptor, subfamily B1 [LLRB1]), adhesion molecules (CD2, CD11b/CD18, CD11c/CD18, CD31, CD96, CD49A/CD29, CD226), and costimulatory receptors (CD244 and NKG2D).[6–8] Elimination of target cells expressing inadequate or no MHC class I molecules on their cell surface is regulated by opposite signals delivered by inhibitory and activating KIRs, of which the expression may be regulated depending on the type of NK-cell stimulus.[9]

KIRs are named according their structural and functional characteristics: the number of extracellular Ig domains (2D or 3D) and the intracellular tail (short [S] or long [L]). Short tails correlate with activating and long tails with inhibitory receptors. Although ligands for activating KIRs are still unknown, the inhibitory KIRs recognize epitopes on human leukocyte antigen (HLA)-C and HLA-Bw4. Ligands for *KIR2DL1* are HLA-C with a lysine residue at position 80 (group 2), for

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KIR2DL2 and *KIR2DL3*, HLA-C with an asparagine residue at position 80 (group 1); and for *KIR3DL1*, HLA-Bw4.

The *KIR* genes are located on chromosome 19q13.4 within the leukocyte receptor complex (LRC). Based on population studies, multiple *KIR* haplotypes have been defined, differing in *KIR* gene content and associated functional properties.[10–12] The *KIR* haplotype A contains 6 inhibitory and 1 activating *KIR* genes, and expression of this haplotype will be associated with functional down-regulation of NK cell activity.[13] The variety of *KIR* haplotypes B all contain a mixture of functional activating and inhibiting KIRs.[11,14]

In previous reports on hematopoietic stem cell transplantation, significant associations were found between *KIR* genes (the presence of specific KIRs, the number of KIRs, mismatching of inhibitory KIRs with their respective HLA ligands) and clinical key parameters, including chronic graft-versus-host disease, which can manifest in the lungs as bronchiolitis obliterans and cytomegalovirus (CMV) reactivation.[15–17]

Recent data demonstrated that in patients with BOS, the number of peripheral blood NK cells is decreased but an increased number are present in transplanted lungs compared with stable patients. These data suggest that NK cells are recruited from blood to the lungs during the process of chronic rejection.[18] However, the role of KIRs responsible for NK cell function after lung transplantation is not elucidated yet. In the present study, we examined patients' *KIR* gene content and hypothesized that KIR ligand incompatibility or *KIR* haplotypes could be associated with the development of BOS or CMV reactivation, or both.

PATIENTS AND METHODS

Subjects

The study included 48 patients who underwent lung transplantation at the Heart Lung Center in Utrecht between October 2001 and March 2006 and survived more than 3 months. To eliminate post-operative complications, we excluded patients who died during the period from transplantation until 3 months after transplantation. According to the standard International Society of Heart and Lung Transplantation criteria, BOS was defined as a decline of the forced expiratory volume in 1 second from the post-operative baseline at 2 distinctive time-points of more than 20% in the absence of infection or other etiology.[19] Patients were monitored for graft function and CMV infections.

Standard immunosuppressive therapy consisted of basiliximab as induction immunosuppression, tacrolimus, mycophenolate mofetil, and prednisone. Patients at risk for CMV reactivation, which is defined as a CMV seropositive donor or patient, were treated with valganciclovir for at least 6 months after transplantation. The study design was approved by the Medical Ethical Committee, and informed consent was obtained from each patient.

Genotyping

Genomic DNA from patients and donors was isolated from peripheral blood lymphocytes, stored, and retrospectively typed. Complementary DNA from donors was typed for HLA-B and HLA-C by polymerase chain reaction (PCR)-specific priming (SSP; Biotest, Dreieich, Germany). HLA-C and HLA-B were segregated into the epitopes groups HLA-C group 1 (HLA-C Asn 80: HLA-Cw1,3,7,8,13,14 alleles), HLA-C group 2 (HLA-Clys80: HLA-Cw2,4,5,6,12,15,17,18 alleles), and HLA-Bw4.[20] DNA from patients was typed for 6 activating *KIR* genes (*2DS1*, *2DS2, 2DS3, 2DS4, 2DS5,* and *3DS1*) and for 8 inhibitory *KIR* genes (*2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2,* and *3DL3*) by LUMINEX SSO (One Lambda Inc, Canoga Park, CA). A missing KIR ligand was defined as the presence of patients' *KIR* genes in the absence of their corresponding HLA on the transplanted lungs (ligand). The following *KIR* genes match with their ligands: *2DL1* corresponds with HLA-C group 2, *2DL2/3* with HLA-C group 1, and *3DL1* with HLA-Bw4.

At the moment, there are 42 *KIR* haplotypes detected based on their gene content. All haplotypes contain the framework genes *3DL3, 2DL4,* and *3DL2* and contain either *2DL2* or *2DL3*, but not both.[10–12] Haplotype A contains framework genes and *2DL3*, *2DL1*, *3DL1*, and *2DS4*. Haplotype B is more variable and contains more than one activating *KIR* gene. The presence of *KIR* genes *2DL5, 2DS1, 2DS2, 2DS3,* or *2DS5* reflects haplotypes B.[14] The presence of *KIR* haplotypes A (homozygous), AB, and B (homozygous) in patients were deducted from the type and number of *KIR* genes detected.[10,12]

Latent CMV Reactivation

The CMV serostatus of patients and donors was determined by enzyme-linked immunoassay (ELISA; VIDAS-biomerieux, Marcy L'Etoile, France). We excluded patients with a negative CMV serostatus before transplantation who received lungs from CMV-negative donors. Monitoring after transplantation for CMV reactivation was performed by PCR. We used the PCR (*Taqman*) whereby a serologic reactivation was defined as a successive assay detecting more than 400 copies/ml in serum. After October 2003, we enlarged our input and used 3 ml of serum instead of 1 ml; therefore, our threshold for a successive assay could be lowered to 50 CMV copies/ml instead of 400 copies/ml. In 6 patients, CMV copies were initially measured with a threshold of 400 copies/ml, and CMV reactivation occurred in 4. Patients with a clinical CMV reactivation, defined as more than 1000 copies/ml were treated with

valganciclovir and reducing immune suppression. Duration and intensity of valganciclovir depended on clinical status and viral load.

Statistical Analysis

The associations between patient characteristics and freedom from BOS and CMV reactivation were analyzed with a log-rank test in the Kaplan-Meier curve. The Fisher exact test was used to compare frequencies. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The median follow-up time of the 48 patients studied was 24 months (range, 9 – 60 months). All but 5 patients (all non-BOS) received a bilateral lung transplant. BOS developed in 7 patients. Two patients died during the course of the study, one of which was associated with BOS. The patients with BOS were similar to those without BOS in age, gender, and underlying disease. Baseline characteristics of the overall study group are summarized in Table 1.

Relationship Between KIR-Ligand Matching and Development of BOS

To examine whether the absence or presence of KIR ligands on transplanted lungs was associated with the development of BOS, we typed patients' *KIR* genes and HLA of the donor. *KIR* genotyping of the 48 patients revealed 47 (98%) were positive for *2DL1* and 44 (92%) for *3DL1*. Forty patients (85%) were positive for *KIR2DL3*, and 19 40(%) were positive for *KIR2DL2*. DNA could not be obtained from 2 donors for HLA typing, but 38 donors (83%) who could be analyzed were typed for HLA-C group 1, 32 (70%) for HLA-C group 2, and 36 (75%) were positive for HLA-Bw4.

An analysis of patients' *KIR* genes with the absence of the corresponding HLA ligands on the transplanted lungs found 28 lung transplants (58%) had HLA ligand(s) missing for patients' KIRs. One KIR ligand was missing in 20 (71%) patients, and 2 ligands were missing in 8 (29%). The distribution of patients missing KIRs and HLA ligands is detailed in Table 2. No significant differences in freedom from BOS were found between patients with and without KIR ligands.

Relationship Between Patients' KIRs and Development of BOS

We also examined whether a relation exists between BOS and the number of activating or inhibitory KIRs in

Table 1. Baseline Characteristics of Overall Study Group

Characteristics	All	BOS	Non-BOS
Number of patients	48	7	41
Median age, years (range)	50 (16–64)	56 (24–60)	50 (16–64)
Underlying disease			
- COPD	25 (52%)	3 (43%)	22 (54%)
- CF	13 (27%)	1 (14%)	12 (29%)
- interstitial lung disease	10 (21%)	3 (43%)	7 (17%)
Gender			
M	23 (48%)	2 (29%)	21 (51%)
F	25 (52%)	5 (71%)	20 (49%)
Recipient-KIR-haplotypes			
- KIR haplotype A (homozygous)	19 (40%)	5 (71%)	14 (34%)
- KIR haplotype A and B	14 (29%)	1 (14%)	13 (31%)
- KIR haplotype B (homozygous)	15 (31%)	1 (14%)	14 (34%)
Median number of activating KIR genes (range)	3 (1–6)	1 (1–5)	3 (1–6)
Median number of inhibitory KIR genes (range)	6 (2–8)	6 (6–8)	7 (2–8)
Median KIR mismatches (range)	1 (0–2)	1 (0–2)	1 (0–2)
CMV status recipient/donor (pre transplant)			
- positive/positive	8 (17%)	2 (29%)	6 (15%)
- positive/negative	15 (31%)	0	15 (37%)
- negative/positive	12 (25%)	3 (43%)	9 (22%)
- negative/negative	13 (27%)	2 (29%)	11 (27%)
CMV reactivation	16/36	3/5	13/30
Median HLA mismatches (range)			
Class I	3 (2–4)	3 (2–4)	3 (2–4)
Class II	1 (0–2)	1 (1–2)	1 (0–2)

Definition of abbreviations: COPD, chronic obstructive pulmonary disease; CF, Cystic Fibrosis; M, Male; F, Female; KIR, Killer Immunoglobulin-like Receptors; HLA, Human Leucocytes Antigen; CMV, cytomegalovirus.

Table 2. Characteristics of Ligand Absence Between Transplanted Lungs and Kir Patient

Ligand absent, number (%)		
Lung (donor)	Recipient	
HLA-C group 1 (HLA-C^{Asn80})	absent for KIR 2DL2/2DL3 patient	10 (22%)
HLA-C group 2 (HLA-C^{Lys80})	absent for KIR 2DL1 patient	15 (33%)
HLA-Bw4 on	absent for KIR 3DL1 patient	12 (25%)
HLA-Bw4 and HLA-C	absent for patient KIR	8 (17%)
No ligand absence		9 (19%)

Definition of abbreviations: KIR, Killer Immunoglobulin-like Receptors; HLA, Human Leucocytes Antigen.

individual patients. The median number of activating KIRs was 3 (range, 1–6), and 13 patients (27%) had 5 or more activating KIRs. The median number of inhibitory KIRs was 6 (range, 2–8), and 24 patients (50%) had 7 or more inhibitory KIRs. No significant relation was found between the number of inhibitory or activating KIRs and the absence or presence of BOS.

Although some individual *KIR* genes (e.g., *2DL2, 2DL5, 2DS1*, and *2DS2*) appeared to differ in their frequencies in BOS patients compared with non-BOS patients, this did not reach statistical significance (Figure 1). The *KIR2DS5* gene, however, was present in 14 non-BOS patients (34%) but absent in patients with BOS, which was significantly associated with the development of BOS (hazard ratio, 0.00; $p = 0.04$; Figure 2).

KIR Haplotypes and BOS

Nineteen patients (40%) were homozygous for *KIR* haplotype A, 15 (31%) had a copy of *KIR* haplotypes A and B, and 14 (29%) were homozygous for *KIR* haplotype B. No association was found between *KIR* haplotypes and underlying lung diseases. A comparison of *KIR* haplotypes between BOS and non-BOS patients found that 5 (71%), 1 (14%), and 1 patients (14%) with BOS had *KIR* haplotypes A, AB, or B, respectively, whereas 14 (34%), 14 (34%), and 13 (32%) from the non-BOS patients contained these haplotypes. The presence of *KIR* haplotype A resulted in a significant higher risk of BOS development (hazard ratio, 4.9; $p = 0.03$; Figure 3).

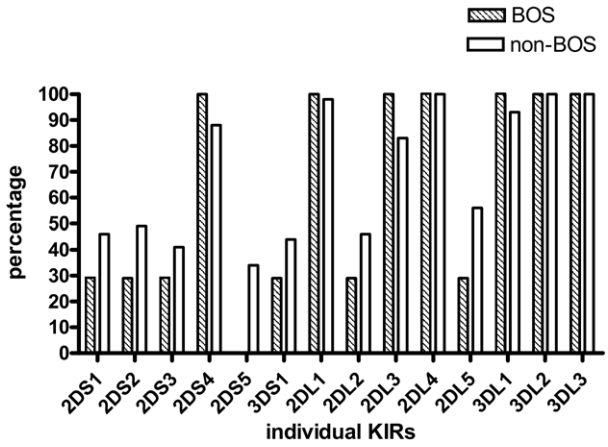

Figure 1. Frequency of individual killer immunoglobulin-like receptors (*KIR*) genes in patients with and without bronchiolitis obliterans syndrome (BOS) was determined as described in the Materials and Methods section. Shown are the frequencies of individual *KIR* genes found in patients with (gray bars) or without (white bars) BOS.

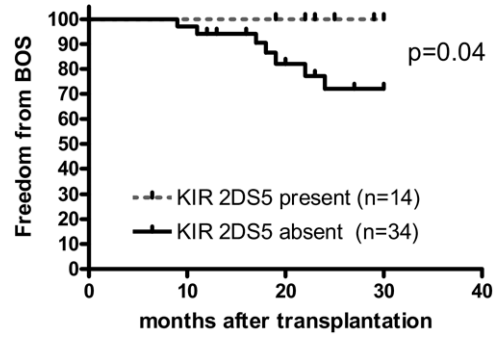

Figure 2. Kaplan-Meier analysis shows of freedom from bronchiolitis obliterans syndrome (BOS) was significantly different between the 34 patients with killer immunoglobulin-like receptor (*KIR*) gene *2DS5* absent (solid line) and the 14 patients with *KIR2DS5* present (dashed line). Log-rank test, $p = 0.04$.

Influences of Missing KIR Ligands, Individual *KIR* Genes, and *KIR* Haplotypes on CMV Reactivation

To examine whether CMV reactivation was related to *KIR* gene content, we studied CMV serostatus in donors and patients before transplantation and linked them to individual *KIR* genes. This analysis indicated that in 12 transplantations, CMV was neither present in donor nor patient. From the 36 remaining patients at risk for a CMV reactivation, a reactivation actually occurred in 16 patients despite prophylactic use of ganciclovir. CMV

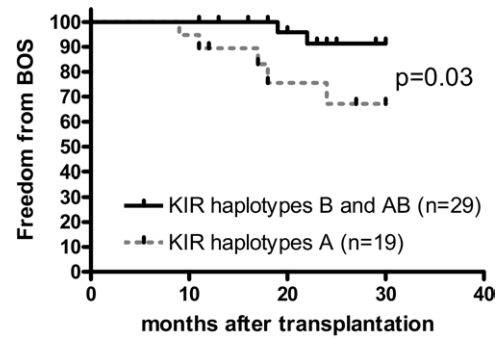

Figure 3. Influence of killer immunoglobulin-like receptor (*KIR*) haplotype A on freedom from bronchiolitis obliterans syndrome (BOS) by Kaplan-Meier analysis showed a significant difference between the 19 patients with *KIR* haplotype A (homozygous, dashed line) and the 29 patients with *KIR* haplotype AB and B (homozygous, solid line). Log-rank test, $p = 0.03$.

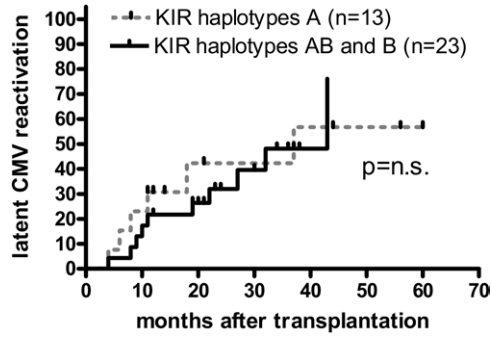

Figure 4. Kaplan-Meier analysis of cytomegalovirus (CMV) reactivation showed no significant difference between the 13 patients with killer immunoglobulin-like receptor (*KIR*) haplotype A (homozygous, dashed line) and the 23 patients with *KIR* haplotype AB and B (homozygous, solid line). CMV reaction was defined as a successive assay in the polymerase chain reaction after transplantation in those patients who had a seropositive CMV status before transplantation or who received lungs from a CMV seropositive donor.

reactivation occurred at a median of 11 months (range, 4–43 months) after transplantation.

KIR genotyping of the 36 patients at risk for CMV reactivation showed that 35 patients (97%) were positive for *KIR2DL1*, all (100%) for *KIR2DL2* or *KIR2DL3* (53% for *KIR2DL2*, 86% for *KIR2DL3*), and 34 (94%) were positive for *KIR3DL1*. Eighteen of the transplanted lungs (69%) were typed for HLA-C group 1, 27 (75%) for HLA-C group 2, and 34 (94%) for HLA-Bw4. Twenty-two patients with CMV reactivation received a lung with KIR ligands missing, whereas in the other 14 patients, all ligands were present on the transplanted lungs. Studying the influence of missing KIR ligands on CMV reactivation showed no significant differences in patients with or without a missing KIR ligand. In addition, no significant relation could be found between the number of inhibitory or activating *KIR* genes, neither between individual *KIR* genes nor CMV reactivation. Furthermore, no significant correlation was found between the *KIR* haplotypes of patients and CMV reactivation (Figure 4).

DISCUSSION

Stimulated by the reported associations between KIRs and several clinical features after hematopoietic stem cell transplantation, we hypothesized that *KIR* gene content may be related to chronic allograft rejection and CMV reactivation after lung transplantation. To our knowledge, this is the first study to analyze the influence of individual *KIR* gene contents, their corresponding ligands, and haplotypes on the results of lung transplantation. We demonstrate that *KIR* haplotype A and the absence of *KIR2DS5*, presumably the lack of activating KIRs, are predictive for development of chronic allograft rejection.

As inhibitory signals derived from KIRs are primarily regulated by ligation with their corresponding HLA-ligands, we first analyzed *KIR2DL1, KIR2DL2, KIR2DL3,* and *KIR3DL1* from all patients, and HLA-Cw and HLA-Bw4 in the donors. An effect of KIR-ligand matching on chronic rejection after lung transplantation was not detected. These data are in line with a previous report showing no effect of KIR ligand-matching on the 10-year allograft survival in 2,757 patients receiving cadaver kidney transplantation.[21] In a recent study of 69 kidney transplants, no association was found between individual *KIR* genes or *KIR* haplotypes and the occurrence of acute rejection when tapering their immunosuppressive regimen.[22] However, Kunert et al[23] demonstrated in 224 kidney transplants that patients without acute rejections had higher frequencies of *KIR2DL2/KIR2DS2* although no association was found between acute rejections and *KIR* haplotypes.

In the present study we have found that both *KIR* haplotype A and the absence of *KIR2DS5* are significantly associated with BOS. This finding is unexpected when the viewpoint is taken that NK cells are simply killer cells guided by their cell-surface receptors. Because NK cells containing the homozygous *KIR* haplotype A express a phenotype mainly encoded by inhibitory KIRs, this *KIR* haplotype should be associated with *less* reactivity against donor cells recognized on lung allografts, and thus absence from BOS, instead of the association found in the present study. It is possible that the differences observed are a random effect due to a limited sample size. Nevertheless, if these findings are confirmed in a larger study, one could speculate that NK cells play a more regulatory role in the alloresponse (e.g., by dendritic cell editing) in the period of months and years after transplantation when allograft damage is persistently induced by the immune system.[24] In the lung, immature dendritic cells are located in airway epithelium, alveolar septa, and around pulmonary vessels. These immature dendritic cells are sensitive to killing by NK cells, especially by those expressing activating KIRs and Toll-like receptors. When immature dendritic cells become activated and mature, they down-regulate secretion of chemokines (CCR1, CCR5, and CCR6), up-regulate expression of CCR7,[25,26] and become insensitive to lysis by NK cells.[27,28] The efficiency with which activated NK cells kill immature dendritic cells may differ between those containing the *KIR* haplotype A vs AB or B haplotypes, which will result downstream in differences in alloantigen presentation of dendritic cells to T cells.

A couple of arguments made the study analyzing the influence of NK cells on CMV reactivation are appealing. The first is that NK cells are important in the control of viral infection, and second is the association between viral infections, especially CMV, with the

development of BOS.[19,29] Studies in patients with human immunodeficiency virus have shown that the combination of the activating *KIR3DS1* and its presumed ligand HLA-Bw4 is associated with a delayed progression to acquired immunodeficiency syndrome, whereas the presence of *3DS1* in absence of Bw4 is associated with a rapid progression. These data suggest that virus elimination is facilitated upon binding of an activating KIR to its ligand.[30]

After hematopoietic stem cell transplantation, missing ligands for inhibitory KIRs did not influence CMV reactivation, but results of such studies in organ transplantation have not been reported yet.[31] In our study on lung transplantation, we did not observe any relation between missing KIR ligands or in the number of activating KIRs or individual KIRs on CMV reactivation. After allogeneic stem cell transplantation, however, patients with *KIR* haplotypes AB and B—and thus with more activating KIRs—showed a lowered incidence of CMV reactivation compared with those with *KIR* group A haplotypes.[16,31] Because CMV reactivation after lung transplantation is associated with BOS, and the KIR haplotypes AB and B are associated with lack of BOS, we expected a protective influence of activating KIRs on CMV reactivation, but did not find any.

Next to this difference, CMV reactivation occurred 10 to 20 days after stem cell transplantation in 55% to 63% of patients vs 4 months after lung transplantation in 44% ($n = 16$), despite prophylactic use of ganciclovir. Apparently, in the stem cell transplantation setting where myeloablative therapy was given and the immune system was reconstituting under immunosuppression, the influence of KIRs on CMV reactivation differs from that in organ transplantation in which a fully functional immune system is only reduced in activity by immunosuppressive therapy.

Our results show that the *KIR* gene content of recipients, especially activating KIRs, influences chronic allograft rejection after lung transplantation, possibly through editing of dendritic cells. We are aware that our study is hampered by the small patient population and a relatively short follow-up. Our findings need to be confirmed in larger studies elucidating the role of KIR usage by NK cells in chronic allograft rejection. We propose a detailed analysis of NK cells infiltrating lung allografts, including the influence of different immunosuppressives on their function. A challenging issue will be to further explore the mechanism of NK cells activation by characterizing activating ligands and their expression on the lung allograft.

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