Title

CD16+ natural killer cells in bronchoalveolar lavage are associated with antibody-mediated rejection and chronic lung allograft dysfunction

Journal

American Journal of Transplantation, 23(1)

ISSN

1600-6135

Authors

Calabrese, Daniel R
Chong, Tiffany
Singer, Jonathan P
et al.

Publication Date

2023

DOI

10.1016/j.ajt.2022.10.006

Peer reviewed

American Journal of Transplantation xxx (xxxx) xxx

Contents lists available at ScienceDirect

American Journal of Transplantation

journal homepage: www.journals.elsevier.com/american-journal-of-transplantation

Original Article

CD16$^+$ natural killer cells in bronchoalveolar lavage are associated with antibody-mediated rejection and chronic lung allograft dysfunction

Daniel R. Calabrese [1,2,*], Tiffany Chong [1], Jonathan P. Singer [1], Raja Rajalingam [3], Steven R. Hays [1], Jasleen Kukreja [3], Lorriana Leard [1], Jeffrey A. Golden [1], Lewis L. Lanier [4], John R. Greenland [1,2]

[1] Department of Medicine, University of California, San Francisco, California, USA
[2] Medical Service, Veterans Affairs Health Care System, San Francisco, California, USA
[3] Department of Surgery, University of California, San Francisco, California, USA
[4] Department of Microbiology and Immunology and the Parker Institute for Cancer Immunotherapy, University of California, San Francisco, California, USA

ARTICLE INFO

Keywords:
biomarker
inflammation
innate immunity
acute lung injury

ABSTRACT

Acute and chronic rejections limit the long-term survival after lung transplant. Pulmonary antibody-mediated rejection (AMR) is an incompletely understood driver of long-term outcomes characterized by donor-specific antibodies (DSAs), innate immune infiltration, and evidence of complement activation. Natural killer (NK) cells may recognize DSAs via the CD16 receptor, but this complement-independent mechanism of injury has not been explored in pulmonary AMR. CD16$^+$ NK cells were quantified in 508 prospectively collected bronchoalveolar lavage fluid samples from 195 lung transplant recipients. Associations between CD16$^+$ NK cells and human leukocyte antigen mismatches, DSAs, and AMR grade were assessed by linear models adjusted for participant characteristics and repeat measures. Cox proportional hazards models were used to assess CD16$^+$ NK cell association with chronic lung allograft dysfunction and survival. Bronchoalveolar lavage fluid CD16$^+$ NK cell frequency was associated with increasing human leukocyte antigens mismatches and increased AMR grade. Although NK frequencies were similar between DSA+ and DSA− recipients, CD16$^+$ NK cell frequencies were greater in recipients with AMR and those with concomitant allograft dysfunction. CD16$^+$ NK cells were associated with long-term graft dysfunction after AMR and decreased chronic lung allograft dysfunction–free survival. These data support the role of CD16$^+$ NK cells in pulmonary AMR.

1. Introduction

Chronic lung allograft dysfunction (CLAD) occurs in approximately 50% of lung transplant recipients by 5 years and is the major barrier to long-term survival and quality of life.[1] Although T cell–mediated immune responses, in the form of acute cellular rejection, contribute to the risk of CLAD, humoral immune responses are also CLAD risk factors and can be associated with particularly severe manifestations.[2] Antibodies against donor human leukocyte antigen (HLA) class I and II molecules are observed in lung allograft recipients at even higher frequencies than in other solid organ transplant cohorts.[3] Recipients with preexisting, de novo donor-specific antibodies (DSAs) or autoantibodies against lung parenchymal self-antigens have greater rates of acute rejection, CLAD, and death than those that remain free of allograft-specific antibodies.[4,5]

Antibody-mediated rejection (AMR) is an acute process that occurs when an antibody binds to the lung allograft, resulting in innate immune infiltration and tissue injury. In severe clinical manifestations, these pathologic findings are accompanied by lung allograft dysfunction. The pathophysiology of AMR is believed to result from the binding of graft-specific antibodies to vascular endothelial cells leading to the activation of the complement pathway.[6] However, the observation of patients with lethal AMR and no evidence of complement activation implies that injury also occurs via a complement-independent pathway.[7]

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AMR, antibody-mediated rejection; BAL, bronchoalveolar lavage; CLAD, chronic lung allograft dysfunction; CMV, cytomegalovirus; DSA, donor-specific antibody; HLA, human leukocyte antigens; HR, hazard ratio; IgG, immunoglobin G; IVIG, intravenous immunoglobulin; KIR, killer cell immunoglobulin-like receptor; MFI, mean fluorescent intensity; NK, natural killer; UCSF, University of California San Francisco.

* Corresponding author. 4150 Clement St, San Francisco, CA 94121, USA.
E-mail address: daniel.calabrese@ucsf.edu (D.R. Calabrese).

https://doi.org/10.1016/j.ajt.2022.10.006
Received 2 June 2022; Received in revised form 12 September 2022; Accepted 10 October 2022
Available online xxxx
1600-6135/Published by Elsevier Inc. on behalf of American Society of Transplantation & American Society of Transplant Surgeons.

Antibody-dependent cell cytotoxicity (ADCC) occurs when innate immune cells bind to the Fc component of immunoglobin G (IgG) antibodies, resulting in target cell death, independent of complement activation. Natural killer (NK) cells are innate immune cells increasingly recognized as important in lung transplant outcomes.[8-10] NK cells express the activating Fc receptor, FcγRIIIa (CD16).[11] Upon ligation, CD16 triggers NK cell–mediated cytokine production and cytotoxicity via granzymes and perforin.[12] NK cells are the dominant source of interferon gamma within the lung.[13] When cultured with primary cells bound with antibody, their secretion of interferon gamma is reduced by blocking the CD16 receptor.[14]

The extent to which ADCC mediates lung transplant AMR is unknown. In contrast to other Fc receptors on macrophages and neutrophils, CD16 is exclusively activating. Thus, NK cells are likely the dominant effector cells in ADCC.[15] Therefore, we hypothesized that NK cells, as measured in the bronchoalveolar lavage (BAL), would be associated with acute and chronic injury in pulmonary AMR.

2. Materials and methods

2.1. Study population

Consenting adult participants who underwent lung or heart-lung transplantation between January 22, 2014, and August 15, 2016, were considered for inclusion in this study, which was approved by the University of California San Francisco (UCSF) institutional review board. BAL was prospectively collected as part of routine clinical care, and excess fluid and cells were analyzed. Participants were included if at least 1 adequate BAL sample, defined as >50 NK cells, was collected within the study period. Bronchoscopies were performed for clinical indication or for allograft surveillance at 0.5, 1, 2, 3, 6, 12, 18, and 24 months after transplantation and then annually.[16]

2.2. Clinical data and outcomes

UCSF lung transplant clinical protocols, including induction and maintenance immunosuppression regimens and antifungal and cytomegalovirus (CMV) prophylaxis strategies, have been previously reported.[9,16,17] Participant clinical data were obtained from chart review of medical records and the United Network for Organ Sharing database. We prospectively recorded all transbronchial biopsy pathology findings, BAL microbiology results, and vital status.

HLA was typed as part of routine clinical care, and mismatches between donor and recipient were categorized across A, B, C, DRB1, DRB3, DRB4, DRB5, DQB1, DQA1, DPA1, and DPB1 loci. The HLA class I and II antibodies were measured using a Luminex-based single-antigen bead assay (One Lambda Inc).[18] Serum samples were pretreated with dithiothreitol to prevent aggregation of high titer antibodies. Interassay variation was limited using a high throughput liquid handling system (LABXpress, One Lambda). DSAs were defined as antibodies against the donor's mismatched HLA reaching a mean fluorescent intensity (MFI) of >1000 on a Luminex single-antigen bead immunoassay (One Lambda). DSAs were assessed at the time of surveillance bronchoscopy during the first 2 years after transplantation and for cause in the setting of multimodal clinical investigation of allograft dysfunction. Persistent DSAs were characterized as those detected for at least 3 consecutive months. Participants with DSA assays above 3000 MFI without the evidence of graft dysfunction were treated with monthly infusions of intravenous immunoglobulin (IVIG) until 3 months following clearance.

Transbronchial biopsies were reviewed clinically by experienced pulmonary pathologists, graded for acute rejection, and stained for complement component 4d (C4d) with clinical or pathologic suspicion.[19] AMR was graded using international criteria into 4 categories: no AMR, possible AMR, probable AMR, and definite AMR.[20] AMR was further classified as clinical or subclinical based on the presence of acute allograft dysfunction, defined as an acute decrease in forced vital capacity or forced expiratory volume in 1 second by at least 10% from posttransplant baseline or as the presence of diffuse chest radiographic abnormalities. Other causes of allograft dysfunction were characterized based on pathogens identified in BAL, the presence of pleural effusion, acute cellular rejection, or an increase in recipient weight. The initial treatment of AMR consisted of corticosteroids and IVIG, with subsequent therapy dependent on clinical consensus opinion and the severity of AMR clinical manifestations. Following the diagnosis of clinically meaningful acute rejection, bronchoscopy was repeated 1 month later or after treatment.

CLAD was defined as an unresolved 20% decline in forced expiratory volume in 1 second or forced vital capacity lasting for >30 days, as previously described.[21-23] CLAD-free survival was quantified as years of freedom from CLAD or death.

2.3. BAL immunophenotyping

Fresh BAL samples were collected, processed, and stained with antibodies for enumeration of NK cell subsets by flow cytometry (Supplementary Fig. S1). Antibodies and staining strategies were performed as described previously.[9] After processing, samples were acquired using a Beckman Coulter Navios cytometer, and data were analyzed with the Kaluza software package (Beckman Coulter). Live lymphocytes were identified by side and forward light scatter properties, CD45 positivity, and absence of viability dye. NK cells were defined as $CD3^{-}CD56^{+}$ lymphocytes and stratified by CD16 expression. Absolute quantities of NK cells were derived from total lymphocyte counts on paired clinical BAL cell differentials.

2.4. Statistical analysis

The clinical outcomes of interest were DSAs and AMR. Comparisons between NK cells and nonordinal outcomes in longitudinal data were made using generalized linear models, adjusted with generalized estimating equations with an exchangeable covariance matrix to account for repeated observations within participants. Comparisons between NK cells and the ordinal variable of AMR grade employed cumulative-linked mixed models adjusted for repeat observations within participants. All regression models were also adjusted for recipient age and CMV serostatus, and models using longitudinal data were adjusted for time after transplantation. Pairwise differences in these models were assessed with estimated marginal means and adjusted for multiple comparisons using Benjamini-Hochberg corrections.

Cox proportional hazards models were used to assess the difference in CLAD-free survival hazard ratios (HRs) between the AMR grade, median percentage of $CD16^{+}$ NK cells among AMR participants, and median percentage of $CD16^{+}$ NK cells across all participants. We applied generalized type 1 censoring for all participants who survived for 6 months after transplantation. All Cox proportional hazards models were adjusted to include participant characteristics frequently associated with poor transplant outcomes or NK cell frequencies: recipient age at transplantation, sex, ethnicity, donor and recipient CMV serostatus, lung allocation score indication grouping (A: obstructive, B: pulmonary vascular, C: cystic fibrosis, and D: fibrotic, as referent group), and the type of lung transplantation (double, single, or heart-lung). All analyses were adjusted for time-dependent covariates and repeat measures among participants. Time-dependent analyses treated each bronchoscopy as an individual occurrence such that covariate values of participants who experienced an event were compared to all others at risk at that time.[24,25] Bronchoscopies were coded according to intervals of time relative to CLAD-free survival, and bronchoscopies occurring after an event did not contribute to the analysis. Survival data were displayed with extended Kaplan-Meier plots.[26]

Statistical analyses and graphics were conducted in R (R Foundation for Statistical Computing) using packages "survminer," "gee," "lsmeans," "ggplot," "survival," "dplyr," "ordinal," and "ggbeeswarm."

3. Results

3.1. Participant characteristics

Characteristics of the 195 participants included in the study are displayed in the Table 1, and a flow diagram detailing inclusion and exclusion criteria is presented in Figure 1. UCSF is a referral center for interstitial lung diseases; as such, more double lung transplants and fewer lung transplants for cystic fibrosis are performed at our center, compared with other centers. A total of 795 bronchoscopies were performed within the study dates, with 508 samples included in the final analysis. Notably, 287 samples were excluded because of lack of collection, low sample quality, or too few cells to enumerate populations. Supplementary Table. S1 illustrates the indication and time of collection for the included bronchoscopy samples.

3.2. $CD16^+$ NK cells are mature and more proliferative than CD16- NK cells

We characterized the phenotypes of BAL NK cells expressing the CD16 Fc receptor and those that did not express the CD16 Fc receptor. The gating strategy and representative histograms are shown in Supplementary Figure S1. Cell proliferation, inhibitory receptors, and activating receptors were measured in these NK cells (Fig. 2A). The median absolute number of $CD16^+$ NK cells in lavage was 2.6×10^5 cells/L (IQR, 8.1×10^4 to 7.3×10^5 cells/L), and the median percentage of NK cells with CD16 was 31% (IQR, 20%-51%). Compared with $CD16^-$ NK cells, $CD16^+$ NK cells were more proliferative ($P < .0001$), had more maturation-associated killer cell immunoglobulin-like receptor (KIR) expression ($P < .0001$), and had less inhibitory receptor NKG2A (Fig. 2B, $P < .0001$). The CMV-specific receptor, NKG2C, was present on 9% (IQR, 2%-17%) of $CD16^+$ NK cells, compared with 4% (IQR, 1%-12%) of $CD16^-$ NK cells ($P < .0001$).

Because immunosuppression and chronic inflammation may influence the NK cell phenotype, we characterized NK cell subsets by time after transplantation. Notably, percentages of $CD16^+$ NK cells (Supplementary Fig. S2A) and the absolute quantity of $CD16^+$ NK cells (Supplementary Fig. S2B) did not change over time. However, there was a small positive correlation between time after transplantation and KIR^+ NK cells (Supplementary Fig. S2C) as well as with $NKG2C^+$ NK cells (Supplementary Fig. S2E).

Table 1
Baseline characteristics for the 195 participants with bronchoscopy results.

Variable	Value
Recipient age (y), median (IQR)	58 (49-64)
Male sex, n (%)	122 (63)
Transplant type, n (%)	
Double lung	176 (90)
Single lung	16 (8)
Heart-lung	3 (2)
Ethnicity, n (%)	
Caucasian	138 (71)
African American	20 (10)
Hispanic	28 (14)
Asian/Pacific American	9 (5)
Transplant group, n (%)	
COPD	31 (16)
Pulmonary hypertension	3 (2)
Cystic fibrosis	18 (9)
Interstitial lung disease	143 (73)
CMV serostatus, n (%)	
R−/D−	31 (16)
R−/D+	54 (28)
R+/D−	38 (19)
R+/D+	69 (36)
Unknown	3 (2)

CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease.

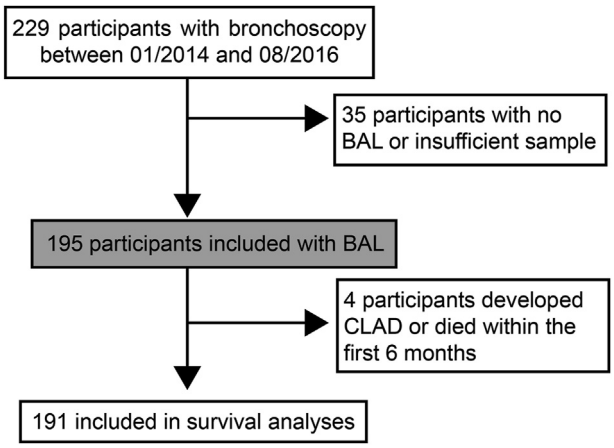

Figure 1. Inclusion and exclusion criteria for study participants. BAL, bronchoalveolar lavage; CLAD, chronic lung allograft dysfunction.

3.3. Percentage of $CD16^+$ BAL NK cells are positively associated with donor and recipient HLA mismatches

Participants with increasing donor and recipient HLA mismatches are at increased risk of AMR, CLAD, and death.[27-29] Additionally, increased HLA mismatches would be expected to confer less inhibitory NK cell receptor engagement.[10] Therefore, we sought to assess differences in NK cells in BAL by HLA mismatch. We found that the median number of HLA mismatches between donor and recipient was 9 (IQR, 7-11). Figure 3A illustrates the percentage of $CD16^+$ NK cells in BAL by the number of HLA mismatches. We found that each HLA mismatch was associated with a 0.7% increase in BAL NK cell CD16 percentage (95% CI, 0.1%-1.3%; multivariable $P = .04$). On average, a participant in the highest quartile of HLA mismatches would have 8% more NK cells with CD16 in BAL compared with a participant the lowest quartile. Absolute numbers of $CD16^+$ NK cells were increased but did not reach our pre-specified level of significance (3.5×10^4 [95% CI, 3.1×10^4 to 1.5×10^5 cells/L]; $P = .3$).

3.4. There was no difference in NK cell frequency or phenotype in the presence of de novo DSA alone

Posttransplant de novo recipient antibodies specific to donor HLA class I and II are associated with poor short- and long-term lung transplant outcomes.[5] We hypothesized that NK cells with the CD16 Fc receptor would be increased in BAL of participants with circulating de novo DSAs at the time of bronchoscopy. There were 44 participants with DSAs during the study period, and there were 84 bronchoscopies performed at the time of positive DSAs. Most DSAs were against HLA class II (n = 48) or HLA class I (n = 27), but there were 9 instances with both HLA class I and II antibodies. The median MFI was 2428 (IQR, 1551-3818), and antibodies against HLA DQ were the most prevalent (n = 39). We found no differences in the percentage of CD16 on NK cells in participants with DSAs during the time of bronchoscopy, compared with bronchoscopies performed in the absence of DSAs (Fig. 3B, $P = .4$). The CD16 percentage also did not differ among recipients with transient or persistent DSAs (Fig. 3C). Total $CD16^+$ NK cells were not different between bronchoscopies performed in the presence of DSAs vs those performed without DSAs (Fig. 3D; $P = 1$) nor were they different between bronchoscopies performed in the setting of transient or persistent DSAs (Fig. 3E). This suggests that DSAs alone may not be a consequential indicator of BAL NK cell inflammation.

3.5. BAL $CD16^+$ NK cells are increased in lung transplant AMR

Of the 508 included bronchoscopies, 92 (n = 51 participants) were graded by international criteria with some form of AMR. There were 372

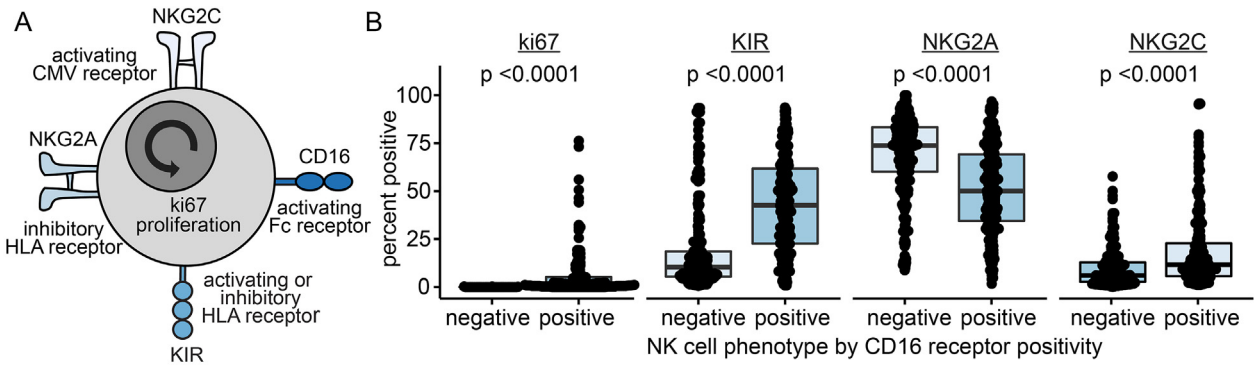

Figure 2. CD16$^+$ NK cells are mature and proliferative. (A) Natural killer (NK) cell action is determined by the integration of signals from a range of activating and inhibitory receptors. The CD16 receptor produces a potent activating signal after binding the Fc component of antibodies. NKG2C recognizes cytomegalovirus-infected cells through human leukocyte antigen (HLA) E. Killer cell immunoglobulin-like receptors (KIRs) bind HLA and can send activating or inhibiting signals. NKG2A ligation of HLA-E sends an inhibitory signal. (B) Flow cytometry phenotypes for CD16$^-$ and CD16$^+$ NK cells in 506 bronchoscopy samples were analyzed. Individual data points are shown bound by boxes at 25th and 75th percentiles, and medians are depicted with bisecting lines. Compared with CD16$^-$ NK cells, CD16$^+$ NK cells were more proliferative (ki67, $P < .0001$), had increased KIR expression ($P < .0001$), had decreased NKG2A ($P < .0001$), and had increased NKG2C ($P < .0001$). *P* values were generated with generalized estimating equation models adjusted for age and cytomegalovirus serostatus.

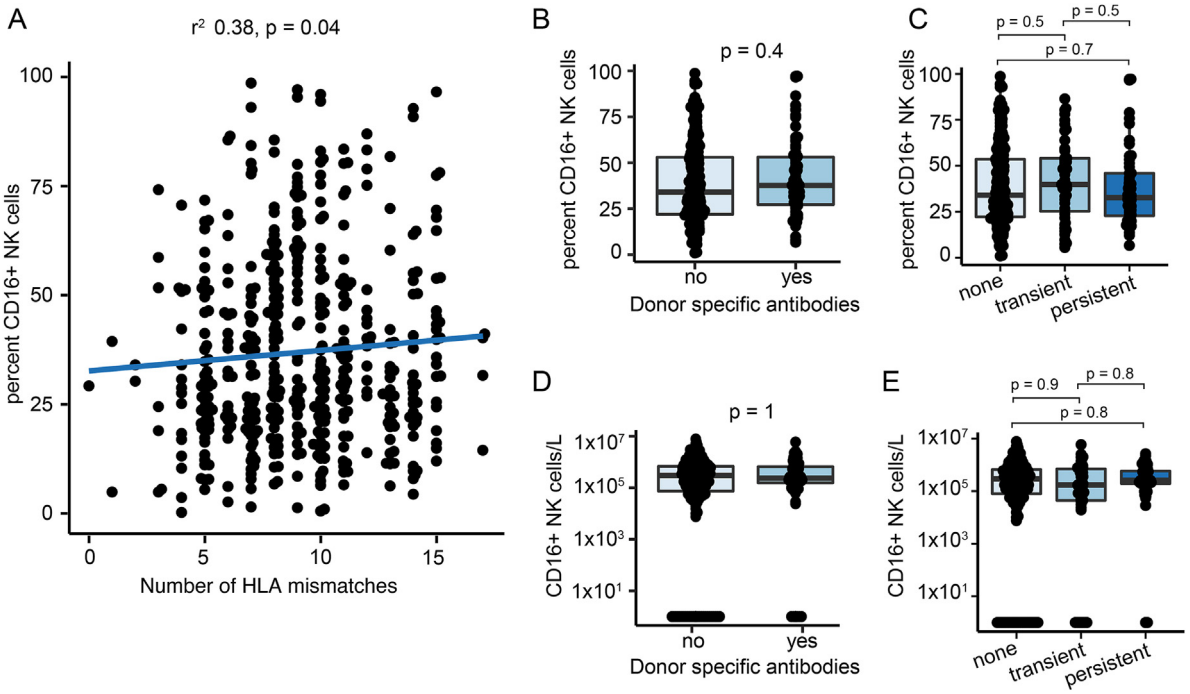

Figure 3. The percentage of CD16$^+$ natural killer (NK) cells increases with increasing donor and recipient human leukocyte antigen (HLA) mismatches but not donor-specific antibodies (DSAs). NK cells were measured in bronchoalveolar lavage (BAL) and quantified by absolute numbers and as percentage positive for CD16. (A) With increasing donor and recipient HLA mismatches, the percentages of CD16 on CD3$^-$CD56$^+$ NK cells were increased. (B) Percentage of NK cells expressing CD16 on BAL NK cells at the time of positive (n = 84) or negative DSAs (n = 484). (C) Percentage of NK cells expressing CD16 during transient or persistent DSAs. (D) The absolute numbers of CD16$^+$ NK cells among recipients with and without DSAs. (E) The absolute numbers of CD16$^+$ NK cells among recipients by DSA persistence. Individual data points are shown bound by boxes at 25th and 75th percentiles, and medians are depicted with bisecting lines. *P* values were generated with generalized estimating equation models adjusted for age, cytomegalovirus serostatus, and time after transplant. Pairwise differences were adjusted for multiple comparisons using Benjamini-Hochberg corrections.

cases with no evidence of AMR, 40 subclinical possible, 24 clinical possible, 11 subclinical probable, 13 clinical probable, and 4 cases of clinical definite AMR.[20] Figure 4 shows the percentage of NK cells expressing CD16$^+$ and absolute CD16$^+$ NK cells across the 4 grades and 2 subtypes of lung transplant AMR. Compared with bronchoscopies with no AMR (30% [IQR, 21%-49%]), the percentage of NK cells with CD16 increased in those with possible AMR (32% [IQR, 21%-45%]), probable AMR (46% [IQR, 28%-57%]), and definite AMR (54% [IQR, 45%-64%]) ($P = .002$) (Fig. 4A). Absolute CD16$^+$ NK cells were not different in the BAL by AMR grade but were increased in an analysis of definite AMR (2×10^6 cells/L [IQR, 1.6×10^5 to 2.1×10^6 cells/L]) ($P < .0001$), compared with BAL counts among participants without AMR (5.1×10^5 cells/L [IQR, 7.4×10^4 to 6.1×10^5 cells/L]) (Fig. 4B).

3.6. NK cells differentiate between subclinical and clinical AMR

We hypothesized that CD16$^+$ NK cells would generally be increased in participants with more severe forms of AMR. We stratified the population by episodes of AMR, 1 (n = 27), 2 (n = 19), or 3 (n = 9). The median time between episodes was 64 days (IQR, 30-142 days). We

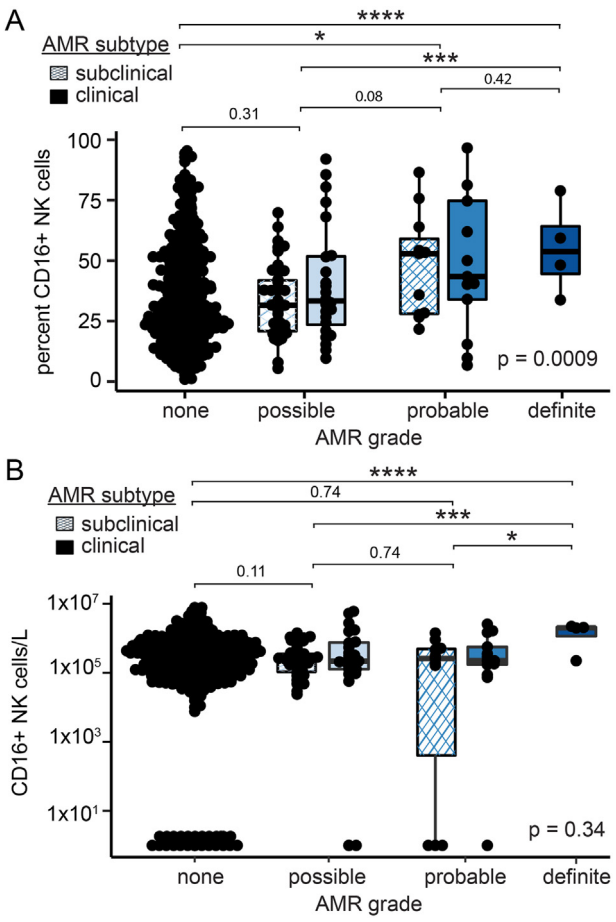

Figure 4. CD16$^+$ natural killer (NK) cells are increased during antibody-mediated rejection (AMR). AMR for participants was graded during serial bronchoscopies. (A) The percentage of NK cells expressing CD16 within bronchoalveolar lavage was increased across AMR grades and subtypes. (B) The absolute numbers of bronchoalveolar lavage CD16$^+$ NK cells were increased in participants with definite AMR only. Individual data points are shown bound by boxes at 25th and 75th percentiles, and medians are depicted with bisecting lines. Analyses employed cumulative-linked mixed models of AMR as an ordinal variable adjusted for repeat observations among study participants, age, cytomegalovirus serostatus, and time after transplant. Pairwise differences were adjusted for multiple comparisons using Benjamini-Hochberg corrections. *P* values: * < .05; *** < .001, **** < .0001.

found that participants with multiple biopsies displaying AMR had increased CD16$^+$ NK cells in their lavage at any time (Fig. 5A). Notably, there were no differences in CD16$^+$ NK cells when multiple AMR episodes were characterized as recurrence or persistence of disease, although the percentage of CD16$^+$ NK cells increased during AMR and at follow-up (Supplementary Fig. S4A-D). We examined whether NK cells and CD16 were different in participants with allograft dysfunction. Figure 5B shows that participants with allograft dysfunction had increased NK cells with CD16 (40% [IQR, 27%-62%]; $P = .01$) than participants without allograft dysfunction (32% [IQR, 23%-48%]). This did not hold true for total CD16$^+$ NK cells (Fig. 5C). These data suggest that differences in NK cell phenotypes were observed across clinical outcomes.

3.7. Increased CD16$^+$ NK cells was associated with worse CLAD-free survival

Finally, we examined whether increased CD16$^+$ NK cells in BAL would be associated with worse CLAD-free survival. Figure 6A shows an extended Kaplan-Meier survival plot representative of findings from the Cox proportional hazards model that showed participants with AMR had an increased risk of CLAD or death with time-dependent analyses (HR, 3; 95% CI, 1.7-5.2, ; adjusted $P < .001$). Notably, the risk was not different between participants with clinical or subclinical grades of AMR (adjusted $P = .4$). Next, we tested whether CD16 would differentiate between AMR outcomes. Indeed, we identified that participants with AMR and higher than the median percentage of NK cell CD16 had significantly increased risk of CLAD or death (HR, 2.9; 95% CI, 1.5-5.6; adjusted $P = .002$) (Fig. 6B). In another time-dependent analysis across all participants, higher than the median percentage of NK cell CD16 conferred a nearly 2.3-fold increased risk of CLAD or death (95% CI, 1.3-4; adjusted $P = .005$) (Fig. 6C). These models were adjusted for recipient baseline characteristics and CMV serostatus.

We previously found that NKG2C$^+$ NK cells were increased in BAL following CMV infection and were associated with poor long-term outcomes.[9] To test whether this NK cell association was mediated by CMV infection, we used the marker of CMV specificity, NKG2C, to exclude the CMV-specific proportion of NK cells. Although NKG2C$^+$ NK cells represent a minority of CD16$^+$ NK cells, we investigated whether NKG2C$^-$CD16$^+$ NK cells were also associated with worse CLAD-free survival. Indeed, we observed an adjusted HR for CLAD or death of 1.2 (95% CI, 1.03-1.5; $P = .05$) per increased quartile of BAL NKG2C$^-$CD16$^+$ NK cells.

4. Discussion

In this large observational single-center study, we describe an association between NK cells and pulmonary AMR. We found that CD16$^+$ NK cells within BAL from lung transplant recipients were mature and proliferative, compared with CD16$^-$ NK cells. The frequency of NK cells expressing CD16 increased in the setting of clinical AMR, and the absolute numbers of CD16$^+$ NK cells were also increased during severe clinical AMR. Importantly, increased BAL CD16$^+$ NK cells were found during AMR-associated acute lung allograft dysfunction. Higher percentages of CD16$^+$ NK cells differentiated the risk of CLAD or death among recipients with AMR and, to a lesser degree, across the entire cohort, irrespective of AMR pathology.

These observational data are consistent with those of previous reports implicating NK cells in other antibody-dependent processes. Studies of biopsies from participants with renal allograft dysfunction demonstrate increased expression of CD16 and NK cell gene transcripts.[30] NK cell action through ADCC is critical in mediating tumor clearance in some situations.[31,32] High-affinity FCGRIIIA (CD16 receptor) genotypes confer increased success of antitumor monoclonal antibody therapy.[33,34] In a gene expression study of acute lung allograft dysfunction, genes involved in NK cell–mediated cytotoxicity were some of the most differentially expressed.[35] Further study may show whether increased NK cells or NK cell receptor transcripts may prove useful as an adjunctive test in the diagnosis of pulmonary AMR.

The findings that increased CD16$^+$ NK cells were observed during AMR and allograft dysfunction suggest that NK cells play a role in acute lung allograft injury. We, and others, have previously reported that NK cells are present in the lung during other acute injury syndromes, such as acute cellular rejection and during CMV infection.[9,23,36,37] NK cells may be prominent in transplant lung injury syndromes because they are less susceptible to standard immunosuppression regimens, stemming from their storage of cytotoxic granules in the cytoplasm or scavenging of interleukin 2 after interleukin 2 receptor blockade.[38,39] Supporting this, we found across the cohort that CD16$^+$ NK cells were at their highest early after lung transplant. The finding that CD16$^+$ NK cells increase the risk for CLAD supports growing evidence that activated NK cells contribute to chronic pulmonary injury.[9,40] The causal link between CD16$^+$ NK cells and AMR is further supported by the finding that high-affinity CD16A (FCGRIIIA) genotypes are associated with increased risk of acute rejection, CLAD, and death.[41,42] Additional preclinical studies are needed to determine whether targeting NK cell activation or

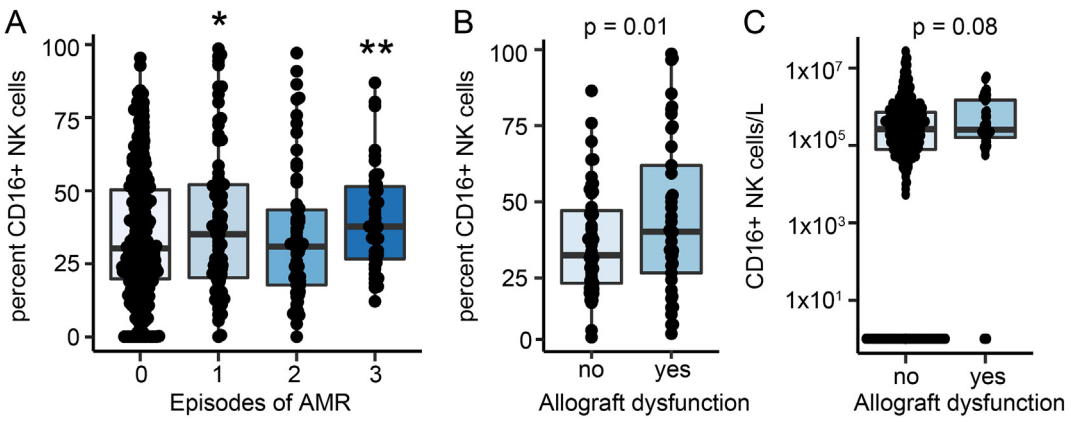

Figure 5. Bronchoalveolar lavage CD16^{+} natural killer (NK) cells are increased by antibody-mediated rejection (AMR) recurrence and graft dysfunction. (A) The percentage of NK cells expressing CD16 according to the frequency of AMR detections (1 episode [bronchoscopy n = 78], 2 episodes [bronchoscopy n = 60], and 3 episodes [bronchoscopy n = 41], compared with no AMR. (B) The percentage of NK cells expressing CD16 in AMR stratified by participants with and without allograft dysfunction. (C) The absolute counts of NK cells expressing CD16 in AMR stratified by participants with and without allograft dysfunction. Individual data points are shown bound by boxes at 25th and 75th percentiles, and medians are depicted with bisecting lines. Associations were established with logistic regression models adjusted for repeat measures, age, cytomegalovirus serostatus, and time after transplant. *P* values: * < .05; ** < .01.

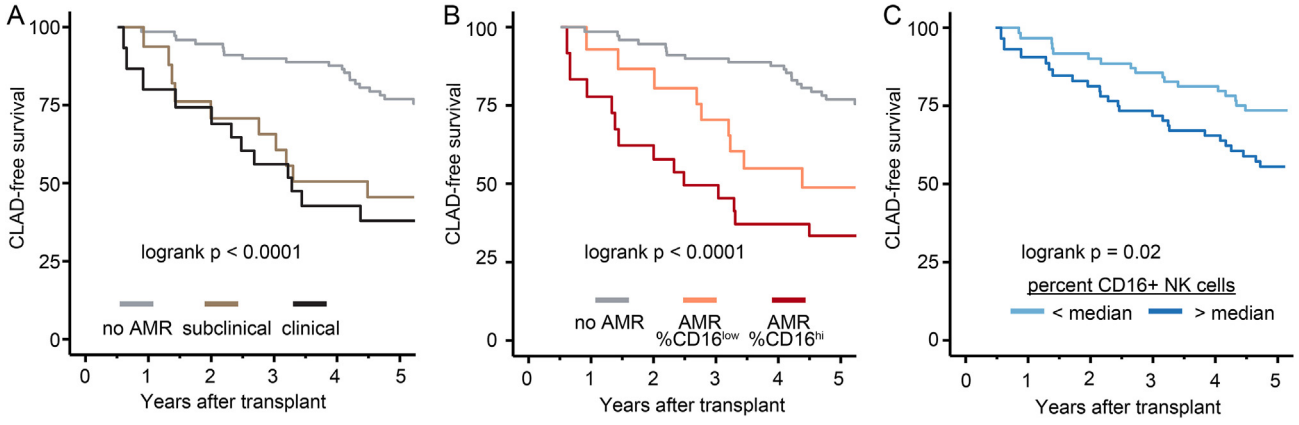

Figure 6. Percentage of bronchoalveolar lavage (BAL) CD16^{+} natural killer (NK) cells predict chronic lung allograft dysfunction (CLAD)–free survival. Extended Kaplan-Meier plots are shown in each panel, left censored at 6 months. (A) CLAD-free survival according to AMR grade. (B) Time to CLAD or death stratified by median NK cell CD16 percentage among participants with antibody-mediated rejection (AMR). (C) Time to CLAD or death stratified by median NK cell CD16 percentage across all participants. *P* values represent results from log rank tests.

effector functions may be an additional approach to the treatment of pulmonary AMR or CLAD.[43]

We found no difference in NK cell quantity or phenotype in the presence of DSAs. Human IgG has 4 subclasses that bind complement and Fc receptors with varying affinities.[44] A limitation of the standard bead-based DSA assay employed here is that it does not differentiate among IgG subclasses.[45,46] It may be that improved classification of donor-specific antibody IgG subclasses, specifically delineating those that have an affinity for complement or Fc, would inform the likelihood of complement-mediated vs cell-mediated pathways. Additionally, NK cell action is dependent on the integration of multiple receptor signals. DSA's may represent 1 signal, and we would expect to observe increased allograft NK cells in participants with AMR with a secondary priming insult, such as a viral infection.

It is possible that CD16^{+} NK cells may mediate ADCC by connecting the binding of the DSA on the allograft to NK cell-mediated cytotoxic responses independent of the complement system (Fig. 7). At the same time, the number of BAL NK cells may be a marker of injury via recruitment.[47,48] Consequently, NK cell activation in the context of AMR may be augmented by recognition of CMV by NKG2C, identification of missing self with decreased inhibition through KIR or NKG2A, response to damage or hypoxia signals via NKG2D, or CD16 binding of antibodies to autoantigens (Major Histocompatibility Complex class I polypeptide-related sequence A, collagen V or k-alpha tubulin).[49,50] There are also regulatory signals, such as NK cell inhibition in response to complement binding through C3a anaphylatoxin chemotactic receptor (C3aR).[51]

There are some limitations to our study. Although our center employed a uniform protocol for managing acute rejection and DSAs, we were unable to control individual recipient immunosuppression doses. The effect of IVIG on NK cells is incompletely understood, but IVIG treatment may reduce the incidence of NK cell ADCC.[52] Further, CMV remains an important driver of allograft inflammation.[53] Although our models adjusted for CMV serostatus, this study lacked the power to investigate if NK cell response to AMR or CLAD was different among CMV donor and recipient serologic groups. Not all bronchoscopies were captured in this study. Because AMR participants are generally more ill, they may be more likely to have been deemed too sick for research participation, which would be expected to have biased the results to the null hypothesis. Additionally, there was some clinical heterogeneity in the staining of biopsy samples to complement C4d, which may have impacted the ascertainment of AMR. Because this was a single-center study, these findings may not apply in centers where participant characteristics or posttransplant immunosuppression and monitoring protocols differ.

Despite limitations, these data have significant strengths. This is the largest study of pulmonary AMR graded by the International Society for

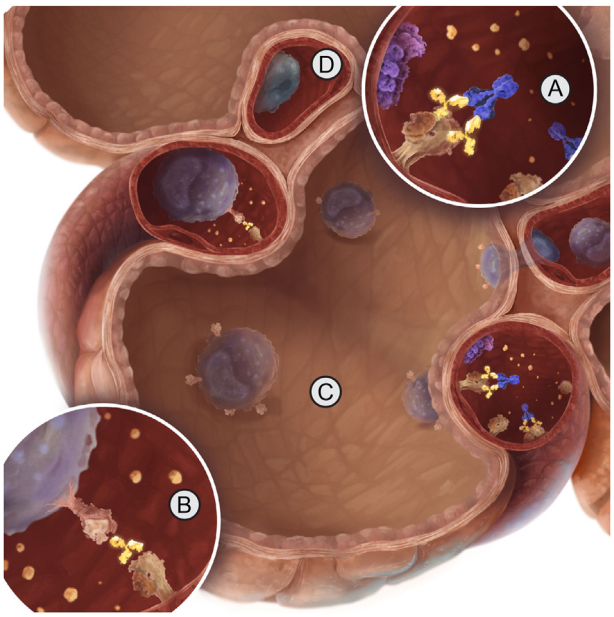

Figure 7. Pulmonary antibody-mediated rejection (AMR) occurs through complement and Fc-dependent mechanisms. (A) After the antibody binds to the allograft, complement can be activated, leading to C4d deposition and cell death via the complement cascade. (B) Through the CD16, CD32, and CD64 Fc receptors, effector cells can recognize bound antibodies in the process of antibody-dependent cellular cytotoxicity (ADCC). (C) Neutrophils are common AMR pathologic features and may become activated through the Fc receptors. (D) Of the Fc receptors, CD16 has a high affinity and is an activating-only receptor. The finding of $CD16^+$ NK cells in the bronchoalveolar lavage of AMR participants supports this alternate mechanism of lung allograft injury.

Heart and Lung Transplantation criteria. The collection and analysis of fresh BAL cells minimized the variability associated with cryopreservation and thawing of samples. The prospective study design allowed for the evaluation of CLAD and survival outcomes. Finally, the large sample size allowed for thorough modeling of potentially confounding participant characteristics.

In conclusion, we identified $CD16^+$ NK cells in BAL as a marker of acute and chronic lung allograft dysfunction associated with AMR. A further mechanistic study is needed to contextualize the findings from this observational cohort. NK cell–specific diagnostic tools and therapies may deserve scrutiny as supplements to current clinical approaches.

Funding

D. Calabrese was supported with funding from the International Society for Heart and Lung Transplantation's Joel D. Cooper Career Development Award, Cystic Fibrosis Foundation Harry Shwachman Career Development Award (CALABR19Q0), and VA Office of Research and Development Career Development Award 2 (IK2BX005301). J. Greenland was supported by the VA Office of Research and Development Merit (CX002011); UCSF Nina Ireland Program for Lung Health Innovative Grant program; and the National Heart, Lung, and Blood Institute (R01HL151552 and R01HL161048).

Author contributions

Study design: D.R.C., J.R.G., L.L.L., and S.R.H.; experiments: D.R.C., T.C., and R.R.; analysis of results: D.R.C., J.R.G., and J.P.S.; vital data/interpretation: D.R.C., J.R.G., L.L.L., and R.R.; manuscript preparation: D.R.C. and J.R.G. with input from all authors. The data that supports the findings of this study are available in the supplementary material of this article or are available upon reasonable request from the corresponding author.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Acknowledgments

The authors thank William Karlon for advice and support, as well as Chiyo Uchida, Brandon Lim, Ryan Tsiao, Brandon Cowan, and Malori Mindo for aid in collecting and processing study samples. The authors also thank Kirk Jones, Anatoly Urisman, Mary Ellen Kleinhenz, Rupal Shah, Aida Venado, Nick Kolaitis, and the other members of the clinical lung transplant team for their care of our patients and the design of the clinical protocols. Nicholas Bezio of Bezio Studios (nickbezio.com) created the illustration for Figure 7.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2022.10.006.

ORCiD

Daniel R. Calabrese https://orcid.org/0000-0002-0596-3434
Jonathan P. Singer https://orcid.org/0000-0003-0224-7472
Raja Rajalingam https://orcid.org/0000-0001-8821-7877
John R. Greenland https://orcid.org/0000-0003-1422-8367

References

1. Meyer KC, Raghu G, Verleden GM, et al. An international ISHLT/ATS/ERS clinical practice guideline: diagnosis and management of bronchiolitis obliterans syndrome. *Eur Respir J*. 2014;44(6):1479–1503.
2. Le Pavec J, Suberbielle C, Lamrani L, et al. De-novo donor-specific anti-HLA antibodies 30 days after lung transplantation are associated with a worse outcome. *J Heart Lung Transplant*. 2016;35(9):1067–1077.
3. Hachem RR, Kamoun M, Budev MM, et al. Human leukocyte antigens antibodies after lung transplantation: primary results of the HALT study. *Am J Transplant*. 2018;18(9):2285–2294.
4. Angaswamy N, Saini D, Ramachandran S, et al. Development of antibodies to human leukocyte antigen precedes development of antibodies to major histocompatibility class I-related chain A and are significantly associated with development of chronic rejection after human lung transplantation. *Hum Immunol*. 2010;71(6):560–565.
5. Hachem RR, Yusen RD, Meyers BF, et al. Anti-human leukocyte antigen antibodies and preemptive antibody-directed therapy after lung transplantation. *J Heart Lung Transplant*. 2010;29(9):973–980.
6. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. *Nat Rev Immunol*. 2005;5(10):807–817.
7. Valenzuela NM, McNamara JT, Reed EF. Antibody-mediated graft injury: complement-dependent and complement-independent mechanisms. *Curr Opin Organ Transplant*. 2014;19(1):33–40.
8. Calabrese DR, Lanier LL, Greenland JR. Natural killer cells in lung transplantation. *Thorax*. 2019;74(4):397–404.
9. Calabrese DR, Chong T, Wang AS, et al. NKG2C natural killer cells in bronchoalveolar lavage are associated with cytomegalovirus viremia and poor outcomes in lung allograft recipients. *Transplantation*. 2019;103(3):493–501.
10. Greenland JR, Sun H, Calabrese D, et al. HLA mismatching favoring host-versus-graft NK cell activity via KIR3DL1 is associated with improved outcomes following lung transplantation. *Am J Transplant*. 2017;17(8):2192–2199.
11. Gleason MK, Verneris MR, Todhunter DA, et al. Bispecific and trispecific killer cell engagers directly activate human NK cells through CD16 signaling and induce cytotoxicity and cytokine production. *Mol Cancer Ther*. 2012;11(12):2674–2684.
12. Seidel UJ, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. *Front Immunol*. 2013;4:76.
13. Abboud G, Tahiliani V, Desai P, et al. Natural killer cells and innate interferon gamma participate in the host defense against respiratory vaccinia virus infection. *J Virol*. 2016;90(1):129–141.
14. Toyoda M, Ge S, Suviolahti E, et al. IFNgamma production by NK cells from HLA-sensitized patients after in vitro exposure to allo-antigens. *Transpl Immunol*. 2012;26(2-3):107–112.
15. Resch T, Fabritius C, Ebner S, Ritschl P, Kotsch K. The role of natural killer cells in humoral rejection. *Transplantation*. 2015;99(7):1335–1340.
16. Calabrese DR, Florez R, Dewey K, et al. Genotypes associated with tacrolimus pharmacokinetics impact clinical outcomes in lung transplant recipients. *Clin Transplant*. 2018;32(8), e13332.

17. Calabrese DR, Wang P, Chong T, et al. Dectin-1 genetic deficiency predicts chronic lung allograft dysfunction and death. *JCI Insight*. 2019;4(22), e133083.
18. Roll GR, Webber AB, Gae DH, et al. A virtual crossmatch-based strategy facilitates sharing of deceased donor kidneys for highly sensitized recipients. *Transplantation*. 2020;104(6):1239–1245.
19. Stewart S, Fishbein MC, Snell GI, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant*. 2007;26(12):1229–1242.
20. Levine DJ, Glanville AR, Aboyoun C, et al. Antibody-mediated rejection of the lung: a consensus report of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant*. 2016;35(4):397–406.
21. Verleden GM, Raghu G, Meyer KC, Glanville AR, Corris P. A new classification system for chronic lung allograft dysfunction. *J Heart Lung Transplant*. 2014;33(2):127–133.
22. Greenland JR, Jones KD, Hays SR, et al. Association of large-airway lymphocytic bronchitis with bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med*. 2013;187(4):417–423.
23. Greenland JR, Jewell NP, Gottschall M, et al. Bronchoalveolar lavage cell immunophenotyping facilitates diagnosis of lung allograft rejection. *Am J Transplant*. 2014;14(4):831–840.
24. Kragh Andersen P, Pohar Perme M, van Houwelingen HC, et al. Analysis of time-to-event for observational studies: guidance to the use of intensity models. *Stat Med*. 2021;40(1):185–211.
25. Cleary SJ, Kwaan N, Tian JJ, et al. Complement activation on endothelium initiates antibody-mediated acute lung injury. *J Clin Invest*. 2020;130(11):5909–5923.
26. Snapinn SM, Jiang Q, Iglewicz B. Illustrating the impact of a time-varying covariate with an extended Kaplan-Meier estimator. *Am Stat*. 2005;59(4):301–307.
27. Lobo LJ, Aris RM, Schmitz J, Neuringer IP. Donor-specific antibodies are associated with antibody-mediated rejection, acute cellular rejection, bronchiolitis obliterans syndrome, and cystic fibrosis after lung transplantation. *J Heart Lung Transplant*. 2013;32(1):70–77.
28. Quantz MA, Bennett LE, Meyer DM, Novick RJ. Does human leukocyte antigen matching influence the outcome of lung transplantation? An analysis of 3,549 lung transplantations. *J Heart Lung Transplant*. 2000;19(5):473–479.
29. Todd JL, Neely ML, Kopetskie H, et al. Risk factors for acute rejection in the first year after lung transplant: a multicenter study. *Am J Respir Crit Care Med*. 2020;202(4):576–585.
30. Parkes MD, Halloran PF, Hidalgo LG. Evidence for CD16a-mediated NK cell stimulation in antibody-mediated kidney transplant rejection. *Transplantation*. 2017;101(4):e102–e111.
31. Giles AJ, Hao S, Padget M, et al. Efficient ADCC killing of meningioma by avelumab and a high-affinity natural killer cell line, haNK. *JCI Insight*. 2019;4(20), e130688.
32. Lo Nigro C, Macagno M, Sangiolo D, Bertolaccini L, Aglietta M, Merlano MC. NK-mediated antibody-dependent cell-mediated cytotoxicity in solid tumors: biological evidence and clinical perspectives. *Ann Transl Med*. 2019;7(5):105.
33. Gavin PG, Song N, Kim SR, et al. Association of polymorphisms in FCGR2A and FCGR3A with degree of trastuzumab benefit in the adjuvant treatment of ERBB2/HER2-positive breast cancer: analysis of the NSABP B-31 trial. *JAMA Oncol*. 2017;3(3):335–341.
34. Sun H, Martin TG, Marra J, et al. Individualized genetic makeup that controls natural killer cell function influences the efficacy of isatuximab immunotherapy in patients with multiple myeloma. *J Immunother Cancer*. 2021;9(7), e002958.
35. Weigt SS, Wang X, Palchevskiy V, et al. Usefulness of gene expression profiling of bronchoalveolar lavage cells in acute lung allograft rejection. *J Heart Lung Transplant*. 2019;38(8):845–855.
36. Hoegl S, Ehrentraut H, Brodsky KS, et al. NK cells regulate CXCR2+ neutrophil recruitment during acute lung injury. *J Leukoc Biol*. 2017;101(2):471–480.
37. Harpur CM, Stankovic S, Kanagarajah A, et al. Enrichment of cytomegalovirus-induced NKG2C+ natural killer cells in the lung allograft. *Transplantation*. 2019;103(8):1689–1699.
38. Eissens DN, Van Der Meer A, Van Cranenbroek B, Preijers FW, Joosten I. Rapamycin and MPA, but not CsA, impair human NK cell cytotoxicity due to differential effects on NK cell phenotype. *Am J Transplant*. 2010;10(9):1981–1990.
39. Meehan AC, Mifsud NA, Nguyen TH, et al. Impact of commonly used transplant immunosuppressive drugs on human NK cell function is dependent upon stimulation condition. *PLoS One*. 2013;8(3), e60144.
40. Kawakami T, Ito K, Matsuda Y, et al. Cytotoxicity of natural killer cells activated through NKG2D contributes to the development of bronchiolitis obliterans in a murine heterotopic tracheal transplant model. *Am J Transplant*. 2017;17(9):2338–2349.
41. Sun H, Greenland JR, Kopchaliiska D, et al. OR24 Recipient FCGR3A-158V homozygous genotype is associated with an increased risk of chronic lung allograft dysfunction. *Hum Immunol*. 2016;77:19–20.
42. Paul P, Pedini P, Lyonnet L, et al. FCGR3A and FCGR2A genotypes differentially impact allograft rejection and patients' survival after lung transplant. *Front Immunol*. 2019;10:1208.
43. Vadstrup K, Bendtsen F. Anti-NKG2D mAb: a new treatment for Crohn's disease? *Int J Mol Sci*. 2017;18(9):1997.
44. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. 2014;5:520.
45. Tait BD. Detection of HLA antibodies in organ transplant recipients – triumphs and challenges of the solid phase bead assay. *Front Immunol*. 2016;7(570):570.
46. Tait BD, Süsal C, Gebel HM, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. *Transplantation*. 2013;95(1):19–47.
47. Crinier A, Milpied P, Escalière B, et al. High-dimensional single-cell analysis identifies organ-specific signatures and conserved NK cell subsets in humans and mice. *Immunity*. 2018;49(5):971–986. e5.
48. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol*. 2013;31:227–258.
49. Shah PD, McDyer JF. Viral infections in lung transplant recipients. *Semin Respir Crit Care Med*. 2010;31(2):243–254.
50. Culley FJ. Natural killer cells in infection and inflammation of the lung. *Immunology*. 2009;128(2):151–163.
51. Nandagopal S, Li CG, Xu Y, Sodji QH, Graves EE, Giaccia AJ. C3aR signaling inhibits NK-cell infiltration into the tumor microenvironment in mouse models. *Cancer Immunol Res*. 2022;10(2):245–258.
52. Ruiz JE, Kwak JY, Baum L, et al. Effect of intravenous immunoglobulin G on natural killer cell cytotoxicity in vitro in women with recurrent spontaneous abortion. *J Reprod Immunol*. 1996;31(1-2):125–141.
53. Beam E, Lesnick T, Kremers W, Kennedy CC, Razonable RR. Cytomegalovirus disease is associated with higher all-cause mortality after lung transplantation despite extended antiviral prophylaxis. *Clin Transplant*. 2016;30(3):270–278.