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NKG2C natural killer cells in bronchoalveolar lavage are associated with cytomegalovirus viremia and poor outcomes in lung allograft recipients

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DISCLOSURES

The authors of this manuscript have no conflicts of interest to disclose.

AUTHORSHIP CONTRIBUTIONS

DRC and JRG participated in research design, manuscript writing, performance of research, and data analysis. JAG, JK, SRH, LLL, QT, and JPS participated in research design, interpretation of the results, and manuscript editing. TC, AW, and MG participated in performance of research.

ABBREVIATIONS

BAL, bronchoalveolar lavage

CI, confidence interval

CLAD, chronic lung allograft dysfunction

CMV, cytomegalovirus

FEV1, forced expiratory volume 1 second

FVC, forced vital capacity

IQR, interquartile range

KIR, killer cell immunoglobulin-like receptors

NK, natural killer

PBMC, peripheral blood mononuclear cell

QPCR, quantitative polymerase chain reaction assay

VL, viral load

ABSTRACT

Background: Cytomegalovirus (CMV) infection is a risk factor for chronic lung allograft dysfunction (CLAD), which limits survival in lung allograft recipients. Natural killer (NK) cells that express the NKG2C receptor mediate CMV-specific immune responses. We hypothesized that NKG2C$^+$ NK cells responding to CMV in the lung allograft would reduce CMV-related inflammation and would improve CLAD-free survival.

Methods: We prospectively followed 130 subjects who underwent lung transplantation from 2012 to 2016. Bronchoalveolar lavage (BAL) NK cells were immunophenotyped for NKG2C, maturation, and proliferation markers. CMV viral load, serologies, serial spirometry, and mortality were recorded from medical records. NK cell subset association with CMV endpoints were made using generalized estimating equation-adjusted linear models. BAL NKG2C$^+$ NK cell association with CLAD-free survival was assessed by Cox proportional hazards modeling.

Results: NKG2C$^+$ NK cells were more mature and proliferative than NKG2C- NK cells and represented a median of 7.8% of BAL NK cells. The NKG2C$^+$ NK cell proportion increased prior to the first detection of viremia and was nearly tripled in subjects with high level viremia (>1000 copies/ml) compared with no detected viremia. Subjects with increased BAL NKG2C$^+$ NK cells, relative to the median, had a significantly increased risk for CLAD or death (HR 4.2, 95% CI 1.2 – 13.3).

Conclusions: The BAL NKG2C^{+} NK cell proportion may be a relevant biomarker for assessing risk of CMV viremia and quantifying potential CMV-related graft injury that can lead to CLAD or death.

INTRODUCTION

Lung transplantation is a potentially life-prolonging therapy for patients with end-stage lung disease. However, lung allograft recipients have some of the poorest survival rates among solid organ transplants[1]. Chronic lung allograft dysfunction (CLAD) is the most common cause of death after the first year following lung transplant[2,3]. While alloimmune responses are believed to be central to the pathologies seen in CLAD, cytomegalovirus (CMV) infection is a well-established risk factor[2,4].

Among solid organ transplant recipients, lung allograft recipients have the highest rates of CMV infection and disease[5,6]. In the era of effective and safe CMV antivirals, the risk of CMV complications has been reduced. However, CMV infection continues to contribute to lung transplant morbidity both directly and indirectly[7-9]. CMV infection has been linked to risk of bacterial, herpesvirus, and fungal infections, as well as diabetes and vasculopathy[10]. The chronic immune responses required to control CMV infection may result in graft injury via direct antibody- and cell-mediated cytotoxicity, generation of heterologous alloimmune responses, and proinflammatory cytokines[11-13].

Natural killer (NK) cells are cytotoxic lymphocytes that are becoming increasingly recognized as important in the immune response to allografts[14,15]. For example, NK cells have been shown to drive rejection pathology in a mouse model of lung transplantation, even in the absence of T or B cells. In humans, NK cells are found in higher numbers in the settings of both acute cellular rejection and CLAD[16]. NK cells can also respond to nonself HLA antigens, regulate antigen presentation, and contribute to antibody-mediated rejection[17-22]. In contrast with T cells, whose

specificity is determined by diverse T cell receptors, NK cell effector function is dependent on integration of germline-encoded activating and inhibitory receptors[23,24]. As NK cells mature they undergo changes in their receptor repertoire, such as upregulating the low-affinity Fc fragment of IgG IIIa receptor, CD16, and killer cell immunoglobulin-like receptors (KIR), and downregulating the inhibitory receptor NKG2A[25].

NK cells impact CMV infection by direct cytotoxicity to CMV-infected cells, secretion of cytokines that modulate T and B cell responses to CMV, and by mediating antibody-dependent cellular cytotoxicity (ADCC) against CMV-infected cells coated with anti-CMV IgG antibodies[17,26,27]. NKG2C$^+$ NK cells, in particular, have been shown to expand following CMV viremia in solid organ transplant recipients and may control CMV viremia through a memory-like response[28-31]. This NKG2C receptor covalently bonds with the CD94 glycoprotein and noncovalently associates with the DAP12 signaling adapter to form a receptor complex that recognizes the invariant HLA-E protein as a ligand. This NKG2C signaling complex activates many of the same intracellular signaling pathways as a T cell receptor. Lung transplant subjects homozygous for the expressed allele in the *KLRC2* gene encoding NKG2C have less CMV viremia and disease compared to those with the null allele, and there is evidence for expanded NKG2C$^+$ NK cells in peripheral blood of lung transplant subjects with CMV+ allografts after CMV viremia[32,33]. We hypothesized that the proportion of BAL NKG2C$^+$ NK cells relative to total NK cells would increase in association with CMV infection, and that this cell proportion would be associated with subsequent decreased risk for CLAD or death.

MATERIALS AND METHODS

Study Population and Clinical Data

The UCSF institutional review board approved this study under protocol 13-10738. Bronchoalveolar lavage fluid (BAL) was prospectively collected and analyzed as part of routine clinical care for subjects who underwent transplantation between 11/15/2012 and 7/19/2016. We included all consenting adult subjects who received single lung, bilateral lung, or heart-lung allografts at University of California, San Francisco (UCSF) and received at least 1 adequate BAL sample within 15 months following transplantation. Samples were considered adequate if at least 50 NK cells were identified by flow cytometry. Our institution performs bronchoscopy with lavage and transbronchial biopsies as part of routine surveillance at 0.5, 1, 2, 3, 6, 12, 18, and 24 months after transplantation and then annually. Additional bronchoscopy procedures were performed when clinically indicated for suspicion of acute infection or rejection.

Chronic lung allograft dysfunction (CLAD) was defined according to established criteria as an unresolving 20% decline in FEV_1 or FVC lasting over 30 days[34] and determined from clinical records, as previously described[21,35]. CLAD-free survival was quantified as years of freedom from CLAD or death. Plasma CMV viral loads were measured in a CLIA-certified laboratory by PCR on peripheral blood samples as part of routine clinical care. Subject clinical data, obtained by chart review of UCSF records and from the United Network for Organ Sharing (UNOS) database, included recipient age at the time of transplantation as well as recipient ethnicity, sex, CMV serostatus, transplant indication, valgancyclovir dosing, acute rejection pathology findings,

BAL microbiology results and survival. Acute rejection was assessed and graded clinically according to established guidelines, and applied A or B scores > 1 applied as dichotomous variables[36]. Allografts were characterized as infected if BAL fluid microbiologic tests identified bacteria, viruses, fungi, or mycobacteria. Bacterial cultures were considered positive if cultures they demonstrated at least moderate growth, corresponding to 10^4 colony-forming units/mL of a dominant bacterial species. Viral infection was assessed by multiplex reverse transcription PCR with primers specific to 12 common viruses including influenza, parainfluenza, adenovirus, and respiratory syncytial virus.

Standard posttransplant induction regimens for all subjects included methylprednisolone, mycophenolate mofetil, and 20 mg intravenous basiliximab intraoperatively and on postoperative day 4. Initial maintenance immunosuppressant therapy included tacrolimus, prednisone, and mycophenolate mofetil. Tacrolimus was started on postoperative day 1 and dosed to a target trough level of 10-14 ng/ml for the first 3 months following transplantation. Tacrolimus troughs of 8-10 ng/ml were targeted during the first 3 to 12 months after transplant and 6-10 ng/ml thereafter. All subjects were started on 20 mg of prednisone daily after induction therapy. Prednisone was reduced at 3 months after transplant if surveillance bronchoscopies were negative for acute rejection to a goal dose of 0.1 mg/kg by 1 year. Mycophenolate mofetil at a target dose of 1000 mg twice daily was started on all subjects. Lower doses, alternate formulations, or azathioprine were used if subjects could not tolerate mycophenolate mofetil. Antifungal prophylaxis with posaconazole or voriconazole was initiated on postoperative day 1 and stopped after 90 days of treatment if bronchoalveolar lavage (BAL) surveillance fungal cultures were negative.

CMV protocol and definitions

Subjects were classified clinically as high risk (CMV Recipient-/Donor+), moderate risk (CMV R+/D+ or R+/D-), or low risk (CMV R-/D-) for CMV infection. All subjects received prophylaxis with intravenous ganciclovir or oral valgancyclovir. High and moderate risk subjects additionally received cytomegalovirus immune globulin intravenously following transplantation for 7 doses (150 mg/kg week 0, 100 mg/kg on weeks 2, 4, 6, 8, and 50 mg/kg on weeks 12 and 16)[37]. While the goal duration of prophylaxis was lifelong for all subjects, some subjects received shorter durations or interruptions when clinically indicated, such as in the case of leukopenia[38].

BAL lavage samples were analyzed for CMV by shell vial culture[39]. Plasma CMV monitoring by quantitative polymerase chain reaction assay (QPCR) was performed monthly per protocol, or more or less frequently at the providers' discretion. CMV QPCR laboratories were collected during the same clinical visits as the surveillance bronchoscopies. CMV QPCR results were categorized as negative, low (<1000 copies/mL), or high (>1000 copies/mL) consistent with prior published classifications[40]. Subjects that developed CMV infection were monitored weekly for CMV viral load and treated with at least 14 days of treatment-dose valgancyclovir or ganciclovir until 2 negative CMV QPCR tests. CMV infection was categorized as transient if CMV QPCR was elevated once and returned to negative following treatment during the first 2 years following transplant. CMV infection was categorized as persistent if CMV QPCR was elevated again following a transient infection.

BAL Immunophenotyping

We phenotyped NK cell subsets by flow cytometry in fresh BAL samples, as described previously[21]. A subset of 40 paired peripheral blood mononuclear cell (PBMC) samples selected for an even distribution of microbiologic study results that had been cryopreserved were thawed according to established protocols and phenotyped using the same protocol[41]. Samples were pretreated with saturating concentrations of human aggregated immunoglobulins to block nonspecific binding and viability dye (eBioscience, South San Francisco, CA, #65-0863-18). NK cell subsets were identified using: AL488-conjugated anti-NKG2C (clone 134591, R&D systems, Minneapolis, MN, #FAB138G-100), R-phycoerythrin (PE)-conjugated anti-KIR2D (clone NKVFS1, Miltenyi Biotec, San Diego, CA, #130- 092-688), PE-conjugated anti-KIR3D (clone Z27.3.7, Beckman Coulter, Brea, CA, #IM3292), peridinin-chlorophyll-protein complex (PerCP)-conjugated anti-KIR3DL1 (clone DX9, BioLegend, San Diego, CA, #312718), PE-Cy7-conjugated anti-CD56 (clone HCD56, BioLegend, #318318), AL700-conjugated anti-CD45 (clone HI30, BioLegend, #304024), and allophycocyanin-cyanine 7 (APC-Cy7)-conjugated anti-CD3 (clone SK7, BioLegend #344818). After washing, samples were acquired with a Beckman Coulter Navios cytometer and data were analyzed with the Kaluza software package (Beckman Coulter). Negative thresholds were set based on peripheral blood no-fluorochrome controls. Viable lymphocytes were identified by viability dye exclusion, $CD45^+$ expression, and forward scatter and side scatter size. NK cells were identified as $CD56^+CD3^-$ lymphocytes and categorized as immature ($CD16^-NKG2A^+$), double positive ($CD16^+NKG2A^+$), and mature ($CD16^+NKG2A^-$).

Statistical Analysis

NKG2C$^+$ NK cell proportion was defined as NKG2C$^+$ NK cells over total NK cells. Differences in the mean frequency of NK cell subsets expressing the NKG2C molecule and NK cells not expressing the NKG2C molecule were assessed using paired Student's *t* tests and graphically represented with violin and box and whisker plots. The distributions of data and regression coefficients were assessed by visual inspection, and log-normally distributed data were log transformed before statistical analysis. BAL and PBMC NK cells and NKG2C$^+$ NK cell subsets were analyzed with paired Student's *t* tests and Pearson product-moment correlation coefficients (ρ). CMV outcomes of interest were donor and recipient serostatus, maximum viral load, time from first detection of viremia, and transient versus persistent viremia. Comparisons between the proportions of NK cells expressing NKG2C and CMV outcomes of interest were made using generalized linear models (GLM), adjusted with generalized estimating equations (GEEs) with an exchangeable covariance matrix to account for repeated observations within subjects. The analyses of persistence and timing relative to first CMV viral load detection were restricted to subjects with high CMV viral loads of >1000 copies/mL.

Kaplan Meier plotting and Cox proportional hazards models were used to assess the difference in CLAD-free survival hazard ratios between subjects with higher than the median average frequency of NKG2C$^+$ NK cells compared to subjects with lower than the median average frequency of NKG2C$^+$ NK cells. The Schoenfeld test and visual inspection of the Kaplan Meier plots were used to assess for violations of proportional hazards. We applied generalized Type I censoring for all subjects. To prevent time-dependent bias of outcome variables, the survival data

were left truncated at 450 days after transplantation and subjects that died or developed CLAD before this interval were not included in the survival analysis. Models were adjusted to include subject characteristics frequently associated with poor transplant outcomes or NK cell frequencies: age at transplantation, recipient gender, recipient ethnicity, donor CMV serostatus, lung allocation score indication grouping (A: obstructive, B: pulmonary vascular, C: cystic fibrosis, and D: fibrotic, as referent group), CMV viremia and serostatus, allograft infection and acute cellular rejection. A Fine and Gray model was also applied to assess the competing risks of CLAD and death in this population. Missing data were imputed using 5-fold multiple imputation via chained equations for up to 5.5% of the covariates.

Statistical analyses and visualization were performed in R (R Foundation for Statistical Computing, Vienna, Austria) using packages "mgcv," "gee," "ggplot," "mice," and "ggpubr."

RESULTS

Population characteristics

Figure 1 shows the flow diagram of study enrollment. There were 307 bronchoscopies included in this study, which occurred a median 92 (interquartile range, IQR 84-323) days after lung transplant. The characteristics of the 130 subjects who were included in the study can be found in Table 1. There were 106 subjects with high or moderate risk for CMV infection based on donor and recipient serostatus, and only 2 subjects had CMV risk that could not be determined because serologic data were unavailable. Forty-two subjects developed CMV viremia during the course

of the study with 50% exceeding viral loads >1000 copies/mL. The median peak CMV VL (viral load) in the 21 subjects over the 1000 copies/mL threshold was 4900 (IQR 1800-50 000) copies/mL. The median time to first high level CMV viremia (>1000 copies/mL) was 379 (IQR 254-880) days with 12 subjects developing transient viremia and 9 subjects with persistent viremia. Only 2 subjects had positive BAL CMV viral cultures during the study period.

Mature and immature NK cells were prevalent in BAL

$CD56^{+}CD3^{-}$ NK cells represented 9% (IQR 5-15%) of the isolated viable lymphocytes. Among NK cells, 7.2% (IQR 3-16%, mean 14.1%) were $NKG2C^{+}$. CD16 and NKG2A defined 4 distinct populations of BAL NK cells. Figure 2 illustrates $NKG2C^{+}$ and $NKG2C^{-}$ NK cells stratified by these markers. Compared to the 46.5% (IQR 31-59%) of total NK cells that were immature ($NKG2A^{+}CD16^{-}$), 14% (IQR 7-26%) of the population were mature ($NKG2A^{-}CD16^{+}$). KIR2D/3D receptors were most strongly expressed on mature cells, and 18% of the NK cell population expressed high levels of KIR. Mature cells were also the most proliferative subset with 2% (IQR 0-6%) expressing Ki67.

$NKG2C^{+}$ NK cells were more active and proliferative

Relative to $NKG2C^{-}$ NK cells, the percentage of $NKG2C^{+}$ NK cells that were mature was greater (25.8% vs 18.4%, $p < 0.0001$). No difference was observed in the proportion of NKG2C on double positive NK cells ($NKG2A^{+}CD16^{+}$), but the percent of $NKG2C^{+}$ NK cells that were immature was decreased (31.1% vs 45.9%, $p < 0.0001$) relative to $NKG2C^{-}$ NK cells. Further, an

increased proportion of NKG2C$^+$ NK cells stained positive for the proliferation marker Ki67 (12.6% vs 8.1%, p<0.001) and had high levels of KIR expression (37.4% vs 20.8%, $p < 0.0001$).

NKG2C$^+$ NK cells in peripheral blood and BAL were positively correlated but phenotypically distinct

Figure 3 depicts a paired analysis of BAL and peripheral blood NK cell immunophenotypes in a subset of 40 subjects. We found no difference in the overall frequency of NK cells in BAL (10.5%) compared to PBMC (9.2%, $p = 0.1$, Figure 3A) and no correlation between these 2 populations of cells ($\rho = 0.22$, 95% CI -0.1 – 0.5). Similarly, there was no difference observed in frequencies of NKG2C$^+$ NK cells in BAL (14.6%) and PBMC (Figure 3B, 16.7%, $p = 0.5$), but these 2 populations were strongly correlated ($\rho = 0.63$, 0.4 – 0.8).

Phenotypically, NKG2C$^+$ NK cells in PBMC were relatively more mature (CD16$^+$NKG2A$^-$) (Figure 3C). Correspondingly, BAL NKG2C$^+$ NK cells were relatively more immature (CD16$^-$ NKG2A$^+$), as shown in Figure 3D. There were significant correlations in frequencies of mature NKG2C$^+$ cells ($\rho = 0.68$, 0.5 – 0.8) and immature NKG2C$^+$ NK cells ($\rho = 0.33$, 0.1 – 0.6) between BAL and PBMC. Expression of KIR molecules on NKG2C$^+$ NK cells did not differ between the 2 groups (Figure 3E, $p = 0.7$), but were highly correlated ($\rho = 0.76$, 0.6 – 0.9). Finally, there was less cell proliferation as measured by Ki67 in BAL (0.4%) compared to PBMC (2%, p=0.001, Figure 3F).

NKG2C$^+$ NK cell frequencies increased in association with CMV exposure

We found no difference in the number of subjects who developed viremia while taking valgancyclovir and those with viremia while off valgancyclovir (χ^2= 2.8, p = 0.25). Compared to subjects who never demonstrated CMV viremia (NKG2C proportion 10.2%), we observed increased frequencies of NKG2C$^+$ NK cells in subjects with maximum CMV viral loads >1000 copies/mL (29%, 95% CI 15.8 – 42.1%, p = 0.0001, Figure 4A). No difference in NKG2C proportion were observed between subjects with maximum viral loads <1000 copies/mL (15.5%, 95% CI 6.1 – 24.9%, p = 0.15) and those with high CMV viral loads (>1000 copies/mL) or no CMV viremia. Similarly, 16% of BAL NK cells from subjects with CMV positive donor allografts were positive for NKG2C compared to an average of 10.6% of NK cells in subjects with CMV-negative allografts (p = 0.05, Figure 4B).

We examined the relationship between NKG2C$^+$ NK cell proportion and recurrent CMV infection. There was no difference in NKG2C$^+$ NK cell proportion between subjects with a single episode of CMV viremia and the subjects that had recurrent episodes of CMV viremia (Figure 4C, p = 0.29). In the 21 subjects that developed high levels of viremia, the proportion of NKG2C$^+$ NK cells increased by 12.3% per absolute number of years from the time of first viremia (Figure 4D, 95% CI 7.8% – 16.7%, p < 0.0001), with increases in NKG2C$^+$ NK cell frequencies largely preceding detection of viremia.

Increased BAL NKG2C is associated with decreased CLAD-free survival

Figure 5 shows a Kaplan-Meier survival plot demonstrating differential CLAD-free survival for subjects stratified based on the average proportion of $NKG2C^+$ NK cells detected in BAL as higher or lower than the median of 7.8% (log-rank $p = 0.01$). The group with the higher $NKG2C^+$ NK cell proportion had a hazard ratio for CLAD or death of 4.2 (95% CI 1.2 – 13.3) adjusting for baseline characteristics, CMV viremia and serostatus, graft infection, and acute rejection. We also assessed $NKG2C^+$ NK cell proportion as a continuous variable and observed an adjusted hazard ratio for CLAD or death of 1.2 (95% CI 1.05-1.43) per 10 percent increase in maximum BAL $NKG2C^+$ NK cell proportion. A Fine and Gray cumulative incidence model of CLAD with death as a competing risk, including baseline characteristics, showed an almost identically increased risk of CLAD in the subjects with higher median $NKG2C^+$ NK cells (HR 4.2, 1.4-12.1, $p = 0.009$). CMV viremia was also associated with an increased risk of CLAD or death in univariable analysis (HR 2.1, 1-4.5, $p = 0.05$), but no difference in CLAD-free survival was observed based on donor CMV serostatus (1.2 HR, 0.5 – 2.8, $p = 0.71$).

DISCUSSION

In summary, we found that $NKG2C^+$ NK cells were prevalent in BAL fluid and that these cells were more mature than $NKG2C^-$ NK cells. Frequencies of BAL and PBMC $NKG2C^+$ NK cells were correlated, but BAL $NKC2C^+$ NK cells were less mature and proliferative than $NKG2C^+$ NK cells from PBMC. The NK $NKG2C^+$ cell percentage was elevated in many cases before the

detection of CMV viremia, and higher BAL NKG2C$^+$ NK cell percentage portended an increased risk of CLAD or death.

These data are consistent with previous reports implicating the importance of the NKG2C$^+$ subset of NK cells in the control of CMV infection[29,31,42]. Prior studies show expansion of this subset following detection of viremia in the blood. The detection of increasing NKG2C$^+$ NK cells in BAL prior to detection of viremia raises the possibility that viremia could be secondary to an inadequate CMV immune response in the allograft. Indeed, CMV replication in the lung allograft is common and increased rates of replication have been associated with increased risk for CLAD[4].

We had postulated that increased NKG2C$^+$ NK cell responses might identify patients with superior CMV control, perhaps for whom CMV prophylaxis could be withdrawn early, based on studies in other contexts showing an association between higher numbers of these cells in the peripheral blood and decreased risk of recurrence of CMV viremia[32,43]. However, these data suggest the opposite: that subjects with a high BAL NKG2C$^+$ NK cell proportion were at significant risk of both new and recurrent CMV viremia. As CMV serostatus and CMV viremia are imprecise markers of the CLAD and death risk associated with CMV disease, the proportion of NKG2C$^+$ NK cells may be better at predicting CLAD or death risk than CMV serostatus or viremia alone[44,45]. Quantifying BAL NKG2C$^+$ NK cells as a potential marker of CMV-associated disease burden may be important for identifying lung transplant subjects who could even benefit from additional antiviral medication, as CMV BAL QPCR measurements do not reliably predict

CMV viremia and early CMV control may protect from episodes of acute and chronic rejection[46,47].

The correlations between BAL and PBMC NKG2C$^+$ NK cell frequency and maturation status implies that PBMC analyses have the potential to provide similar information with respect to CMV-specific immune responses. However, BAL NKG2C$^+$ NK cells were substantially less mature, when compared with PBMC. This difference may reflect BAL-specific suppression of NK cell activation, as has been previously attributed to high concentrations of TGF-β found in lavage fluid[48]. Ki67 staining was also significantly lower in BAL compared with PBMC, although higher on NKG2C$^+$ versus NKG2C$^-$ NK cells, when present. These maturation and proliferation data would be consistent with a model where ligation of NKG2C on immature bone marrow-derived NK cells occurs in the lung, but activation and expansion are delayed until these cells egress into the periphery. At the same time, NK cell populations within the allograft parenchyma could differ from those found in BAL fluid. As PBMC were cryopreserved, we cannot exclude an effect of storage conditions on the observed differences between compartments.

Our center uses a lifelong CMV antiviral dosing strategy when possible, which, while not unique, differs from consensus guidelines[49,50]. This strategy and other center-specific protocols, may limit the generalizability of these findings and might explain the relatively low frequency of CMV viremia. The significance of a potent CMV-specific NK cell response in the BAL could even be exaggerated with less aggressive CMV prophylaxis protocols. Similar rates of viremia between subjects on and off valganciclovir may indicate reduced efficacy of prolonged CMV

prophylaxis or reflect a greater hesitancy in higher risk subjects to reduce prophylaxis in the face of side effects.

These data have some additional limitations: Our modeling of $NKG2C^+$ NK cell effect on CLAD-free survival may be incomplete as we did not control for some known confounders such as primary graft dysfunction or gastroesophageal reflux, in addition to potential unknown confounders. Non-CMV infections could also potentially contribute to $NKG2C^+$ NK cell activation; though, we did not see a difference in CLAD-free survival in our models controlling for graft infections. $NKG2C^+$ NK cells have been reported to expand in the setting of other infections such as HIV and Hantavirus, but only in individuals who were also CMV infected[51,52]. This cohort has some heterogeneity in follow-up of CMV QPCR timing relative to the BAL surveillance as providers may have modified the timing of CMV QPCR measurements outside of the bronchoscopy visits. CMV-associated complications at our center are historically infrequent, which differs from other centers' reports and may reflect specific differences in our treatment or monitoring regimens[38]. Finally, CMV BAL QPCR has better test characteristics and would have been a more sensitive assay than CMV shell vial culture for assessing $NKG2C^+$ NK cell associations with lavage CMV burden.

At the same time, these data also have noteworthy strengths. The prospective study design minimized bias in subject selection and the long follow-up period allowed for analyses of CLAD and survival outcomes. Multiple bronchoscopy specimens were available for most subjects throughout the duration of the posttransplant period which allowed for modeling of NK cell phenotypes and CMV outcomes over time.

In summary, we identified NKG2C$^+$ NK cells as a potential marker of CMV allograft replication and immune activation. If these findings are confirmed in other cohorts, measurement of NKG2C$^+$ NK cells from BAL may have the potential to identify lung allograft recipients who are at increased risk for poor long-term outcomes potentially related to CMV and who may benefit from additional antiviral therapy or closer monitoring.

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FIGURE LEGENDS

Figure 1: Consort diagram showing study enrollment and follow up.

Figure 2: $NKG2C^+$ NK cells are more mature than $NKG2C^-$ NK cells. NK cell immunophenotypes are shown with $NKG2C^+$ cells in blue and $NKG2C^-$ cells in red. (A) NK cells were identified as $CD56^+CD3^-$ viable lymphocytes. (B) NK cells were subsequently stratified as mature ($CD16^+NKG2A^-$) or immature ($CD16^-NKG2A^+$). Histograms show representative cell counts versus level of Ki67 (C) and KIR2D or KIR3D expression (D), stratified by NKG2C status. Violin plots compare $NKG2C^+$ and $NKG2C^-$ distributions across the entire cohort for the proportions of (E) immature ($CD16^-NKG2A^+$), (F) mature ($CD16^+NKG2A^-$), (G) proliferative ($Ki67^+$), and (H) KIR2D or KIR3D expressing NK cells. P values were determined by Student's *t* test. *Ki67 percentages were log transformed, which normalized the data and removed zero values, prior to statistical comparison. Ki67 percentages across NKG2C strata were not different by nonparametric testing.

Figure 3: Paired comparisons of NK cell frequency and phenotypes between BAL (dark blue) and PBMC (light blue). (A) NK cell frequencies did not differ between BAL and PBMC (p = 0.1). (B) Frequencies of $NKG2C^+$ NK cells were also similar between the 2 compartments (p = 0.5). (C) There were less mature cells in BAL compared to PBMC (p = 0.01), and (D) more immature $NKG2C^+$ NK cells in BAL compared to PBMC (p <0.001). (E) KIR expression on $NKG2C^+$ NK cells was similar between BAL and PBMC (p = 0.7). (F) There were less $Ki67^+$ $NKG2C^+$ NK cells in BAL compared to PBMC (p = 0.001).

Figure 4: Association between NKG2C$^+$ NK cell proportion and CMV status. (A) The percentage of NK cells expressing NKG2C is shown versus maximum viral load of undetected, <1000 copies/mL (low), and >1000 copies/mL (high). The BAL NKG2C$^+$ NK cell proportion was increased in subjects with high vs. no viremia (p = 0.001). (B) The proportion of BAL NKG2C$^+$ NK cells are shown stratified by pretransplant donor and recipient anti-CMV antibody status. The comparison between donor seropositive and donor seronegative is shown (p = 0.05) For subjects who experienced high-level viremia, the proportion of BAL NKG2C$^+$ NK cells are shown stratified by whether viremia was observed on 1 occasion (transient) or detected multiple times (persistent, p = 0.29). (D) Locally weighted scatterplot with smoothing (LOWESS) showing NKG2C$^+$ NK cell proportion relative to the first time of high level CMV viremia. NKG2C$^+$ NK cells in BAL increased inversely with time from first detection of viremia (p<0.0001).

Figure 5: Time to CLAD or death stratified by average BAL NKG2C$^+$ NK cell proportion within the first 15 months as greater (red) or less (blue) than the median. Subjects with greater than the median proportion of NKG2C$^+$ NK cells had significantly decreased CLAD-free survival compared to subjects with a lower BAL NKG2C$^+$ NK cell proportion (log rank p = 0.01).

Figure 1

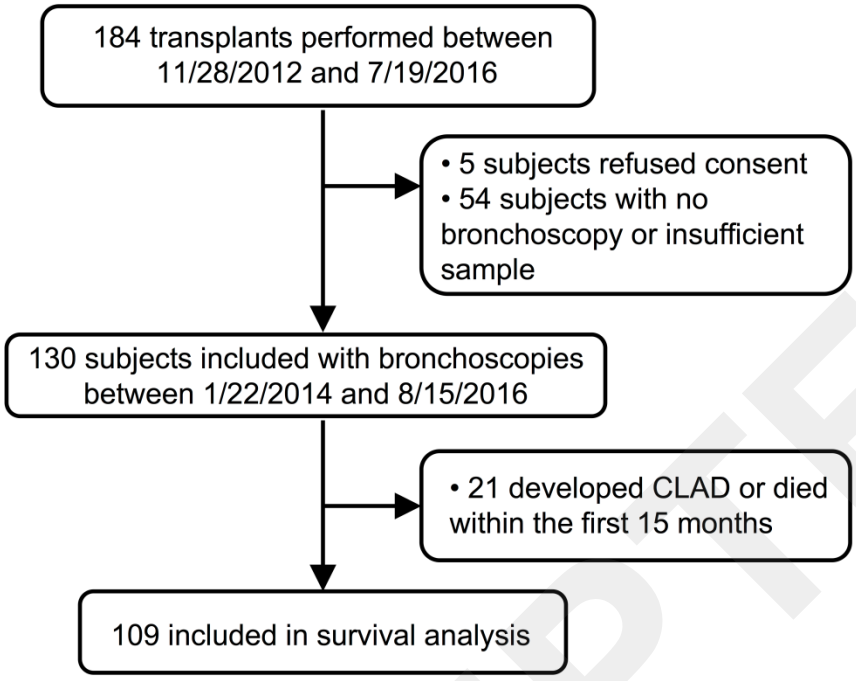


Figure 2

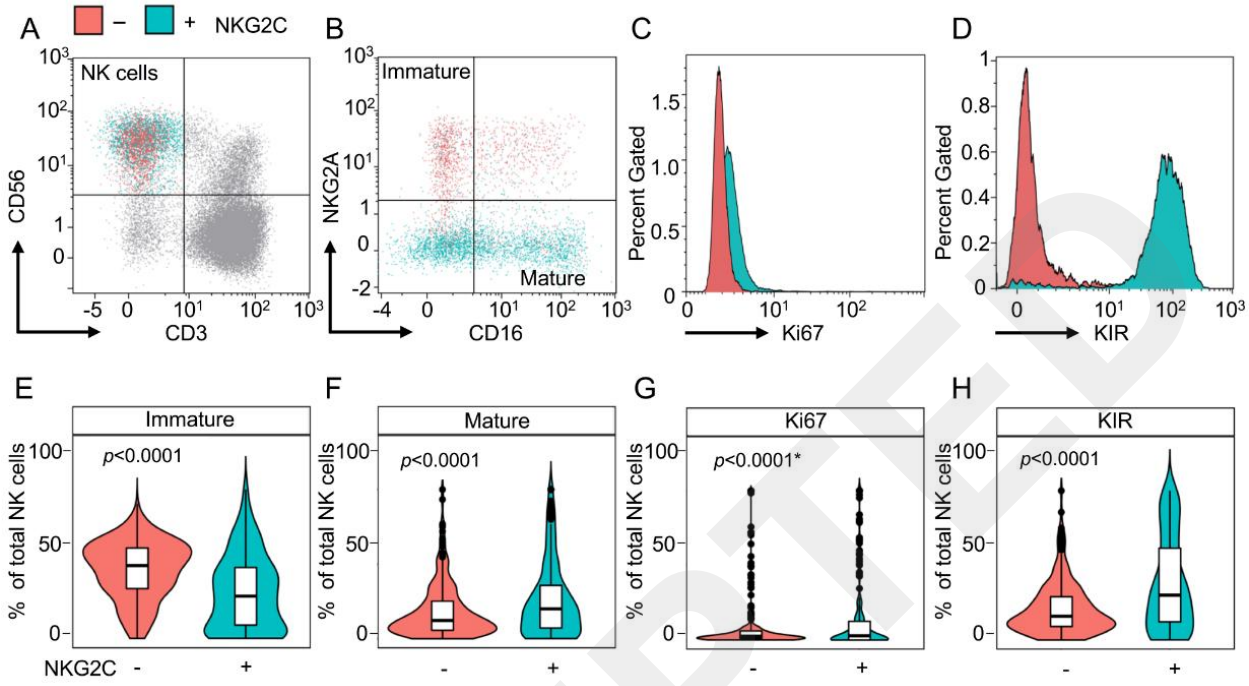


Figure 3

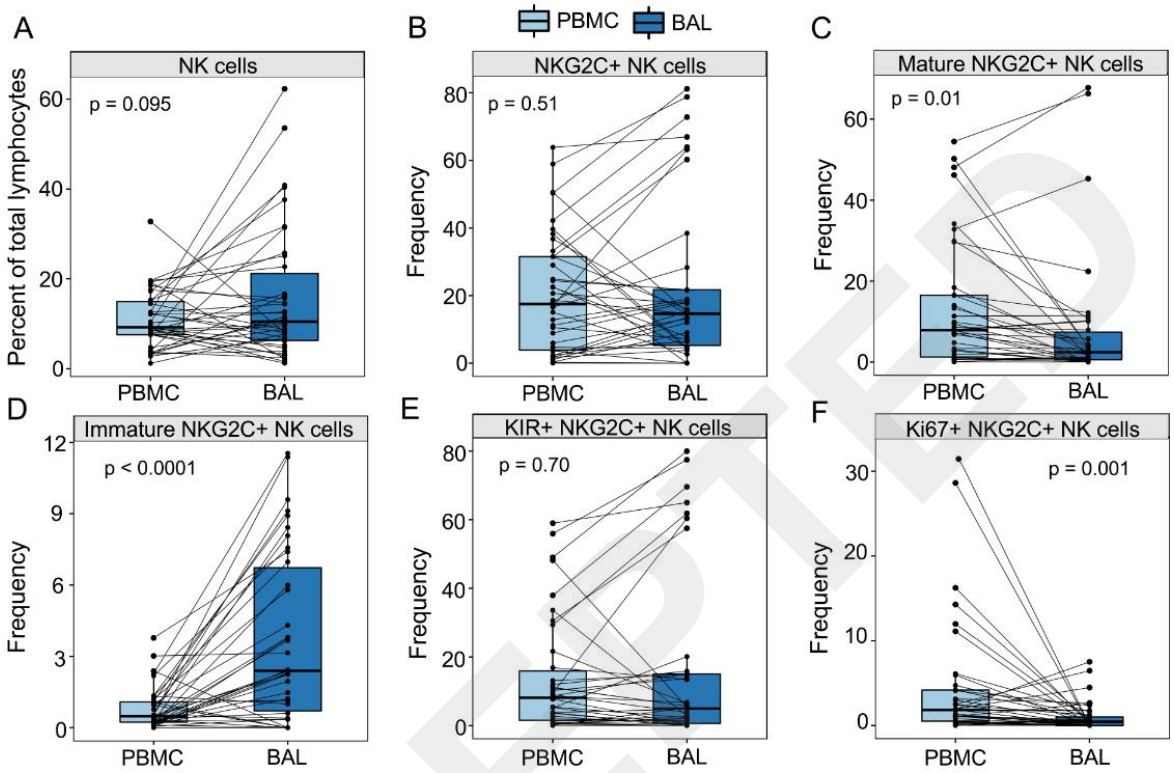


Figure 4

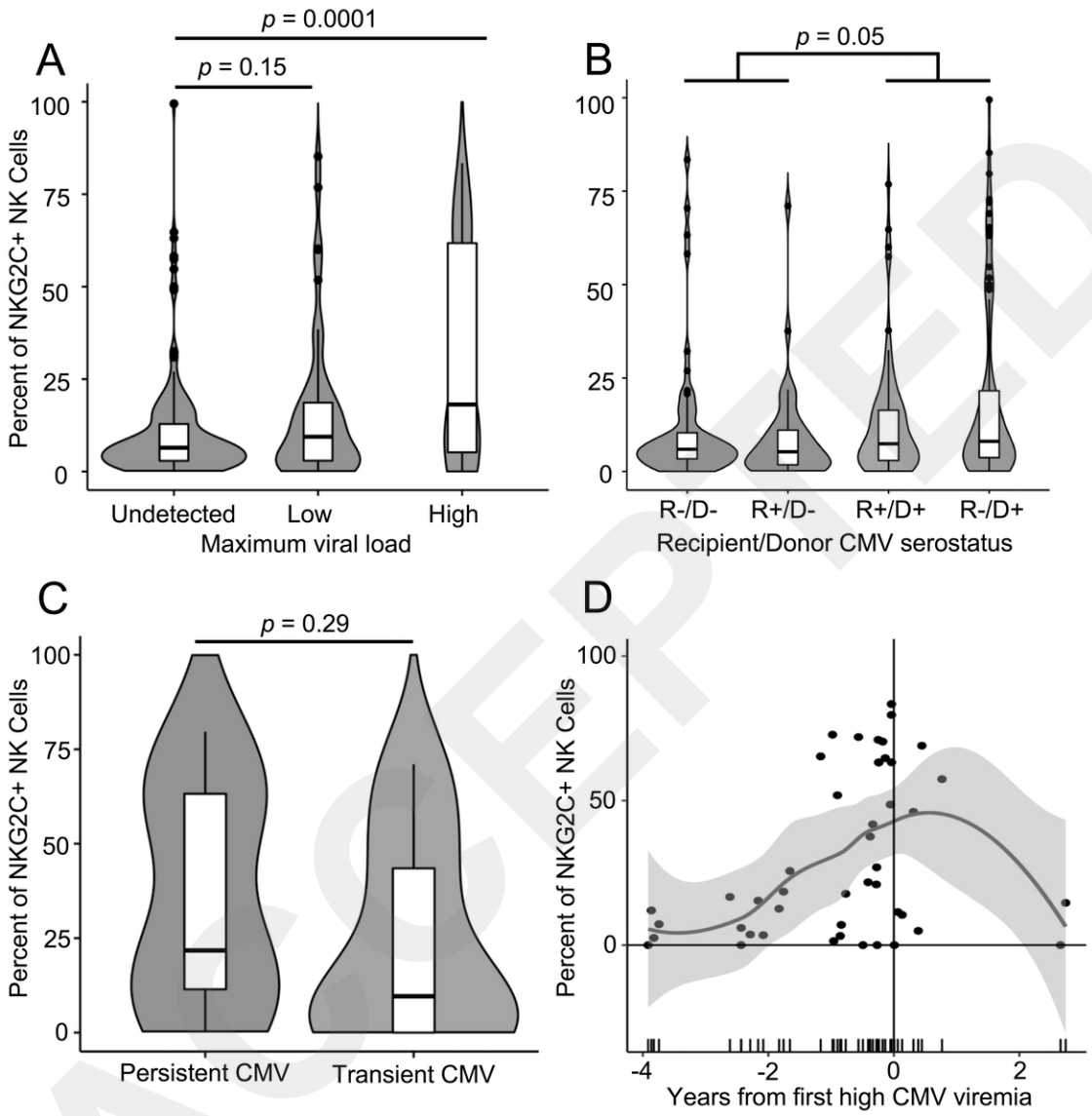
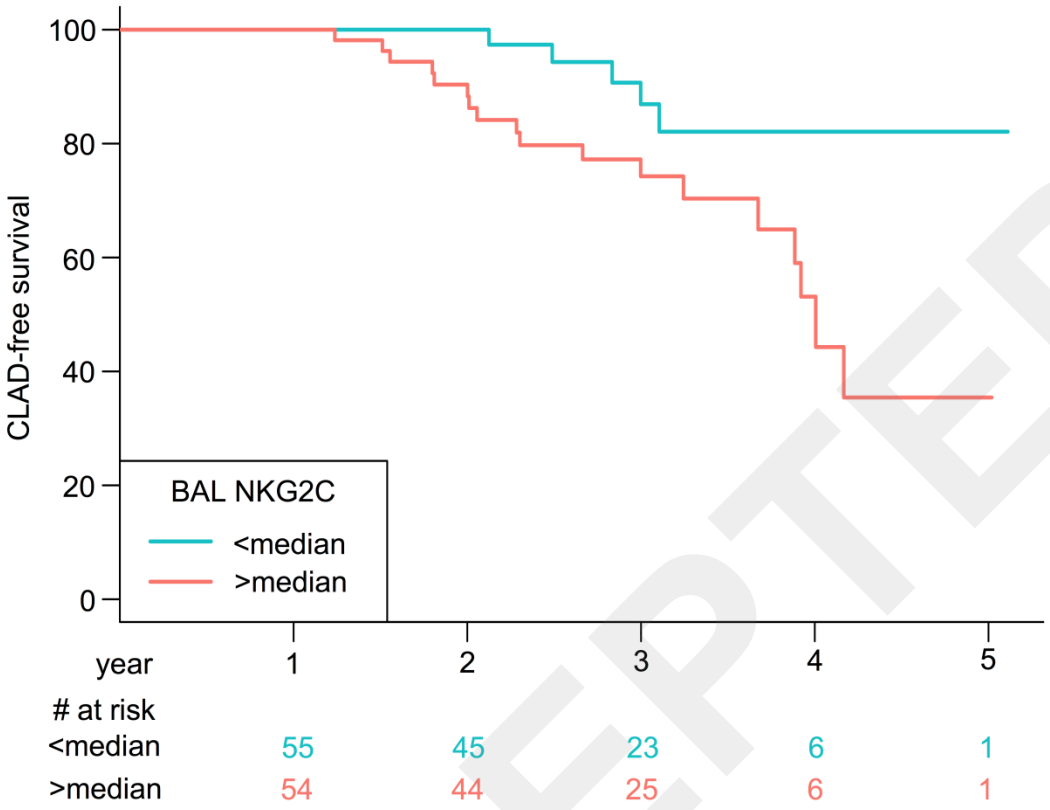


Figure 5



TABLES

Table 1: Subject baseline characteristics

Subjects (n)	130
Age at transplant, mean ± SD (years)	56 ± 12
Male sex (%)	61
Transplant type: N (%)	
Double	116 (89.2)
Heart and Lung	12 (9.2)
Single	2 (1.5)
Race/Ethnicity: N (%)	
Caucasian	93 (71.5)
African American	15 (11.5)
Hispanic	18 (13.9)
Other	4 (3.1)
Indication: N (%)	
A (COPD)	16 (12.3)
B (Pulmonary Hypertension)	3 (2.3)
C (CF)	15 (11.5)
D (Pulmonary Fibrosis)	96 (73.9)
CMV status: N (%)	
R-/D-	22 (16.9)
R+/D+	42 (32.3)
R+/D-	26 (20)
R-/D+	38 (29.2)
Unknown	2 (1.5)
Mean valgancyclovir dose: mg/d	488
CMV peak viral load: N, median (IQR)	
<1000 copies/mL	21, 326 (175 – 569)
>1000 copies/mL	21, 4927 (1818 – 50400)
Frequency of CMV VL: median (IQR)	75 (53 – 127)