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Author manuscript

*Am J Transplant.* Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

*Am J Transplant.* 2019 December ; 19(12): 3284–3298. doi:10.1111/ajt.15505.

## Absence of evidence that respiratory viral infections influence pediatric lung transplantation outcomes: results of the CTOTC-03 study

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### Abstract

Based on reports in adult lung transplant recipients, we hypothesized that community acquired respiratory viral infections (CARV) would be a risk factor for poor outcome after pediatric lung transplant. We followed 61 pediatric lung transplant recipients for 2+ years or until they met a composite primary endpoint including bronchiolitis obliterans syndrome (BOS)/obliterative bronchiolitis, re-transplantation or death. Blood, bronchoalveolar lavage and nasopharyngeal specimens were obtained with standard of care visits. Nasopharyngeal specimens were obtained with respiratory viral symptoms. Respiratory specimens were interrogated for respiratory viruses using multiplex PCR. Donor-specific HLA antibodies, self-antigens, and Elispot reactivity were also evaluated. Survival was 84% (1 year) and 68% (3 years). BOS incidence was 20% (1 year) and 38% (3 years). The primary endpoint was met in 46% of patients. CARV was detected in 156 patient visits (74% enterovirus/rhinovirus). We did not find a relationship between CARV recovery from respiratory specimens and the primary endpoint (HR 0.64 (0.25, 1.59), P=0.335) or between CARV and the development of allo- or autoimmune humoral or cellular responses. These findings

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Disclosure

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

Data Availability Statement

The data supporting this publication will be available at ImmPort ([immport.org](http://immport.org)) under study accession SDY960-Viral Triggers of Alloimmunity and Autoimmunity in Pediatric Lung Transplantation<sup>64</sup>

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of this article.

raise the possibility that the immunologic impact of CARV following pediatric lung transplant is different than that observed in adults.

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## Introduction

Advancements in recipient selection, surgical techniques, evolving immunosuppressive regimens and more effective infection prophylaxis over the past 25 years have led to improved perioperative survival in pediatric lung transplant recipients (LTRs). However, long- term graft and patient survival has not improved substantially over the same period and remains significantly worse than outcomes for recipients of heart, liver and kidney allografts. Beyond the first post-transplant year, chronic lung allograft dysfunction (CLAD) is the most common cause of graft failure. The incidence of bronchiolitis obliterans syndrome (BOS), the most common form of CLAD, is 50% at 5 years in children and roughly 80% between 5 and 10 years in adults<sup>1-3</sup>. Between 25 and 40% of lung transplant recipients will die directly or indirectly from CLAD<sup>3</sup>. Although re-transplantation for CLAD is feasible, its utility is limited by organ availability and poorer survival after second transplant<sup>4-6</sup>.

Although the etiology of CLAD remains unclear, a common theme throughout the spectrum of clinical risk factors including primary graft dysfunction, recurrent and/or severe acute cellular rejection (ACR), viral infections including cytomegalovirus (CMV), bacterial infection (particularly *Pseudomonas aeruginosa*), fungal infection and gastroesophageal reflux is allograft injury. Acute and chronic lung allograft injury in adults were also associated with the development of autoantibodies (AutoAb) in cross sectional studies<sup>7</sup>. Given accumulating data suggesting a role for both donor- specific anti-human leukocyte antigen (HLA) antibodies (DSA) and autoantibodies reactive to collagen type V (ColV) and k-alpha 1 tubulin (K $\alpha$ 1T) in the development of CLAD, it is possible that the development of DSA and AutoAb in response to allograft injury serves as a common pathway.<sup>7,8</sup> The mechanistic link between antibody formation/binding and chronic graft injury remains unclear, although complement mediated injury, cytokine/macrophage mediated injury, and direct effects of antibody binding on endothelial or epithelial growth/proliferation may be involved<sup>9-13</sup>.

Community acquired respiratory viral infections (CARV) are frequently reported after lung transplantation and cause significant morbidity. Studies of viral isolation from adult LTRs have identified infection with both established respiratory viruses such as rhinovirus, respiratory syncytial virus (RSV), parainfluenza virus and influenza virus<sup>14-18</sup>, as well as emerging respiratory viruses, including human metapneumovirus and human coronavirus<sup>17,19,20</sup>. CARV are associated with poor long-term outcomes in adult LTRs<sup>21-25</sup>.

Based on these findings we hypothesized that CARV infections directly activate the immune system by inducing a pro-inflammatory environment and thus could a) directly damage the lung allograft, b) augment innate immunity leading to direct lung allograft damage, c) augment innate immunity leading to enhanced adaptive cellular and humoral allo- and or auto-immunity and resultant lung allograft injury, and, d) induce anti-viral immunity that could cross react with lung allograft antigens to mediate graft injury (heterologous immunity).

In spite of accumulating evidence in adults, the role CARV play in the long-term outcome of pediatric LTRs remains uncertain. A retrospective analysis of CARV within the first year after transplantation found an association with death but not BOS in a cohort of nearly 600 pediatric LTRs <sup>26</sup>. Moreover, there have been no reports supporting a relationship between T cell responses and the development of DSA or AutoAb in pediatric LTRs, or a relationship between DSA and/or AutoAb and CLAD in pediatric LTRs.

We conducted a prospective, longitudinal study of pediatric LTRs to test the hypothesis that CARV increase the risk of meeting a composite endpoint consisting of BOS, obliterative bronchiolitis (OB), death or re-transplantation in pediatric LTRs. A companion mechanistic study assessed the relationship between CARV and immunologic events known to be associated with CLAD development.

## Methods

### Study Development and Oversight

The Clinical Trials in Organ Transplant in Children study (CTOTC-03, ) enrolled patients at six pediatric institutions in the United States from 2009 to 2013. CTOTC-03 was the first prospective, longitudinal study of pediatric LTRs. This prospective multicenter observational trial had a target accrual of 80 pediatric lung or heart-lung transplant recipients (78 enrolled). The CTOTC-03 protocol development team was led by Drs. Sweet and Danziger-Isakov. Clinical and/or laboratory data were contributed by Drs. Heeger, Mohanakumar and Storch. Medical safety oversight was provided by Dr. Jonah Odum. Data analysis was the responsibility of Karen Kesler and Hyunsook Chin (with the CTOTC-03 team). Data were collected by the investigators and coordinators at each study site. All authors vouch for data accuracy and completeness. Each site participated under the auspices of its Institutional Review Board. An independent, NIAID-appointed Data Safety Monitoring Board was responsible for periodic safety review. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) ().

### Subjects

Candidates for first single or bilateral lung or heart-lung transplant who were less than or equal to 19 years of age at enrollment were considered for inclusion pending appropriate informed consent. We excluded multi-organ transplant recipients (aside from heart-lung) and recipients with a condition or characteristic which, in the opinion of the local primary investigator, made the participant unlikely to complete the study.

### Endpoints

We tested the hypothesis that CARV increase the risk of meeting a composite endpoint consisting of BOS, obliterative bronchiolitis (OB), death or re-transplantation in pediatric LTRs. We chose this composite endpoint to increase the power to detect an impact, postulating that CARV result in direct and indirect allograft injury leading to expanded graft-reactive T and B cell immunity and the development of autoimmunity through the unmasking of cryptic autoantigens. We also evaluated the impact of risk factors associated with outcomes in adults such as primary graft dysfunction (PGD) and acute cellular rejection (ACR).

Subjects were followed for at least two years or until they met one or more components of the composite endpoint which included death, re-transplantation or meeting criteria for BOS grade 0-p or 1<sup>27</sup> or the finding of OB on lung biopsy. The endpoint included BOS<sup>27</sup> because this study was performed prior to CLAD becoming accepted terminology<sup>28</sup>, but all patients identified using the 2001 BOS criteria would meet current CLAD criteria. Subdivision between those with obstructive and restrictive CLAD was not performed.

## Study Design

Clinical data (including pulmonary function testing and imaging studies) was collected at scheduled visits including: pre-transplant, at transplant (relevant donor demographics were also collected at transplant) and at post-transplant weeks 2, 4, 6, 8 and months 3, 6, 9, 12, 18 and 24. Blood, NP and BAL specimens were obtained at these visits for viral testing and assessment of immunity. Subjects who developed clinical symptoms consistent with CARV had a nasopharyngeal (NP) swab obtained as well as blood and BAL samples if bronchoscopy was performed as part of clinical care. Standard of care included sending BAL specimens (obtained during bronchoscopies performed for routine surveillance or with symptomatic episodes) to the local laboratory to assess for routine bacterial, fungal, mycobacterial, viral and opportunistic organisms. When BAL was not performed with a symptomatic episode, standard of care generally included sputum culture.

Subjects underwent routine pre-transplant evaluation. Post-transplant management followed International Pediatric Lung Transplant Collaborative (IPLTC) guidelines (see supplementary materials). Use of an induction agent was at the discretion of the transplant center: two centers utilized an IL-2 antagonist (42 subjects), two used rabbit anti-thymocyte globulin (10 subjects), one used both an IL-2 antagonist and rabbit anti-thymocyte globulin (6 subjects), and one administered no induction therapy (3 subjects). All subjects received triple drug maintenance immunosuppressive therapy with tacrolimus, mycophenolate and steroids, with standardized levels for tacrolimus. Pneumocystis, fungal and CMV prophylaxis were determined by the local center. Treatment of viral infection detected locally was at the discretion of the treating physician. Viral testing of specimens sent to the central laboratories was performed in batches. Therefore, the clinical sites were not informed of these results.

Clinical data was collected from each site using standardized forms. Primary graft dysfunction was determined based on published criteria<sup>29</sup>. Bacterial, fungal, viral and mycobacterial systemic and respiratory tract Infections meeting International Society for Heart and Lung Transplantation (ISHLT) working formulation criteria<sup>30</sup> were captured based on local clinical data interpretation and intervention as determined by the clinical site primary investigator (PI). After verifying adequate correlation of local biopsy readings with readings made by two core pathologists, local pathology readings were used for histologic determination of acute, humoral and chronic rejection. Centers submitted all pulmonary function data to the data coordinating center (including those obtained during scheduled visits and with acute events). Based on all PFT data, a tentative BOS grade was assigned using an algorithm based on published criteria<sup>27</sup>. The local site PI was asked to verify that no concomitant conditions excluding BOS were present before finalizing the BOS grade.

## Laboratory Studies

**Virology PCR**—BAL and NP swab samples obtained at the time points specified above were analyzed in the CTOTC-03 virology core laboratory using the xTAG Respiratory Virus Panel manufactured by Luminex Corp. (Austin, TX) following the manufacturer's instructions. A research use only panel was used, which included assays for the following viruses: influenza A, influenza A H1, influenza A H3, influenza B, respiratory syncytial virus A and B, parainfluenza 1–4, enterovirus/rhinovirus, coronaviruses OK43, 229E, NL63, and 229E, metapneumovirus, and adenovirus. Samples with mean fluorescence index (MFI)

1000 were considered positive. Samples with MFI 300–999 were retested and considered positive if the repeat MFI was  $\geq$  300. If the MFI of the repeat test was  $<$ 300, the test was repeated a second time, and the sample was considered positive if the MFI of the second repeat test was  $\geq$  300. CMV and Epstein-Barr virus in whole blood and BAL were also assessed using PCR.<sup>31</sup> for inclusion in the assessment of the relationship between immunosuppression and viral infection.

**Assessment of Humoral Immunity**—Anti-HLA alloantibody testing was performed using Luminex assays.<sup>32</sup> DSA were determined by comparing HLA antibody specificities to the donor HLA. Detection of autoantibodies to type V collagen (colV) and K-alpha 1 tubulin (K $\alpha$ 1T) were performed by ELISA methodology using vimentin and myosin as controls.<sup>8</sup>

**Assessment of Cellular Immunity**—Cellular alloreactivity was assessed by cytokine (interferon gamma, IFN $\gamma$ ) enzyme linked immunosorbent spot assays (ELISPOT) using donor B cell lines expanded using CD40L transfected fibroblasts and IL-4 as described<sup>33</sup>. Specimens with  $>$  25 positive cells per 300,000 PBL were considered positive. For T cell autoreactivity, peripheral blood mononuclear cells (PBMCs) were tested against recombinant colV (Chemicon/ Millipore, Billerica, MA) and K $\alpha$ 1T (recombinant protein expressed in the Mohanakumar laboratory as published)<sup>34</sup> using cytokine ELISPOT as well<sup>35</sup>.

**Methods – Statistical Analyses**—Continuous variables were summarized with the means and standard deviations and categorical variables with counts and percentages. Subject characteristics were compared using chi-square, Fisher's Exact, or Cochran-Mantel-Haenszel tests for categorical variables and t-tests for continuous variables. Time-dependent Cox proportional hazard models were used to examine the associations between viral infections and the composite endpoint as well as between viral infections and the selected mechanistic endpoints. Hazard ratios and corresponding 95% confidence intervals were reported. Survival curves were estimated using the Kaplan-Meier method. Because subjects' infection status could transition between positive and negative, and due to the presence of the endogenous time-varying aspect of viral infection, a sensitivity analysis was performed using multi-state and joint survival models<sup>36</sup>. Log<sub>10</sub> transformations were applied as necessary to satisfy normal distribution assumptions. All statistical analyses were performed using SAS Version 9.4 (SAS Institute, Inc., Cary, NC).

## Results

### Subject Population

Seventy-eight subjects were enrolled in the study from which 61 underwent transplant (Figure 1). Twenty-nine (48%) subjects had Cystic Fibrosis, 11 (18%) had pulmonary hypertension, 9 (15%) had obliterative bronchiolitis, 6 (10%) had interstitial lung disease, 4 (7%) had congenital disorders of surfactant metabolism and 2 (3%) had other diseases leading to transplant. Mean age at transplant was 11.3 years, ranging from 9 months to 18 years. (Table 1). Patients were followed for an average of 20 (+/- 8) months following transplant.

Primary graft dysfunction was common among subjects in this cohort: 33% of the 55 subjects with PGD data available met criteria for grade 2 or 3 PGD within 72 hours.

Twenty of the 61 subjects died prior to the end of the study period. Survival probability was 84% at 1 year and 68% at 3 years (Figure 2A). Chronic rejection (40%) was the most common cause of death followed by infection (20%). Death from primary graft dysfunction (10%) or post-transplant lymphoproliferative disease (5%) was uncommon in this population. Other causes of death (1 each) included renal failure, liver failure, heart failure, drug overdose and multisystem organ failure of unknown etiology.

**Infection**—Lower respiratory tract infections (LRTI) were reported in nearly all subjects (Table 2). For bacterial LRTI, 22 of the 38 subjects had CF and accounted for 68% of the infections. None of the bacterial LRTI were the primary cause of mortality. Thirteen episodes of bacteremia were identified in 10 subjects (16%); 5 of the subjects had bacteremia within the first 90 days after transplant. Bacteremia contributed to 2 of the 4 deaths attributable to infection.

Of the locally identified viral LRTI, adenovirus (7) was the most common virus reported followed by parainfluenza (5). CMV pneumonitis was reported in four patients. Adenovirus infection contributed to 2 of the 4 deaths attributable to infection. In 20 of 22 (91%) of the locally identified viral infections for which a matching specimen was sent to the CTOTC-03 virology core laboratory the same virus was identified. Approximately 40% of the locally identified viral LRTI did not have a central specimen submitted to the core lab.

Consistent with what was previously reported,<sup>37</sup> none of the fungal LRTI were contributors to mortality.

**Rejection**—A Kaplan-Meier analysis showed the probability of having at least one acute cellular rejection (ACR) grade A2 or greater was 21% at 1 year and 30% at 2 years. There were 19 ACR grade A2 or greater in 12 subjects within 1 year and 6 ACR episodes in 6 subjects between 1 year and 2 years. Thirteen episodes of antibody-mediated rejection (AMR) were identified in eleven subjects (18%) during the 2-year follow-up period.

The probability of developing either BOS grade greater than 0-p or a histologic diagnosis of OB was 20% at 1 year and 38% at 3 years (Figure 2B)



## Composite Endpoint

Twenty-eight subjects (46%) met one or more of the components of the composite endpoint: death, re-transplantation or development of BOS or bronchiolitis obliterans (Figure 1). Recipient diagnosis, age, sex and race/ethnicity were not associated with the composite endpoint (Table 1). Neither was type of transplant, transplant time, ischemic time, the use of induction immunosuppressant medications, nor the development of PGD, ACR or AMR. Univariate analyses showed that recipients receiving organs from a female donor were less likely to meet the composite endpoint, particularly when the recipient was female as well ( $P=0.028$ ). No other donor factors were associated with the composite endpoint. No relationship was found among demographic factors and the separate components of the composite outcome (supplementary tables S1 and S2).

## Mechanistic markers and the composite endpoint

We analyzed serum DSA and autoantibodies in this cohort because the presence of these antibodies has been associated with poor lung transplant outcomes in adults<sup>8,38-40</sup>. Twenty-eight subjects in the cohort developed de novo DSA. Twenty-one subjects without detectable serum autoantibodies pre-transplant developed post-transplant autoantibodies to CoIV and 26 subjects developed de novo post-transplant autoantibodies to K $\alpha$ 1T. We did not detect relationships among any of the antibodies and the composite endpoint. We also quantified pre- and post-transplant, donor-reactive and K $\alpha$ 1T-reactive immunity in PBMCs by IFN $\gamma$  ELISPOT. While ten subjects developed de novo post-transplant donor reactive cellular responses and 20 subjects developed *de novo* K $\alpha$ 1T-reactive cellular immunity, we did not observe relationships between either of these parameters and the composite endpoint (Table 3). Nor did we observe significant correlations between the composite endpoint (or its components) and the presence of post-transplant humoral or cellular reactivity to either allo- or autoantigens whether *de novo* or not. (Table 3)

## Viral Infection and the composite endpoint

The virology core laboratory evaluated 397 BAL and 480 NP specimens for viral pathogens. Of these, CARV were detected in 156 subject visits. In 40 of these, both BAL and NP specimens were positive. In 26, the BAL alone was positive while 90 had only a positive NP sample (this includes symptomatic episodes where no BAL was obtained). Of the CARV identified in positive specimens, in 116 (74%) of the visits the specimen was positive for enterovirus/rhinovirus. Eight subjects had successive samples positive for enterovirus/rhinovirus, suggestive of persistent infection.

To investigate the association between CARV and composite endpoint, we considered only CARV that occurred on or before the subject's known endpoint status. Of subjects who had at least one BAL or NP specimen positive for CARV, 38% (17/45) met the composite endpoint (11 BOS/OB; 5 Death; 1 re-transplant). In contrast, two thirds of subjects who did not test positive for CARV (10/15) met the composite endpoint (6 BOS/OB; 4 Death). When CARV with symptoms was evaluated, this difference was more pronounced. Only 20% of subjects with symptomatic CARV (4/20) met the composite endpoint. (Table 4).

However, recognizing that the subject's CARV status could change over time, we utilized a time-dependent Cox analysis. First, we focused on the transitions from positive to negative or negative to positive. We found no relationship between the presence of CARV by PCR and the composite endpoint (Table 5). Second, when the presence of CARV was treated as an absorbent state (i.e. a subject is considered permanently positive for CARV after the initial exposure), the hazard ratio was 0.8 with a 95% CI of 0.3–2.5 and a p-value of 0.73. These findings, which were contrary to the primary hypothesis, led us to suspect competing risk bias in the simpler Chi-square and K-M analyses, so to support the Cox model we added a sensitivity analysis of multi-state and joint survival models. As with the Cox model, no relationship between the presence of CARV and the composite endpoint was found (data not shown).

No relationship was found when we limited the analysis to paramyxovirus, influenza and adenovirus episodes, when we looked for relationships between the timing of the viral episode (occurring within the first 3 months or the first 6 months) or the number of independent viral episodes and the composite endpoint. We also observed no relationship between presence of CARV by PCR and the individual components of death (HR=0.8, CI=0.2–3.2, p=0.80) and the development of BOS (HR=0.4, CI=0.1–1.7, p=0.20).

In addition, we did not observe statistically significant relationships when we repeated these analyses including viral infection data as reported by the local clinical laboratories (HR=0.5, CI=0.2–1.4, p=0.21).

### **Viral infection and mechanistic endpoints (allo- and autoimmunity)**

We subsequently tested for relationships between viral presence and development of markers of humoral and cellular allo- and autoimmunity. Recovery of CARV from airway specimens was not related to the development of DSA, AutoAb (CoIV and Kα1T), and Kα1T specific ELISPOT. (Table 6). The presence of a CARV was associated with an increased risk for subsequent development of CoIV AutoAb. Because there were multiple CARVs at different times, the time-dependent Cox model was used. (HR=3.1, CI=1.2–8.1, p=0.02).

### **Viral infection as a potential surrogate for degree of immunosuppression**

Finally, we considered the hypothesis that viral infection might be a surrogate marker for the overall level of immunosuppression. We found no relationship between mean or coefficient of variation of tacrolimus trough levels and the composite endpoint (supplementary table S3). Although decreased frequencies of IFN $\gamma$  ELISPOTs to polyclonal stimulus with phytohemagglutinin (PHA) were associated with viral infection (Table 7), we did not detect a relationship between IFN $\gamma$ -producers and the composite endpoint (Table 8).

## **Discussion**

In this first prospective, observational, multicenter study of pediatric LTRs, we obtained clinical and mechanistic data on 61 subjects from 6 pediatric lung transplant centers followed for a minimum of 2 years and found comparable outcomes to the most recent ISHLT registry report<sup>3</sup>. However, in contrast to studies using adult subjects, we did not find relationships among primary graft dysfunction, ACR, or CARV exposure and BOS / OB or



graft loss. Based on the hypothesis that the common link between these different forms of graft injury and chronic allograft dysfunction is stimulation of a cellular and humoral responses directed at the allograft, these observations raise the possibility that the immunologic environment in pediatric lung transplant recipients is different than that of adults.

The indications for lung transplant, incidence of PGD, acute rejection, BOS and overall survival in this population are comparable to those reported in the most recent ISHLT registry report.<sup>3</sup> In contrast to adult LTRs ACR and PGD were not related to the development of BOS/OB or the composite endpoint<sup>41-43</sup>.

We found that identification of common respiratory viruses in NP or BAL specimens was not predictive of poor outcome as measured by a composite endpoint of death, re-transplant or development of CLAD based on OB and BOS criteria. Although subjects with a CARV identified in NP or BAL were less likely to meet the composite endpoint, particularly when the CARV was associated with symptoms, this could be due to a competing risk bias in that subjects with good outcomes had more follow up time to develop more viral infections. The time-dependent Cox analysis partially controls for this bias and when we did not find a statistically significant difference between the groups, we concluded that the competing risk bias was driving these non-intuitive results.

These findings contrast with previous retrospective studies performed in adult populations. Both prospective<sup>21,44,45</sup> and retrospective studies<sup>46</sup> support a link between CARV and chronic rejection, particularly when the infection involves the lower respiratory tract and/or is associated with clinical symptoms<sup>46,47</sup>. Specifically, community acquired respiratory viruses including paramyxoviruses, influenza, and adenovirus have been implicated in OB/BOS.<sup>44,45</sup> Most viral infections in our cohort were asymptomatic, perhaps a reflection of the fact that 75% of the CARV identified in our population were rhinovirus/enterovirus (which was not a risk factor in adult populations). No relationship was found when we limited the analysis to paramyxovirus, influenza and adenovirus episodes but the small number of these episodes limits our power to detect an effect.

Also, in contrast to adult populations, the development of donor specific humoral allo- and autoimmunity to self-antigens was not correlated to meeting the composite endpoint or any of its components. Single- center analyses of adult LTRs have identified anti-donor HLA antibodies,<sup>39,40</sup> as well as CoIV and K $\alpha$ 1T autoantibodies as risk factors for CLAD<sup>8,48</sup>. Similarly, we did not find a correlation between donor-specific alloreactive T cells responsiveness (as assessed by cytokine ELISPOT) in contrast to studies in kidney transplant recipients where ELISPOT reactivity strongly correlates with long term renal graft function<sup>49-55</sup>.

Taken together, the absence of a relationship between among CARV, ACR and outcome as well as the absence of a relationship between donor and/or self-antigen specific immunity and outcome suggests that these events occurring in the context of the developing immune system of a pediatric patient<sup>56</sup> impact transplant outcome differently than they do in adults.

The finding that female subjects, particularly females receiving organs from female donors, were less likely to meet the composite endpoint is difficult to reconcile with the other findings. Although one could hypothesize that females receiving male organs would have increased potential to develop immunity to Y chromosome gene products from the donor, our study population was not adequately powered to evaluate this hypothesis. The impact of gender combinations is not fully studied among LTRs. Indeed, some studies have shown that gender combinations appeared to have a significant impact<sup>57,58</sup>, and others, no effects, on survival of LTRs.<sup>59,60</sup> One single-center, retrospective study of adult LTRs reported a trend toward improved survival in female recipients.<sup>61</sup> Our study was not sufficiently powered to dissect this further.

Although the study population comprised nearly 40% of pediatric lung transplants performed in the United States during the accrual period, it is underpowered. A post-hoc power calculation using the observed frequencies in this cohort would require enrollment of 100 subjects to provide 80% power to detect a hazard ratio of 2.5. Nonetheless, given that we saw *no* relationship between infection and the composite endpoint (including an absence of statistical trends), we feel this study provides strong evidence rejecting the original hypothesis. The absence of a relationship between allo- and autoimmune responses and the composite endpoint may also reflect inadequate power. The relatively short 2-year follow-up period may have also limited our ability to identify the development of BOS, which is often diagnosed beyond 2 years post-transplant. We plan to obtain 5-year clinical follow-up data to assess this possibility. Because we did not collect data about patients who met exclusion criteria, we cannot rule out a recruitment bias in that regard. A final limitation to our study is that it was performed using BOS rather than CLAD criteria. It is possible that our findings would have been different had we collected sufficient data to characterize patients using current CLAD criteria and chosen a composite endpoint based on one of the CLAD subtypes.

Nonetheless, in contrast to the adult population<sup>21,44-46</sup>, respiratory viral infections in pediatric LTRs do not appear to be a predictor for poor outcome. Coupled with the observation that high immunosuppression based on ELISPOT reactivity was associated with viral infection, and the observation that a subset of our subjects had persistent rhinovirus infection<sup>62</sup> it is possible that viral infection was a surrogate for immunosuppression in this population. The finding, in a companion study using samples from this population, that low betatorquevirus levels at 6 weeks and 6 months after transplant were associated with death and the composite outcome supports this hypothesis<sup>63</sup>.

In summary, this comprehensive prospective analysis of pediatric LTRs found comparable outcomes to the most recent ISHLT registry report<sup>3</sup> but did not identify relationships among primary graft dysfunction, ACR, or CARV and graft loss/CLAD. These observations raise the possibility that the immunologic environment in pediatric lung transplant recipients is different than that observed in adults. Further research is needed to determine whether these observations can be correlated with specific immunologic markers and /or warrant changes in screening or immunosuppression therapy in pediatric LTRs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This research was performed as a project of the Clinical Trials in Organ Transplantation in Children, a collaborative clinical research project headquartered at the National Institute of Allergy and Infectious Diseases. The work was supported by Grant U01 AI077810 “Viral Triggers of Alloimmunity and Autoimmunity in Pediatric Lung Transplantation” from the Division of Allergy, Immunology and Transplantation of the National Institutes of Health.

## Abbreviations

<b>ACR</b>	Acute cellular rejection
<b>AMR</b>	Antibody- mediated rejection
<b>AutoAb</b>	Autoantibodies
<b>BAL</b>	Bronchoalveolar lavage
<b>BOS</b>	Bronchiolitis Obliterans Syndrome
<b>CARV</b>	Community acquired respiratory virus infections
<b>CF</b>	Cystic Fibrosis
<b>CLAD</b>	Chronic lung allograft dysfunction
<b>CMV</b>	Cytomegalovirus
<b>ColV</b>	Collagen type V
<b>CTOT-C</b>	Clinical Trials in Organ Transplant in Children
<b>DSA</b>	Donor-specific human leukocyte antigen antibody
<b>ELISPOT</b>	Enzyme linked immunosorbent spot assay
<b>HLA</b>	Human leukocyte antigen
<b>IFN<math>\gamma</math></b>	Interferon gamma
<b>IPLTC</b>	International Pediatric Lung Transplant Collaborative
<b>ISHLT</b>	International Society for Heart and Lung Transplantation
<b>K<math>\alpha</math>1T</b>	K-alpha 1 tubulin
<b>LAS</b>	Lung Allocation Score
<b>LTR</b>	Lung transplant Recipient
<b>LRTI</b>	Lower respiratory tract infection
<b>MFI</b>	Mean fluorescence index

<b>NP</b>	Nasopharyngeal
<b>OB</b>	Obliterative Bronchiolitis
<b>PCR</b>	Polymerase chain reaction
<b>PBMCs</b>	Peripheral blood mononuclear cells
<b>PE</b>	Primary Endpoint
<b>PGD</b>	Primary graft dysfunction
<b>PHA</b>	Phytohemagglutinin
<b>PI</b>	Primary Investigator

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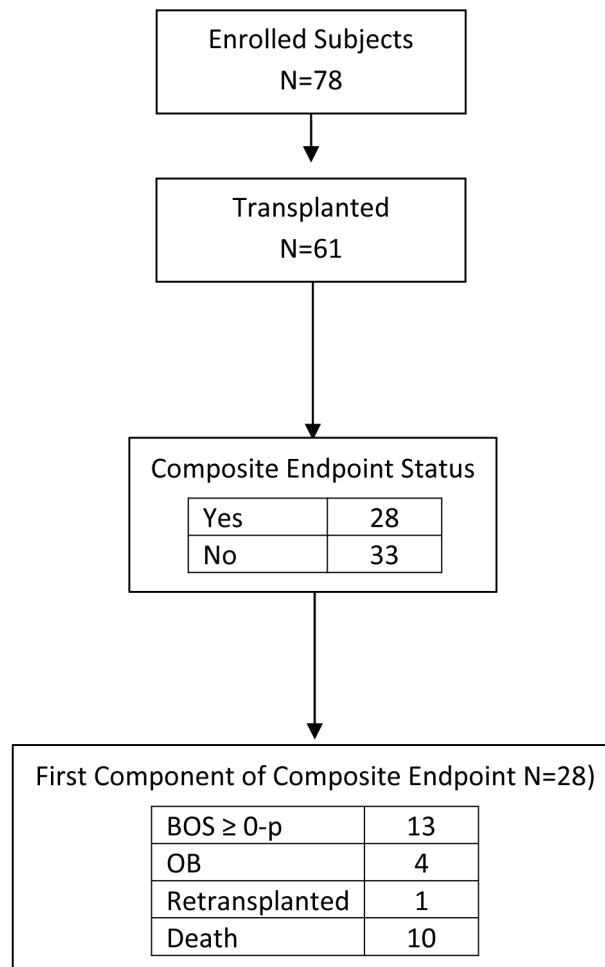
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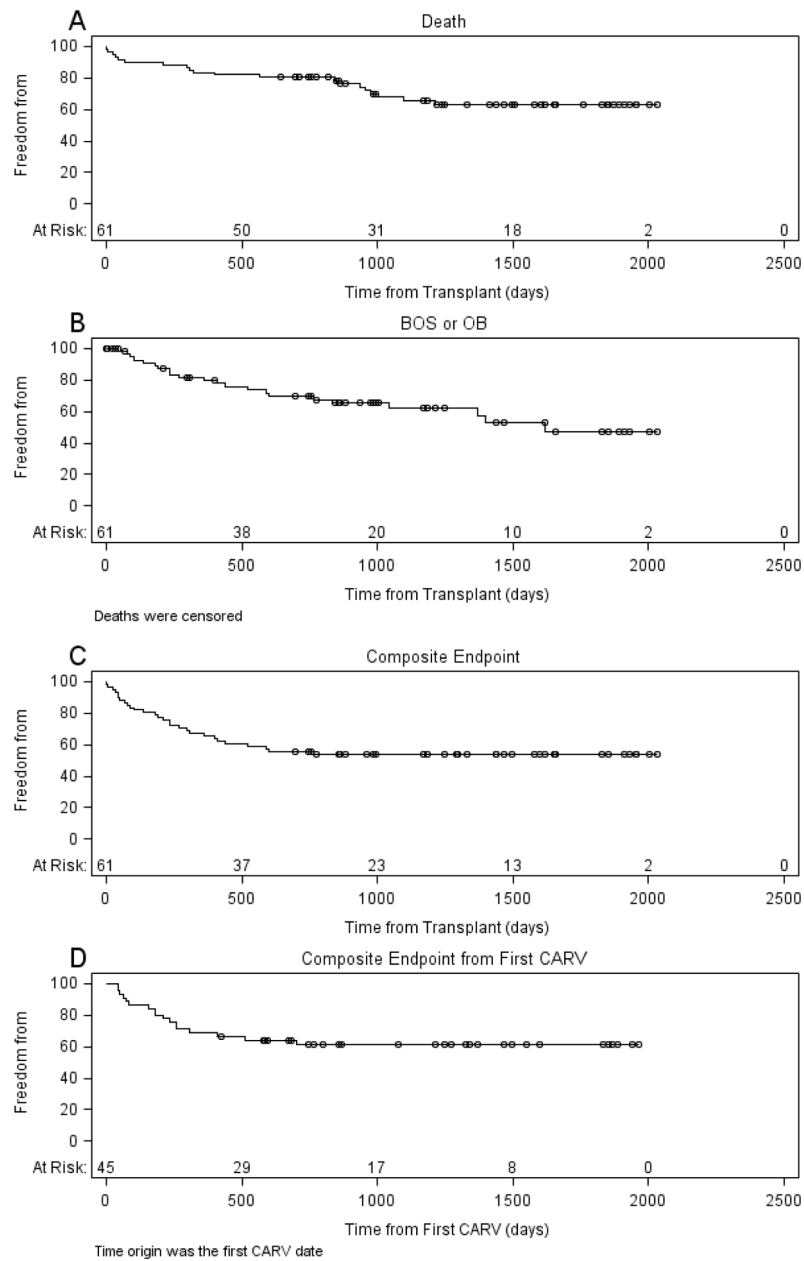


**Figure 1.**

Consort diagram. Consort diagram illustrating the outcome of subjects throughout the course of the study including.

Notes: 5 patients were lost to follow-up: 3 of them met the composite endpoint before being lost to follow-up; 2 were lost to follow-up at the 24 month visit. All 5 patients were included in the analysis. One patient met the composite endpoint without having any viral specimens obtained.

BOS: Bronchiolitis Obliterans Syndrome



**Figure 2.** Probability of freedom from death (A) and Bronchiolitis Obliterans Syndrome (BOS) and Obliterative Bronchiolitis (OB) (B), the composite endpoint (C) and the composite endpoint as a function of time from the first community acquired respiratory virus (CARV) (D). The number of subjects at risk is presented at select time points along the x axis. Death was censored on Figure B.

**Table 1**

Clinical Characteristics and Outcomes in Relation to the Composite Endpoint

	All Subjects (N=61) n (%)	Composite Endpoint Yes (N=28) n (%)	Composite Endpoint No (N=33) n (%)	P-value
<b>Recipient Characteristics</b>				
<b>Lung Allocation Score Diagnosis Group</b>				
Group A	1( 1.6)	1( 3.6)	0	0.758
Group B	11(18.0)	5(17.9)	6(18.2)	
Group C	29(47.5)	13(46.4)	16(48.5)	
Group D	20(32.8)	9(32.1)	11(33.3)	
<b>Age</b>				
N	61	28	33	0.859
Mean (SD)	11.3 (5.46)	11.5 (5.56)	11.2 (5.46)	
Median	13	13	13	
Min, Max	<1, 18	<1, 18	<1, 18	
<b>Gender</b>				
Male	25(41.0)	14(50.0)	11(33.3)	0.187
Female	36(59.0)	14(50.0)	22(66.7)	
<b>Race</b>				
White	50(82.0)	21(75.0)	29(87.9)	0.734
Black or African American	5( 8.2)	3(10.7)	2( 6.1)	
Asian	2( 3.3)	1( 3.6)	1( 3.0)	
More Than One Race	1( 1.6)	1( 3.6)	0	NA
Unknown or Not Reported	3( 4.9)	2( 7.1)	1( 3.0)	NA
<b>Ethnicity</b>				
Hispanic or Latino	5( 8.2)	2( 7.1)	3( 9.1)	>0.999
Not Hispanic or Latino	42(68.9)	19(67.9)	23(69.7)	
Unknown or Not Reported	14(23.0)	7(25.0)	7(21.2)	NA
<b>Type of Transplant</b>				
Double Lung	56(91.8)	25(89.3)	31(93.9)	0.653
Heart-Lung	5( 8.2)	3(10.7)	2( 6.1)	
<b>Donor and Recipient Gender</b>				
Donor Female, Recipient Female	18(29.5)	3(10.7)	15(45.5)	<b>0.028</b>

	All Subjects (N=61) n (%)	Composite Endpoint Yes (N=28) n (%)	Composite Endpoint No (N=33) n (%)	P-value
Donor Female, Recipient Male	13(21.3)	6(21.4)	7(21.2)	
Donor Male, Recipient Female	18(29.5)	11(39.3)	7(21.2)	
Donor Male, Recipient Male	11(18.0)	7(25.0)	4(12.1)	
<b>Induction</b>				
Anti IL-2 antibody	44(72.1)	22(78.6)	22(66.7)	0.241
Thymoglobulin	14(23.0)	6(21.4)	8(24.2)	
None	3( 4.9)	0	3( 9.1)	
<b>Donor Gender</b>				
Male	29(47.5)	18(64.3)	11(33.3)	<b>0.010</b>
Female	31(50.8)	9(32.1)	22(66.7)	
<b>Clinical Events</b>				
<b>Acute Cellular Rejection</b>				
Yes	16(26.2)	8(28.6)	8(24.2)	0.513
No	42(68.9)	17(60.7)	25(75.8)	
<b>Highest Acute Cellular Rejection Grade</b>				
A2 - Mild	14(23.0)	7(25.0)	7(21.2)	>0.999
A3 - Moderate	2( 3.3)	1( 3.6)	1( 3.0)	
<b>Antibody Mediated Rejection</b>				
Yes	11(18.0)	6(21.4)	5(15.2)	0.504
No	47(77.0)	19(67.9)	28(84.8)	

**Table 2**

## Summary of Respiratory Tract Infections

Infection Type	# of Episode	# of Subject
Any infection including CARV, Bacterial, Viral, Fungal, Mycobacterial, and Protozoan	416	57
At Least one infection of CARV, Bacterial, or Fungal LRTI and Respiratory (Unspecified)	268	56
No infection at all	--	4 *
CARV	156	50
Bacterial (LRTI and Respiratory (Unspecified))	117 (103 with LRTI only)	41 (38 with LRTI only)
Fungal (LRTI and Respiratory (Unspecified))	39	25
Bacterial or Fungal (LRTI and Respiratory (Unspecified))	140	46
CARV and either Bacterial or Fungal (LRTI and Respiratory (Unspecified)) at the same time	37	25 **
CARV and Bacterial but no Fungal (LRTI and Respiratory (Unspecified)) at the same time	27	21 **
CARV and Fungal but no Bacterial (LRTI and Respiratory (Unspecified)) at the same time	8	8 **
3 infections at the same time	2	2 **
CARV and Neither Bacterial or Fungal (LRTI and Respiratory (Unspecified)) at the same time	119	46 ***
Bacterial *or* Fungal (LRTI and Respiratory (Unspecified)) but No CARV at the same time	112	42 ***
Bacterial and Fungal (LRTI and Respiratory (Unspecified)) but No CARV at the same time	8	5 ***

\* 3 subjects who did not have any infection died early between 1-27 days post-transplant due to the primary graft failures and cardiac failure. 1 subject completed the study without any infection and not meeting composite endpoint.

\*\* The count reflects the number of unique subjects with CARV, LRT bacterial or fungal infections, regardless of type.

\*\*\* Subjects may be counted in more than one row if the subject experienced multiple types of infection.



**Table 3:**

Relationship between mechanistic endpoints and composite endpoint

Mechanistic Assays	All Subjects (N=61) n (%)	Composite Endpoint Yes (N=28) n (%)	Composite Endpoint No (N=33) n (%)	P-value
<b>Pre to Post-Transplant Antibody Status</b>				
<b>Collagen V</b>				
Neg to Neg	10(16.4)	7(25.0)	3(9.1)	0.135
Neg to Pos	21(34.4)	8(28.6)	13(39.4)	
<b>Ka1Tubulin</b>				
Neg to Neg	3(4.9)	2(7.1)	1(3.0)	0.553
Neg to Pos	26(42.6)	10(35.7)	16(48.5)	
<b>Donor Specific Antibody</b>				
Neg to Neg	28(45.9)	11(39.3)	17(51.5)	0.420
Neg to Pos	28(45.9)	14(50.0)	14(42.4)	
<b>Post-Transplant Antibody Status</b>				
<b>ELISA : Auto Antibodies</b>				
<b>Collagen V</b>				
Positive	46(75.4)	17(60.7)	29(87.9)	0.038
Negative	13(21.3)	9(32.1)	4(12.1)	
Missing	2(3.3)	2(7.1)	0	NA
<b>Ka1Tubulin</b>				
Positive	56(91.8)	24(85.7)	32(97.0)	0.578
Negative	3(4.9)	2(7.1)	1(3.0)	
Missing	2(3.3)	2(7.1)	0	NA
<b>Anti-HLA Antibodies</b>				
<b>Class I</b>				
Positive	33(54.1)	12(42.9)	21(63.6)	0.179
Negative	26(42.6)	14(50.0)	12(36.4)	
Missing	2(3.3)	2(7.1)	0	NA

Mechanistic Assays	All Subjects (N=61) n (%)	Composite Endpoint Yes (N=28) n (%)	Composite Endpoint No (N=33) n (%)	P-value
<b>Class II</b>				
Positive	31(50.8)	13(46.4)	18(54.5)	0.728
Negative	28(45.9)	13(46.4)	15(45.5)	
Missing	2(3.3)	2(7.1)	0	NA
<b>Donor Specific Antibody</b>				
Yes	30(49.2)	14(50.0)	16(48.5)	0.795
No	30(49.2)	13(46.4)	17(51.5)	
<b>ELISPOT</b>				
<b>Pre to Post-Transplant ELISPOT Status</b>				
<b>Donor Specific ELISPOT (&gt;=25)</b>				
Neg to Neg	19(31.1)	10(35.7)	9(27.3)	0.433
Neg to Pos	10(16.4)	3(10.7)	7(21.2)	
<b>Ka.IT Specific ELISPOT (&gt;=5)</b>				
Neg to Neg	3(4.9)	1(3.6)	2(6.1)	>0.999
Neg to Pos	20(32.8)	9(32.1)	11(33.3)	
<b>Post-transplant Donor Specific ELISPOT (&gt;=25)</b>				
Positive	16(26.2)	6(21.4)	10(30.3)	0.368
Negative	21(34.4)	11(39.3)	10(30.3)	
<b>Post-transplant Ka.IT Specific ELISPOT (&gt;=5)</b>				
Positive	52(85.2)	22(78.6)	30(90.9)	>0.999
Negative	6(9.8)	3(10.7)	3(9.1)	

**Table 4**

Relationship between CARV and Composite Endpoint

	Total (N=60) n (%)	Met Composite Endpoint (death, retransplantation or Bronchiolitis Obliterans Syndrome / Obliterative Bronchiolitis) within 24m	
		Yes (N=27) n (%)	No (N=33) n (%)
<b>CARV: (Rhino+Other) BAL or NP</b>			
Yes	45(73.8)	17(63.0)	28(84.8)
No	15(24.6)	10(37.0)	5(15.2)
<b>Symptomatic CARV</b>			
Yes	20(32.8)	4(14.8)	16(48.5)
No	40(65.6)	23(85.2)	17(51.5)

\* One subject met the composite endpoint without having any viral specimens obtained.

CARV: Community Acquired Respiratory Virus

BAL: Bronchoalveolar Lavage

NP: Nasopharyngeal Swab

**Table 5**  
Time Dependent Cox Analysis of relationship with viral infection and Composite Endpoint

Variable	Hazard Ratio (95% Lower CL, Upper CL)	P-value
Any Virus	0.57 (0.26, 1.25)	0.161
Any Virus with Symptoms	0.66 (0.2, 2.2)	0.497
CARV	0.64 (0.25, 1.59)	0.335
CARV with symptoms	0.94 (0.22, 4.03)	0.937

CARV: Community Acquired Respiratory Virus

Table 6.

Relationship between mechanistic endpoints and CARV

Mechanistic Assays	All Subjects (N=60)* n (%)	CARV (N=45) n (%)	No CARV (N=15) n (%)	P-value
<b>Antibody Status Post-Transplant</b>				
<b>ELISA: Auto Antibodies</b>				
<b>Collagen V</b>				
Positive	46(76.7)	37(82.2)	9(60.0)	0.157
Negative	13(21.7)	8(17.8)	5(33.3)	
Missing	1(1.7)	0	1(6.7)	NA
<b>KaITubulin</b>				
Positive	56(93.3)	42(93.3)	14(93.3)	>0.999
Negative	3(5.0)	3(6.7)	0	
Missing	1(1.7)	0	1(6.7)	NA
<b>Anti-HLA Antibodies</b>				
<b>Class I</b>				
Positive	33(55.0)	28(62.2)	5(33.3)	0.081
Negative	26(43.3)	17(37.8)	9(60.0)	
Missing	1(1.7)	0	1(6.7)	NA
<b>Class II</b>				
Positive	31(51.7)	25(55.6)	6(40.0)	0.406
Negative	28(46.7)	20(44.4)	8(53.3)	
Missing	1(1.7)	0	1(6.7)	NA
<b>Donor Specific Antibodies</b>				
Yes	30(50.0)	24(53.3)	6(40.0)	0.493
No	29(48.3)	21(46.7)	8(53.3)	
<b>ELISPOT</b>				
<b>Post-transplant Donor Specific ELISPOT (&gt;=25)</b>				
Positive	16(26.2)	11(24.4)	5(33.3)	0.614

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Mechanistic Assays	All Subjects (N=60)* n (%)	CARV (N=45) n (%)	No CARV (N=15) n (%)	P-value
Negative	21(34.4)	16(35.6)	5(33.3)	
<b>Post-transplant Kα.IT Specific ELISPOT (&gt;=5)</b>				
Positive	52(85.2)	41(91.1)	11(73.3)	0.149
Negative	6(9.8)	3(6.7)	3(20.0)	

\* One subject met the composite endpoint without having any viral specimens obtained.

CARV: Community Acquired Respiratory Virus



**Table 7:**

Relationship between ELISPOT Measures of immunosuppression and any viral Infection

Mechanistic Assays	All Subjects (N=60) <sup>*</sup> n (%)	Any Viral Infection (N=52) n (%)	No Viral Infection (N=8) n (%)	P-value
<b>High immunosuppression per ELISPOT (at least one positive<sup>**</sup> sample)</b>				
Yes	43(71.7)	41(78.8)	2(25.0)	<b>0.003</b>
No	16(26.7)	10(19.2)	6(75.0)	
<b>Number of High immunosuppression samples<sup>**</sup> per Subject</b>				
0	16(26.7)	10(19.2)	6(75.0)	<b>0.055</b>
1	9(15.0)	9(17.3)	0	
2	7(11.7)	6(11.5)	1(12.5)	
3	7(11.7)	7(13.5)	0	
4	11(18.3)	11(21.2)	0	
5	5(8.3)	5(9.6)	0	
6	3(5.0)	2(3.8)	1(12.5)	
10	1(1.7)	1(1.9)	0	

\* One subject met the composite endpoint without having any viral specimens obtained.

\*\* Samples were considered positive for high immunosuppression if the response to positive control (phytohemagglutinin, PHA) was less than 100 spots and cell viability was &gt; 50%.

Relationship between ELISPOT Measures of immunosuppression and Composite Endpoint

**Table 8:**

	All Subjects (N=61) n (%)	Composite Endpoint Yes (N=28) n (%)	Composite Endpoint No (N=33) n (%)	P-value
<b>High immunosuppression per ELISPOT (at least one positive* sample)</b>				
Yes	44(72.1)	18(64.3)	26(78.8)	0.291
No	16(26.2)	9(32.1)	7(21.2)	
<b>Number of high immunosuppression samples* per Subject</b>				
0	16(26.2)	9(32.1)	7(21.2)	0.473
1	10(16.4)	3(10.7)	7(21.2)	
2	7(11.5)	2(7.1)	5(15.2)	
3	7(11.5)	3(10.7)	4(12.1)	
4	11(18.0)	7(25.0)	4(12.1)	
5	5(8.2)	1(3.6)	4(12.1)	
6	3(4.9)	2(7.1)	1(3.0)	
10	1(1.6)	0	1(3.0)	

\* Samples were considered positive for high immunosuppression if the response to positive control (phytohemagglutinin, PHA) was less than 100 spots and cell viability was > 50%.