Recipient-Matching of Passenger Leukocytes Prolongs Survival of Donor Lung Allografts in Miniature Swine

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Abstract

Background—Allograft rejection continues to be a vexing problem in clinical lung transplantation, and the role played by passenger leukocytes in the rejection or acceptance of an organ is unclear. Here we tested whether recipient-matching of donor graft passenger leukocytes would impact graft survival in a preclinical model of orthotopic left lung transplantation.

Methods—In the experimental group (Group 1), donor lungs were obtained from chimeric swine, in which the passenger leukocytes (but not the parenchyma) were MHC-matched to the recipients (n=3). In the control group (Group 2), both the donor parenchyma and the passenger leukocytes were MHC-mismatched to the recipients (n = 3).

Results—Lungs harvested from swine previously rendered chimeric by hematopoietic stem cell transplantation using recipient-type cells showed a high degree of passenger leukocyte chimerism by immunohistochemistry and flow cytometry. The chimeric lungs containing passenger
leukocytes matched to the lung recipient (Group 1) survived on average 107 days (range 80–156). Control lung allografts (Group 2) survived on average 45 days (range 29–64; p<0.05).

Conclusion—Our data indicate that recipient-matching of passenger leukocytes significantly prolongs lung allograft survival.

INTRODUCTION

Lung transplantation is a life-saving measure for patients with end-stage lung disease. However, acute and chronic graft rejection, and the immunosuppression needed to control the alloimmune response, still exact a toll on recipient survival (1). Passenger leukocytes contained within the graft are generally considered to be potent targets for direct allorecognition. However, these leukocytes have also been hypothesized to exert beneficial effects on graft acceptance in certain contexts (2). In human and animal studies, passenger leukocytes have been quantified and characterized (3), but their relative contribution to graft rejection versus graft acceptance is unknown. Understanding the impact of passenger leukocytes on rejection and tolerance is particularly important now that techniques are available to modify this cell population in the lung graft ex vivo.

Using MHC-inbred miniature swine (4), we have previously developed a method to generate chimeric swine through the transplantation of hematopoetic stem cells across a single-haplotype, full MHC mismatch (5). In many cases, the extent of peripheral chimerism achieved in these animals is nearly complete, allowing us to procure grafts in which the passenger leukocyte population is MHC-mismatched to the parenchyma. In this manuscript, we report the impact of passenger leukocyte matching on lung allograft survival.

MATERIALS AND METHODS

Animals

Transplant donors and recipients were selected from our herd of partially inbred miniature swine (age, 3–6 months; weight, 15–30kg). The immunogenetic characteristics of this herd have been described previously (4,6). In the experimental group (Group 1), lungs from chimeric donors bearing recipient-matched passenger cells were transplanted into recipients to achieve a single-haplotype full MHC class I and class II mismatch between donor and recipient and MHC matching between donor passenger leukocytes and recipient (see below). In the control group (Group 2), non-chimeric naïve donor lungs were transplanted into similarly mismatched recipients to achieve single-haplotype full MHC class I and class II mismatch between donor and recipient.

The haplotypes of the animals used in this study were controlled by strict pedigree breeding and confirmed with micro-cytotoxicity assays, by methods previously reported (4,6). Also, all recipients demonstrated significant in vitro anti-donor reactivity on MLR and/or CML, prior to transplantation, by methods previously reported (7). All animal care and procedures were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and conducted in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press.
Chimeric lungs used in Group 1 were harvested from MHC<sup>ad</sup> (class I<sup>ad</sup>/II<sup>ad</sup>) animals that had undergone cytokine-mobilized hematopoietic stem cell (HSC) transplant from MHC<sup>ac</sup> (class I<sup>ac</sup>/II<sup>ac</sup>) animals (HSC donors were MHC-matched to the eventual lung recipient) at least 250 days earlier as described previously (5,7). Stem cell donor animals received a 7-day course of porcine interleukin-3 (pIL-3) and stem cell factor (pSCF), injected intramuscularly at a dose of 100 μg/kg to 30 kg bodyweight, 50 μg/kg for each additional kilogram. Mobilized hematopoietic stem cells were collected from peripheral blood by apheresis over 3 days, starting after the fifth dose of pIL-3/pSCF. Stem cell recipients underwent a reduced intensity conditioning regimen, consisting of 100 cGy total body irradiation on day −2, partial T cell depletion with recombinant CD3-immunotoxin (pCD3-DT390, 50 μg/kg IV) twice daily from day −4 to −1 (8), and a 45-day course of cyclosporine A (target trough 400–800 ng/mL day 0–30 then taper to discontinuation). Unmodified cytokine-mobilized peripheral blood mononuclear cells were transplanted over 2 or 3 days following conditioning (days 0–2) as required to achieve the target dose of 15 × 10<sup>9</sup> cells/kg. A non-MHC-linked marker, pig allelic antigen (PAA), was used to distinguish host (PAA<sup>−</sup>) and HSC donor (PAA<sup>+</sup>) cells (9). HSC engraftment was confirmed in each chimeric lung donor by the presence of donor-derived bone marrow colony-forming units over 14 weeks following transplantation. Chimeric animals had achieved high levels of donor chimerism in the lymphoid and myeloid lineages prior to lung procurement (Table I). Animals which did not initially achieve high-level donor chimerism underwent donor leukocyte infusion to induce conversion to full donor chimerism prior to organ procurement.

**Operative procedure**

Orthotopic left lung transplantation was performed as previously described (10). In brief, after induction of general anesthesia, a left thoracotomy was performed and the recipient hilar structures were isolated. Heparin was administered (400 units/kg) and a pneumonectomy was performed. The donor lung was harvested via median sternotomy. Heparin was administered (400 units/kg). The pulmonary artery was cannulated and flushed in situ with 2 liters of Perfadex (XVIVO Perfusion AB, Göteborg, Sweden) containing prostaglandin E1 (500ug/L). The lung was prepared surgically and immediately transplanted. A thoracostomy tube was placed to evacuate the pleural space and was removed on postoperative day 2.

Two indwelling silastic central venous catheters were placed surgically into the external or internal jugular veins. The catheters facilitated cyclosporine administration and frequent blood sampling for in vitro assays and for monitoring of whole blood cyclosporine levels.

**Rejection monitoring**

Lung allograft survival was monitored by clinical examination of breath sounds, chest x-rays, and lung biopsies. Routine biopsies were performed on all transplant recipients via mini-thoracotomies at predetermined time intervals (POD 20–30, 50–60, 90–100), or whenever indicated by clinical exam or chest radiograph. Allograft rejection was confirmed histologically in all cases.
Immunosuppression

Cyclosporine (Novartis Pharmaceutical Corporation, Hanover, NJ) was mixed and administered as an intravenous suspension according to the specifications of the manufacturer. Cyclosporine was given as a daily intravenous infusion over 1 hour (13 to 16 mg/kg/day with target levels 400 to 800 ng/mL) for 12 consecutive days, starting on the day of transplantation.

Pathology studies

Wedge biopsies were performed on lung allografts. Tissue was fixed in formalin and embedded in paraffin for routine light microscopy (hematoxalin and eosin; periodic acid-Schiff). Separate portions were frozen for immunohistochemical studies. Scoring of acute rejection in the lung allograft was performed by a transplant pathologist without knowledge of the functional status of the graft based on the International Society for Heart and Lung Transplantation System (11). Complete necropsies were performed upon completion of the experiments and tissue was similarly processed for pathological examination.

For assessing tissue chimerism by immunohistochemistry, frozen tissue sections of chimeric and control lungs were stained with antibodies to MHC class I (class I, IgM clone: 16.7.E4.2). All images were evaluated with a Nikon 50i fluorescent microscope at 10x and merged with SpotSoftware Version 4.6 (Diagnostic Instruments Inc.).

Flow cytometry

To characterize the levels of tissue chimerism by flow cytometry, 0.5mm³ biopsies of chimeric lungs were obtained prior to transplantation. Lung tissue was dispersed mechanically in Hanks’ buffered saline solution (Gibco BRL, Grand Island, NY) and then strained through a 40μm filter. The cell suspension was then diluted approximately 1:2 with Hanks’ buffered saline solution and the mononuclear cells were obtained by means of gradient centrifugation with Histopaque (Sigma, St. Louis, MO). The mononuclear cells were washed once with Hanks’ buffered saline solution and re-suspended in phosphate-buffered saline with 0.1% sodium azide at 1×10⁷ cells per ml. Cells were aliquoted into tubes to a concentration of 1×10⁶ cells per tube. 10μL of antibody was added to each tube. Antibodies to the following antigens were used: porcine allelic antigen (PAA, IgM clone: 1038H-10-9 (9)) was detected with fluorescein isothiocyanate or streptavidin allophycocyanin; MHC-DR antigen (biotinylated, IgG clone: TH16) was detected with streptavidin allophycocyanin or fluorescein isothiocyanate; CD11b (IgG clone: M1/70) was detected with fluorescein isothiocyanate or streptavidin allophycocyanin; CD3 (IgG clone: 898H2-6-15) was detected with phycoerythrin. After incubating for 30 minutes at 4°C, the cells were washed twice with 2 ml of phosphate-buffered saline with 0.1% sodium azide and centrifuging for 5 minutes at 2000 rpm. The cells were then re-suspended in 300μl phosphate-buffered saline with 0.1% sodium azide prior to acquisition.

Statistical analysis

Graft survival times were compared using a two-tailed nonparametric Mann-Whitney U test and Kaplan-Meier survival curves. Differences in graft survival time were deemed significant when p < 0.05.
RESULTS

Chimeric lung donors exhibited passenger leukocytes that were matched to the ultimate lung recipient

Lungs were harvested from donors previously rendered chimeric by cytokine-mobilized hematopoietic stem cell (HSC) transplantation from single-haplotype full mismatch donor animals as described elsewhere (5,7). Immunohistochemical analysis of representative lung allograft biopsy specimens that were obtained at the time of organ procurement were performed to determine the relative numbers of HSC donor- versus HSC recipient-derived passenger leukocytes that were present within the donor lung. As shown in Figure 1, the passenger leukocytes in the donor lungs were derived from the HSC donor (i.e. class I+ cells, matched to the ultimate lung recipient), confirming the chimeric nature of the donor lung. At the time of lung procurement, the peripheral blood of the donor also exhibited high levels of lymphoid and myeloid chimerism (Table I). To look more specifically at the levels of tissue chimerism among different cell lineages, lung tissue was dispersed and analyzed by flow cytometry. As seen in Figure 2, CD3+, CD11b+ and MHC class II+ cells from representative donor lung (#21557) contained a significant percentage of PAA+ cells.

Graft survival was prolonged in recipients of chimeric lung allografts

To determine whether MHC-matching between graft passenger leukocytes and the recipient impacted lung allograft survival, three MHCac recipients received lung grafts from chimeric donors across a single-haplotype full MHC mismatched barrier (Table II, Group 1). These grafts were compared to those of three MHCac recipients that received lungs from non-chimeric donors (Table II, Group 2). Lung allografts in Group 1 survived on average 107 days (range 80–156); whereas, grafts in Group 2 survived on average 45 days (range 29–64; p<0.05 by Mann-Whitney U and p=0.02 by Kaplan-Meier). On histopathological analysis, the grafts in Group 2 exhibited severe, diffuse cellular infiltrates with endarteritis at earlier time points, as compared to the chimeric grafts in Group 1 (Figure 3).

Chimeric lung recipients demonstrated variable cell-mediated cytotoxicity (CML) and mixed-lymphocyte reactions (MLR) in vitro

Serial CML and MLR assays were performed to assess the immune competence in recipients of chimeric versus non-chimeric lung allografts (Table III). All recipients in Group 2 demonstrated loss of donor-specific responsiveness by CML by POD30 and remained unresponsive throughout the remainder of the experiment. In contrast, 2 of 3 recipients in Group 2 regained donor-specific responsiveness by MLR at day of rejection (animal #21280 never lost donor-specific response by MLR). Group 1 animals had variable levels of donor-specific responsiveness by CML and MLR. On POD30, animal #21264 had donor-specific response by MLR and animal #21762 had donor-specific response by CML. Both animals had lost donor-specific responsiveness by CML and MLR when assessed on the day of rejection (Table III). CML and MLR data for animal #20557 are unavailable.
Lack of circulating alloantibody production in lung transplant recipients

Lung recipients in Group 2 never showed detectable levels of circulating IgM or IgM alloantibody (Figure 4, solid). One recipient in Group 1 (animal #21264) showed transient elevation of serum IgG, but not IgM, alloantibody whereas Group 1 animal #21762 never showed detectable levels of alloantibody (Figure 4, dotted). Alloantibody data for animal #20557 are unavailable.

DISCUSSION

Passenger leukocytes are donor hematopoietic cells that are transferred to the recipient at the time transplantation. Lungs typically contain approximately $20-40 \times 10^9$ mononuclear cells (3), which can migrate from the graft to the recipient’s lymphoid tissues, where they can be directly recognized by recipient T cells (12,13). However, the role of passenger leukocytes in mediating graft acceptance versus graft rejection in lung transplantation is not completely understood. Here, we demonstrate in a preclinical model that recipient-matching of donor passenger leukocytes significantly prolongs lung allograft survival (average 107 days versus 44 days, p<0.05).

Passenger leukocytes can be viewed as potent targets for direct allore cognition, driving acute rejection and leading to a greater risk of subsequent chronic rejection. In early experiments, the immunogenicity of “ghost” kidneys which had been depleted of donor-type passenger leukocytes could be restored by infusion of donor-type dendritic cells (14). Similarly, transplant immunity and graft rejection were restored to irradiated or cultured thyroid glands by the injection of leukocytes (15). These experiments led to the idea that recipient-matching of passenger leukocytes could be beneficial for allograft survival. Indeed, recipient-matching of passenger leukocytes in donor mouse hearts delayed or prevented acute cardiac allograft rejection (16,17); however, chronic rejection still developed (18). Recipient-matching of passenger leukocytes in donor rat livers also prolonged graft survival (19). In models of lung rejection, graft survival was prolonged when donor rat lung grafts were rendered less immunogenic by depleting lymphocytes and donor-type dendritic cells (20). However, pre-transplant depletion of alveolar macrophages, while decreasing local levels of TNF-alpha and IFN-gamma, had no effect on development of severe acute rejection (21).

In addition to their immunogenic effects, passenger leukocytes also may exert an immunomodulatory effect on allograft survival, favoring graft acceptance through regulatory mechanisms (2,22). Several studies demonstrate that high levels of donor-derived leukocytes within the transplanted lung or peripheral blood can be correlated with better graft outcomes. O’Connell and colleagues assessed chimerism in bronchoalveolar lavage specimens post lung transplantation and found that patients with higher numbers of donor-derived leukocytes in the first 200 days after transplantation had lower incidences of acute or chronic rejection (23). Kruse and colleagues showed that longer lung allograft survival in pigs was correlated with higher levels of intragraft and peripheral donor chimerism (24). In addition, donor passenger leukocytes may contribute to peripheral microchimerism in the recipient following lung transplantation. In a study of 11 patients, stable microchimerism was detected among B cells, T cells, monocytes, and NK cells, but not among dendritic cells.
during the first year after lung transplantation (25,26). The clinical significance of microchimerism is still debatable, as some studies show that microchimerism can be either correlated with allograft dysfunction (27,28), associated with donor-specific hyporesponsiveness (29), or unrelated to graft rejection (30). In our model, donor-type passenger leukocytes seem to have a deleterious effect on graft survival, as replacement with recipient-matched passenger leukocytes prolonged graft survival. However, recipient-matching of passenger leukocytes alone was not able to prevent rejection, suggesting that direct and indirect allore cognition of donor lung parenchymal cells, many of which constitutively express class II antigens (31), also play a role in lung rejection.

One potential caveat to our study is that the recipient-matched passenger leukocytes contained within the lung allograft, having resided in the donor chimera long-term, developed local regulatory activity towards donor alloantigens that is then carried over to the recipient upon transplantation. However, data from chimeric kidney transplants suggest otherwise, as animals receiving chimeric kidneys experienced acute rejection crises in the early postoperative period, likely because, once taken out of the chimeric host, passenger leukocytes expressing MHC$^a$ antigens were alloreactive against the kidney parenchyma expressing MHC$^d$ antigens (manuscript submitted). An additional caveat is that the conditioning protocol used to generate mixed chimeras might have had an impact on the immunogenicity of the chimeric lung allograft, but since chimeric organs were harvested at least 250 days after conditioning, this is unlikely to have a significant impact on graft outcome.

The low levels of circulating alloantibody observed in Group 1 and Group 2 recipients (Figure 4), despite evidence of graft rejection, may be explained by absorption of such antibodies in the allograft (32–34). Lack of in vitro donor responsiveness in recipients at the time when they demonstrated histological signs of rejection (Table III) may have been due to the sequestration of anti-donor, cytotoxic lymphocyte precursors within the rejecting lung allograft. Alternatively, in vitro assays may not reflect in vivo immunobiology.

Our results suggest that therapeutic strategies to reduce the immunogenicity of lung allografts may improve graft survival. There have been a few clinical trials in which donor-type passenger leukocytes were eliminated prior to transplantation in order to reduce the immunogenicity of allografts. In one study, renal allografts were treated with anti-CD45 monoclonal antibody prior to transplantation to deplete antigen-presenting cells, which decreased the incidence of acute rejection (35). Using a different approach, kidney donors have been pre-treated with immunomodulatory drugs such as methotrexate, with mixed results on 5-year graft survival (36–39). The emerging use of ex-vivo lung perfusion circuits, which can facilitate both mechanical and pharmacologic leukocyte depletion, may improve lung allograft survival by reducing the passenger leukocyte load; and recent studies have demonstrated successful removal of passenger leukocytes from human lung allografts (40,41).
In conclusion, our data indicate that recipient-matching of passenger leukocytes significantly prolongs lung allograft survival in a large-animal model. Further studies are needed to see whether reducing passenger leukocyte loads prior to transplantation can promote tolerance induction in preclinical trials currently underway (42).

Acknowledgments

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACR</td>
<td>acute cellular rejection</td>
</tr>
<tr>
<td>CML</td>
<td>cell mediated lympholysis</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed-lymphocyte reaction</td>
</tr>
<tr>
<td>PAA</td>
<td>pig allelic antigen</td>
</tr>
<tr>
<td>POD</td>
<td>postoperative day</td>
</tr>
<tr>
<td>SLA</td>
<td>swine lymphocyte antigen</td>
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</table>

Reference List


Figure 1. Immunohistochemical staining with class Ic antibody to detect donor lung chimerism prior to implantation. (A) MHC^{dd} lung at 10× used as negative control showing no Class Ic staining (#21844). (B) MHC^{cc} lung at 10× used as positive control showing staining of epithelium, endothelium, and resident mononuclear cells (#21506). (C and D) Representative pre-implantation chimeric donor lung (#21557) at 10× and 20× showing only resident mononuclear cells positive for class Ic staining.
Figure 2.
Assessment of lung donor (#21557) tissue chimerism by flow cytometry. PAA+ cells are derived from the hematopoietic stem cell donor. Relative percentages of PAA+ versus PAA− cells in populations of CD3+ cells, CD11b+ cells, and MHC class II+ cells are indicated. PBMC from PAA− and PAA+ animals were stained as negative and positive controls.
Figure 3. Histology from representative lung biopsies. A. POD 29 control lung biopsy (Group 2, #21280), showing acute rejection with confluent alveolar inflammation, subendothelial arteriolar infiltrates, and severe intraepithelial bronchiolar infiltrates (A4B2C1D0). B. POD 35 chimeric lung biopsy (Group 1, #21264), showing no rejection (A1B0C0D0). C. POD 99 chimeric lung biopsy (Group 1, #21264), showing no rejection (A1B0C0D0). D. POD 156 chimeric lung biopsy (Group 1, #21264) showing chronic active rejection with alveolar and endothelial infiltrates, fibrin, and fibrosis (A4B2C XD1).
Figure 4.
Alloantibody response. Levels of circulating IgG and IgM alloantibody were measured by flow cytometry in recipients in Group 1 (dotted) and Group 2 (solid). Data was normalized to the mean fluorescence intensity of negative control values to plot normalized mean fluorescence intensity as a function of postoperative day (POD).
### Table I

Characterization of chimeric lung donors.

<table>
<thead>
<tr>
<th>Animal</th>
<th>MHC recipient</th>
<th>MHC donor cells</th>
<th>Myeloid (% replacement)</th>
<th>Lymphoid (% replacement)</th>
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<tbody>
<tr>
<td>20311</td>
<td>AD</td>
<td>AC</td>
<td>≥98%</td>
<td>≥98%</td>
</tr>
<tr>
<td>20313</td>
<td>AD</td>
<td>AC</td>
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<td>90%</td>
</tr>
<tr>
<td>21557</td>
<td>AD</td>
<td>AC</td>
<td>≥98%</td>
<td>85%</td>
</tr>
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</table>

*a* #20311 was lung donor for #21264; #20313 was lung donor for #20557; #21557 was lung donor for #21762.
### Table II

Histology and survival of lung allografts in recipients of single-haplotype MHC-mismatched lung transplants treated with 12 days of cyclosporine.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal # (recipient)</th>
<th>Animal # (donor)</th>
<th>MHC mismatches</th>
<th>Lung Allograft Histology at Week</th>
<th>Graft Survival (days)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Recipient</td>
<td>Donor</td>
</tr>
<tr>
<td>1</td>
<td>20557</td>
<td>20313</td>
<td>AD parenchyma</td>
<td>1</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>21264</td>
<td>20311</td>
<td>AC</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>21762</td>
<td>21557</td>
<td>AC passengers</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>21516</td>
<td>21610</td>
<td>AD parenchyma</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>21280</td>
<td>21098</td>
<td>AD passengers</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21797</td>
<td>21611</td>
<td></td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
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\(a\) Grading of rejection from 0 (none) to 4 (severe), based on the ISHLT classification of pulmonary allograft rejection (11).
## Table III

Combined CML and MLR data for lung recipients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal # (recipient)</th>
<th>Animal # (donor)</th>
<th>SLA mismatches</th>
<th>CML response at time</th>
<th>MLR response at time</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Recipient</td>
<td>Donor</td>
<td>Pretx</td>
</tr>
<tr>
<td>1</td>
<td>20557</td>
<td>20313</td>
<td>AC</td>
<td>AD parenchyma</td>
<td>+AD</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>21264</td>
<td>20311</td>
<td>AC passengers</td>
<td>−</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>21762</td>
<td>21557</td>
<td>AC</td>
<td>AD parenchyma</td>
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</tr>
<tr>
<td>2</td>
<td>21516</td>
<td>21610</td>
<td>AC</td>
<td>AD passengers</td>
<td>+AD</td>
<td>−</td>
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<tr>
<td></td>
<td>21280</td>
<td>21098</td>
<td>AD</td>
<td>AD passengers</td>
<td>+AD</td>
<td>−</td>
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<tr>
<td></td>
<td>21797</td>
<td>21611</td>
<td>AD</td>
<td>−</td>
<td>+AD</td>
<td>−</td>
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</table>

1. *In vitro* state of donor-specific responsiveness (+) or donor-specific unresponsiveness (−) at time before transplant, days 25–35 after transplant, and at time of lung allograft rejection.

2. Results are unavailable.