Donor-specific cytotoxic lymphocyte activity from bronchoalveolar lavage during acute canine lung allograft rejection

Yasuo Sekine, Takehiko Fujisawa *, Yukio Saitoh, Tsunehiro Takeda, Shigetoshi Yoshida, Norikazu Urabe, Masayuki Baba, Yutaka Yamaguchi

Department of Surgery, Institute of Pulmonary Cancer Research, Chiba University School of Medicine, 1-8-1, Inohana, Chu-ku, Chiba, Japan

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Abstract

Objective: We investigated the relationship between acute lung rejection and donor-specific cytotoxic activity (DSCA) in recipient's lymphocytes obtained from bronchoalveolar lavage (BAL). Methods: A total of 26 mongrel dogs underwent left lung allotransplantation. Dogs received either no immunosuppressive treatment (group I), cyclosporine (group II), or cyclosporine and methylprednisolone for evidence of acute rejection (group III). DSCA was measured by a $^{51}$Cr release assay, using lymphocytes from BAL samples as effector cells and $^{51}$Cr-labeled donor skin fibroblasts as target cells. The pathologic findings of the transplanted lungs were classified according to the working formulation for classification and grading of pulmonary rejection. In addition, the degree of cellular infiltration in the perivascular, peribronchial, interstitial, and intraalveolar areas was determined based on an infiltration score. Results: DSCA in BAL samples was elevated during mild, moderate and severe acute rejection. The accuracy of the diagnosis of mild or moderate rejection was 92.3% at effector:target (E:T) ratios of 100:1 and 50:1. The DSCA in BAL fluid and the total infiltration score were correlated closely with correlation coefficients of 0.859 and 0.828 at E:T ratios of 100:1 in group I and group II dogs, respectively. Lung aeration improved and DSCA decreased with methylprednisolone therapy in three of four dogs with grade 2 rejection. Conclusion: There is a direct relationship between the DSCA in BAL fluid and the degree of tissue damage caused by acute rejection. The DSCA can be detected by a $^{51}$Cr release assay which may hold promise for future clinical applications. © 1997 Elsevier Science B.V.

Keywords: Lung transplantation; Acute rejection; Bronchoalveolar lavage; Cytotoxic T lymphocyte; Skin fibroblast

1. Introduction

Cell-mediated immunity plays a critical role in acute rejection [18] and donor-specific cytotoxic T lymphocytes (CTL) are thought to be important effectors that may attack a graft directly [6,16]. However, there have been few studies to elucidate how CTL participates in the graft injury. In order to study the role of CTL in rejection, it is necessary to obtain lymphocytes from the lung. There are two procedures for harvesting CTL from lung allografts; one is transbronchial lung biopsy (TBLB) and the other is bronchoalveolar lavage (BAL). The former is complicated by pneumothorax and bleeding while BAL is relatively safe and more suitable for harvesting lymphocytes from the graft.

Dal Col et al. measured canine donor-specific lymphocyte cytotoxic activity from cells obtained from BAL and cultured for 4–7 days [3]. This method, however, takes too long to detect acute lung rejection and is therefore inappropriate for clinical application. We have reported previously that donor-specific cytotoxic activity (DSCA) of peripheral blood lymphocytes can be detected directly using donor skin fibroblasts as targets during moderate or severe acute rejection of
canine lung allografts [15]. This direct DSCA assay is completed in 7 h from the time of lymphocyte harvest.

In the present study, we were able to show that sequential measurements of DSCA in BAL is useful for predicting the degree of graft injury caused by rejection. We were further able to show that acute rejection can be reversed by methylprednisolone administration, if it is detected by the DSCA assay.

2. Materials and methods

2.1. Experimental groups

Left unilateral lung allotransplantations were performed in 26 pairs of size-matched mongrel dogs (mean weight: 8.6 kg). All dogs received methylprednisolone (25 mg/kg) intravenously immediately after reperfusion of the transplanted lungs. They also received antibiotics (cefoxitin 50 mg/kg per day) intramuscularly for 5 days postoperatively. Dogs were included in the following three groups to demonstrate DSCA in BAL in dogs treated with cyclosporine or without cyclosporine, and the reversibility of lung rejection by methylprednisolone administration. Group I dogs received no cyclosporine (n = 11). Group II dogs received cyclosporine (20 mg/kg per day) orally for 10 days postoperatively (n = 10). Group III (n = 5) dogs received cyclosporine (20 mg/kg per day) orally for 10 days postoperatively and methylprednisolone (50 mg/kg per day) intravenously for 3 days, if CTL activity was 10% or more, or if there was histologic evidence of acute lung rejection on open chest biopsy. All dogs underwent sequential chest radiographs and BAL of their transplanted left lung every 3–4 days or if there was symptomatic evidence of rejection. Six dogs in group I underwent BAL of the contralateral right lung. All 26 dogs underwent open lung biopsies or necropsies after sacrifice immediately transplanted. The ischemic time for the transplantation procedure ranged from 60 to 75 min. All dogs were cared for in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ published by the National Institutes of Health (NIH publication No. 85–23, revised 1985).

2.3. Bronchoalveolar lavage

BAL was performed via a fiberoptic bronchoscope (Pentax 15H, Asahi Optical Co., Tokyo, Japan) by injecting several 15 ml aliquots of sterile saline (total volume: 10 ml/kg). BAL was performed through the subsegmental bronchus aerating the area of greatest radiographic abnormality, according to the method described elsewhere [17]. The BAL fluid was filtered twice through two layers of sterile gauze and was washed twice with 20 ml of RPMI-1640 medium. The collected BAL was analyzed for cell number and cell differential. The BAL cells were centrifuged in a Ficoll-Hypaque solution (Pharmacia Biotech AB, Uppsala, Sweden) for 20 min and the mononuclear cells were then isolated. These cells then were incubated in a plastic culture flask for 1 h and the non-adherent cells were collected as effector cells.

2.4. Cytotoxic activity test

A 3 × 3 cm skin flap was resected from the donor’s hip prior to harvest of the donor lung. Skin fibroblasts were cultured from the skin flap according to the method described previously [4]. The skin fibroblasts were labeled with 200 μCi/ml Na251CrO4 and 1 × 104 51Cr-labeled target cells in RPMI 1640 medium (100 μl) were coincubated with 100 μl of BAL lymphocytes at an effector to target ratio (E:T ratio) of 100:1, 50:1, 25:1, and 12:1 in 96-well round-bottomed tissue culture plates (Corning, New York, USA). After a 4 h incubation in a humidified 5% CO2 atmosphere (37°C), the plates were centrifuged and aliquots of each supernant (100 μl) were harvested and counted for radioactivity. Spontaneous 51Cr release was determined by incubating the target cells with RPMI-1640 medium alone. Maximal release was determined by incubating the target fibroblasts with 1N HCl. The percentage of cell-mediated lysis (%CML) was determined using the following formula (exp., experimental; spon., spontaneous; max., maximum).

\[
\text{Percent CML} = \left( \frac{\text{exp. release} - \text{spon. release}}{\text{max. release} - \text{spon. release}} \right) \times 100
\]

All tests were performed in triplicate. The time from harvest of the effector cells until the end of the CML measurement was approximately 7 h.
The assay was performed at the postoperative 5–8 days when the grafted lungs were supposed to be rejected in group I. In group II, the assay was performed at the postoperative 10–11 days and at the postoperative 14–18 days to demonstrate the effect of cyclosporine and rejection after the termination of cyclosporine.

2.5. Radiographic findings

Radiographic findings were classified into four grades: grade 0, normal; grade 1, mild infiltrates; grade 2, moderate diffuse infiltrates with air bronchograms; grade 3, severe infiltrates or complete opacity.

2.6. Histopathologic findings

Graft specimens obtained by open chest biopsy or autopsy were stained with hematoxylin and eosin and the severity of acute rejection (ACR) was classified into five grades: grade 0, no significant abnormality (A0); grade 1, minimal acute rejection (A1); grade 2, mild acute rejection (A2); grade 3, moderate acute rejection (A3); and grade 4, severe acute rejection (A4), according to the International Working Formulation [20].

2.7. Cellular infiltration score

Histopathologic findings of cellular infiltration were determined in the four following regions: the perivascular area, peribronchial area, interstitial area and intraalveolar area. The grade lymphocytes infiltration in these areas was scored on a scale from 0 to 3 (Fig. 1). The scoring of lymphocyte infiltration was defined as follows: (0) represented no infiltration and normal histologic findings; (1) represented mild infiltration of less than 50% in each specific area examined; (2) represented moderate infiltration between 50% but less than 100% and (3) represented severe infiltration of the entire area. The infiltration score was defined as the sum of all the scores from each area. Three investigators evaluated the radiographic and histopathologic findings independently and all agreed with the final diagnosis.

Fig. 1. Biopsy specimens illustrating the classification of the degree of cell infiltration in the interstitial area. A. 0, no cell infiltration. B. 1, slight cell infiltration (less than 50% of all areas). C. 2, moderate cell infiltration (between 50% and 100% of all areas). D. 3, severe cell infiltration (all areas of the specimen).
3. Results

3.1. CML of BAL lymphocytes

The CML activity of BAL lymphocytes obtained from the grafted lung was tested against donor targets and third-party targets and revealed high CML against only donor targets. Furthermore, there was no increase in the CML activity of BAL lymphocytes obtained from the contralateral lung against donor targets (Fig. 2). These findings were verified in three sets of independent experiments.

The CML activity of BAL from the right lung was then evaluated, using six dogs undergoing allo-transplantation of left lung but not receiving cyclosporine, in order to determine the cut-off values for CML. The assay was performed 5–8 days postoperatively when there was evidence of mild to moderate rejection. The CML activity in BAL from the contralateral lung (mean ± S.D.) at E:T ratios of 100:1, 50:1, 25:1 and 12:1 were 2.88 ± 2.23, 1.90 ± 2.01, 1.58 ± 1.39, and 0.71 ± 1.30, respectively. Using mean values plus three S.D.s, CML values of 10, 8, 6 and 5% were used as the cut-off values for E:T ratios of 100:1, 50:1, 25:1 and 12:1, respectively, in the following study.

3.2. DSCA in BAL during various rejection stages

Table 1 shows the DSCA in BAL, as well as ACR grade and cellular infiltration score in dogs not receiving cyclosporine postoperatively. Among seven dogs with ACR grade 2, five dogs had demonstrable CTL activity and a total infiltration score of 6 or more. All three dogs with ACR grade 3 demonstrated very high values for both DSCA and total infiltration score.

Table 1
Cytotoxic activity against donor skin fibroblasts of lymphocytes in bronchoalveolar lavage fluids in dogs not treated with cyclosporine

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>POD</th>
<th>E:T ratio</th>
<th>ACR grade</th>
<th>Infiltration score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1.7</td>
<td>−0.8</td>
<td>−0.3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5.8</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>12.3</td>
<td>8.5</td>
<td>8.4</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>14.6</td>
<td>14.8</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>24.6</td>
<td>17.2</td>
<td>2.6</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>40.5</td>
<td>31.1</td>
<td>29.6</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>65.9</td>
<td>51.4</td>
<td>50.5</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>82.8</td>
<td>69.2</td>
<td>54.3</td>
</tr>
<tr>
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<td>34.4</td>
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<tr>
<td>10</td>
<td>8</td>
<td>77.8</td>
<td>64.1</td>
<td>54.8</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>17.4</td>
<td>8.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Mean</td>
<td>7</td>
<td>37.5</td>
<td>27.4</td>
<td>22.2</td>
</tr>
</tbody>
</table>

POD, postoperative day; ACR, acute rejection; Vasc., perivascular area; Br., peribronchial area; Int., interstitial area; I-Al, intraalveolar area.
Table 2
Cytotoxic activity against donor skin fibroblasts of lymphocytes in bronchoalveolar lavage fluids

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>POD</th>
<th>E:T ratio</th>
<th>ACR grade</th>
<th>Infiltration score</th>
<th>E:T ratio</th>
<th>ACR grade</th>
<th>Infiltration score</th>
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<tbody>
<tr>
<td>12</td>
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<td>2.2</td>
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<tr>
<td>13</td>
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<td>1.0</td>
<td>0.1</td>
<td>0.5</td>
<td>24.0</td>
<td>7.2</td>
<td>5.5</td>
</tr>
<tr>
<td>14</td>
<td>11.0</td>
<td>9.3</td>
<td>6.6</td>
<td>1.6</td>
<td>23.0</td>
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<tr>
<td>15</td>
<td>10.0</td>
<td>0.1</td>
<td>1.4</td>
<td>1.9</td>
<td>0.1</td>
<td>18.0</td>
<td>27.1</td>
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<tr>
<td>16</td>
<td>10.0</td>
<td>4.5</td>
<td>0.9</td>
<td>1.3</td>
<td>0.5</td>
<td>15.7</td>
<td>14.4</td>
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<td>17</td>
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<td>11.9</td>
<td>7.6</td>
<td>0.1</td>
<td>1.6</td>
<td>16.8</td>
<td>16.5</td>
</tr>
<tr>
<td>18</td>
<td>11.0</td>
<td>1.9</td>
<td>0.5</td>
<td>2.0</td>
<td>3.5</td>
<td>20.1</td>
<td>16.7</td>
</tr>
<tr>
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<td>5.2</td>
<td>2.8</td>
<td>1.1</td>
<td>0.0</td>
<td>22.4</td>
<td>12.4</td>
</tr>
<tr>
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<td>9.5</td>
<td>4.5</td>
<td>0.2</td>
<td>1.8</td>
<td>72.9</td>
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<tr>
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<td>5.0</td>
<td>2.3</td>
<td>0.8</td>
<td>4.3</td>
<td>48.2</td>
<td>12.3</td>
</tr>
<tr>
<td>Mean</td>
<td>10.3</td>
<td>5.0</td>
<td>2.7</td>
<td>0.9</td>
<td>0.1</td>
<td>25.2</td>
<td>15.9</td>
</tr>
</tbody>
</table>

All measurements made at the termination of cyclosporine (POD 10 or 11) and 4–8 days afterwards (POD 14–18).
POD, postoperative day; E:T, effector:target; ACR, acute rejection; Vasc., perivascular area; Br., peribronchial area; Int., interstitial area; I-Al, intraalveolar area; NT, not tested.

Table 3
Reversibility of acute rejection detected by cytotoxic activity against donor skin fibroblasts of lymphocytes in bronchoalveolar lavage fluids

<table>
<thead>
<tr>
<th>Exp no.</th>
<th>Before methylprednisolone therapy</th>
<th>After methylprednisolone therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POD E:T Ratio ACR grade Infiltr. score C-R grade</td>
<td>POD E:T Ratio ACR grade Infiltr. score C-R grade</td>
</tr>
<tr>
<td>22</td>
<td>16 2.6 1.6 1.8 2.4 1.0 4.0 0.0</td>
<td>18 4.1 0.4 0.7 2.2 0.0</td>
</tr>
<tr>
<td>23</td>
<td>17 17.2 12.6 7.2 4.8 2.6 6.1</td>
<td>25 2.9 2.8 2.2 0.3 0.0</td>
</tr>
<tr>
<td>24</td>
<td>19 10.5 4.3 3.8 1.5 2.6 6.1</td>
<td>24 2.7 3.0 1.1 1.7 0.1</td>
</tr>
<tr>
<td>25</td>
<td>27 38.6 31.8 25.9 5.3 1.2</td>
<td>34 6.5 3.5 5.2 3.8 1.5</td>
</tr>
<tr>
<td>26</td>
<td>18 13 5.6 4.8 1.8 2.8 2.1</td>
<td>21 6.2 1.8 0.8 2.2 0.0</td>
</tr>
<tr>
<td>mean</td>
<td>19.4 16.4 11.2 8.5 5.7 1.8 6.1</td>
<td>24.4 4.5 0.1 0.7 2.0 0.8</td>
</tr>
</tbody>
</table>

POD, postoperative day; E:T, effector:target; ACR, acute rejection; Infiltr., infiltration; C-R, chest roentgenogram.
* P<0.1, ** P<0.05 in comparison to the corresponding values before methylprednisolone therapy.
Among dogs receiving cyclosporine for 10 days postoperatively (group II), only one of the ten dogs had an elevated DSCA while another dog had ACR grade I (Table 2). However, 4–8 days after the termination of cyclosporine (postoperative days 14 to 18), all eight dogs with ACR grade 2 or 3 had elevated values for DSCA at an E:T ratio of 100:1 and a total infiltration score 7 or more.

3.3. Reversibility of ACR detected by DCSA assay

Reversibility of acute lung rejection was examined in five dogs receiving cyclosporine 10 days postoperatively which were followed up by sequential testing of the DSCA in the BAL after discontinuation of the cyclosporine. If the DSCA increased or there was ACR grade 1 or 2 scoring of open lung biopsy specimens, high dose methylprednisolone was administered for 3 days. Histologic examination of the biopsy specimen after methylprednisolone administration showed improvement in the ACR grade of four dogs, as well as a significant decrease in the infiltration score in three of the dogs and evidence of significant aeration of the grafted lung on chest roentgenogram in four (Table 3). One dog (No. 26) had evidence of significant granulocyte infiltration in the grafted lung, consistent with pneumonia.

3.4. Reliability of DSCA in BAL

The reliability of diagnosing grade 2, 3 or 4 acute rejection by measurement of the DSCA in the BAL was investigated, using the results from group I and group II at postoperative days 14–18 and group III before methylprednisolone administration. The specificity, sensitivity, and accuracy of a positive result for ACR grade 2, 3, or 4 were 100% (3/3), 90.0% (20/22), and 92.0% (23/25), respectively, at E:T ratio of 100:1. They were 100% (3/3), 81.8% (18/22), and 84.0% (21/25), respectively, at E:T ratio of 50:1. These results suggest that this test has a high reliability. However, the sensitivity and accuracy decreased to 63.6% and 68.0%, respectively, at E:T ratios of 25:1, and 54.5% and 56%, respectively, at E:T ratio of 12:1.

3.5. Correlation between cellular infiltration score and DSCA in BAL

There was a strong relationship between the total infiltration score and the CTL activity in the BAL with a positive correlation of 0.940 ($P < 0.0001$) at an E:T ratio of 100:1 in group I dogs (Fig. 3). Furthermore, the correlation coefficients at E:T ratios of 50:1, 25:1 and 12:1 were also highly significant at 0.895 ($P = 0.0002$), 0.886 ($P = 0.0003$) and 0.877 ($P = 0.0004$), respectively. The correlation between the CTL activity and the infiltration scores of the perivascular and peribronchial areas were significant at four different E:T ratios ($r = 0.734—0.800, P = 0.01—0.0031$). The correlations between the CTL activity and the infiltration score from the interstitial and intraalveolar areas were significant at an E:T ratio of 100:1 ($r = 0.624, P = 0.0403$ and $r = 0.617, P = 0.0430$, respectively).

Among group II dogs, there was a significant correlation between DSCA and total infiltration score ($r = 0.669, P = 0.0489$) at an E:T ratio of 100:1 (Fig. 4). There were also significant correlations between CTL activity and the infiltration score of the interstitial and intraalveolar areas at an E:T ratio of 100:1 ($r = 0.848, P = 0.0039$ and $r = 0.808, P = 0.0084$, respectively). However, no significant correlations between total infiltration score and DSCA at E:T ratios of 50:1, 25:1 and 12:1 were demonstrated. Further, no significant correlations among four subgroups of cellular infiltration scores and DSCA at E:T ratios of 50:1, 25:1 and 12:1 were also demonstrated.

4. Discussion

Because the main cause of posttransplant acute rejection is a cell-mediated immunologic reaction in which T
lymphocytes play a leading role, immunologic monitoring can help in the understanding of the progression and mechanism of rejection. However, various effector cells can play important intricately interconnected roles during each phase of tissue injury [7,11]. It is therefore important to determine which set of cells is best for monitoring. Furthermore, it is clinically important to distinguish between infection and acute rejection in lung transplant recipients postoperatively. This can be difficult because both share many clinical and radiologic features [8], and it would be extremely helpful to have an objective and specific method to discriminate between these two conditions.

Several investigators [3,10,12–14,21] have reported that the activity of lymphocytes in BAL mirror the immune reactions in the graft. Because of this, BAL is a very useful method for monitoring rejection through use of a proliferative response assay. However, these responses against alloantigen or mitogen require several days to measure. In the present study, we found that the DSCA assay using BAL lymphocytes could reliably diagnose mild to moderate acute rejection with a high sensitivity and specificity. Our present findings are consistent with the previous reports [3,10,12–14,21]. However, our assay requires only 7 h to complete which is considerably shorter than the proliferative response assay.

This assay includes the use of donor skin fibroblasts as the target cells, a method first reported by Benfield et al. [1]. Skin fibroblasts express class I antigens [2] and are easily cultured in vitro. Fibroblasts also can be kept frozen and used for the assay whenever necessary. Furthermore, because the measurement of CML is reproducible when compared to lectin-activated lymphoblast assays [4,15] and because chronic as well as acute rejection relies on the presence of class I antigens [14], this method may be useful for long-term monitoring for evidence of rejection.

In the present study, we also found a strong correlation between tissue injury and DSCA. The tissue injury score in each area, perivascular, peribronchial, interstitial, and intraalveolar areas, increased with DSCA in the BAL. Cellular infiltration in the interstitium and the intraalveolar area correlated significantly with the DSCA in group II dogs, suggesting that such infiltration may be important in influencing the CTL activity in the BAL. We believe that this supports the hypothesis that CTL are the mediators of tissue injury that the degree of tissue destruction can be followed by measurements of CTL activity in the BAL. This would obviate the need for a TBLB. Simultaneous testing of DSCA of bilateral BAL lymphocytes revealed no increase in activity in the contralateral lung, indicating donor-specific cytotoxic T lymphocyte activation occurs only in the graft.

The BAL-derived primed lymphocyte test (PLT) [12,13,21], the mitogen responses of BAL lymphocytes [10] and donor-specific PLT [14] are all immunologic tests that can discriminate between rejection and infection. Because donor-specific CTL activity was assayed in the present study, clear discrimination between infection and rejection was possible. While infection is primarily a nonspecific inflammatory reaction, rejection is predominantly a specific immune reaction, and hence our assay is both sensitive and specific. Furthermore, our assay can permit assessment of the degree of tissue destruction, which is not possible with the other techniques. Because Rabinowich and coworkers [12] found that donor-specific CTL plays an important role in chronic rejection, our assay may be useful in diagnosing chronic rejection or in assessing the degree of tissue injury in relation to clinical findings such as respiratory dysfunction [9].

In conclusion, there is a close correlation between the activation of cytotoxic T lymphocytes and tissue damage during acute lung allograft rejection. Based on this, mild rejection can be detected by DSCA in BAL lymphocytes, and this approach may therefore prove to be clinically useful for following patients after lung transplantation.

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