DETECTION OF ALLOREACTIVE T CELLS BY FLOW CYTOMETRY: A NEW TEST COMPARED WITH LIMITING DILUTION ASSAY

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Background. Frequencies of alloreactive T cells determined by limiting dilution assays (LDA) may not adequately reflect the donor-reactive immune status in transplant recipients. To reevaluate LDA frequencies, we developed a flow cytometry test for direct determination of alloreactive T-cell frequencies and compared these frequencies with classical LDA estimates of frequencies.

Methods. For determination of frequencies by flow cytometry, peripheral blood lymphocytes (or lymphocytes taken from primary mixed lymphocyte culture) were stimulated with either Epstein-Barr virus–transformed lymphoblastoid cell lines or T-cell–depleted spleen cells and stained for intracellular interferon (IFN)-γ production and CD69. In lung transplant recipients, frequencies of IFN-γ alloreactive T cells were compared with LDA frequencies, that is, cytotoxic T lymphocyte precursors and helper T lymphocyte precursors.

Results. With flow cytometry, alloreactive T cells were detected after overnight allostimulation as IFN-γ CD69bright cells (range, 0.1–0.58% and 0.1–0.66% of total CD4 and CD8 cells, respectively). Frequencies increased 25-fold or more when lymphocytes were pre-stimulated in primary mixed lymphocyte culture before testing. After lung transplantation, mean donor-specific IFN-γ CD8 T-cell frequencies did not decrease as mean donor-specific LDA cytotoxic T lymphocyte precursor frequencies, whereas no difference was seen in pretransplantation samples or third-party–specific frequencies at both time points. Mean frequencies of IFN-γ CD4 did not differ from helper T lymphocyte precursors at both time points, but frequencies did not correlate.

Conclusions. The flow cytometry test allows a direct measurement of alloreactive T-cell frequencies and demonstrates a discrepancy between donor-specific IFN-γ CD8 T-cell frequencies and LDA CLTTP after transplantation. This may be a result of the existence of “functional diverse” alloreactive T cells or of acti-
viation-induced cell death of donor-reactive T cells during long (LDA) culturing, which is avoided in the flow cytometry test.

To identify recipients at risk to develop chronic graft dysfunction after solid organ transplantation, in vitro tests measuring the recipient’s T-cell antidonor response have been designed. The most precise and quantitative test used in this respect is the limiting dilution assay (LDA), measuring frequencies of donor-specific T-cell precursors in peripheral blood. Using this test, it has been demonstrated that frequencies of donor-specific cytotoxic T-cell precursors decrease in patients with well-functioning heart (1), kidney (2) and liver transplants (3, 4). However, frequencies have also been found to decrease in recipients of kidney and lung transplants who developed chronic graft dysfunction (5, 6). One explanation may be that donor-specific T-cell precursor frequencies decrease gradually in all recipients irrespective of graft function, possibly as the result of prolonged exposure of T cells to donor graft cells in the absence of costimulation, leading to anergy and apoptosis (5). An alternative explanation, however, may be that alloreactive T-cell frequencies measured by LDA may not always reflect true donor-specific frequencies.

The latter notion has emerged from recent studies using HLA tetramers or interferon (IFN)-γ ELISpot assays as alternative assays to estimate frequencies. These studies suggest that frequencies measured by LDA might underestimate true frequencies, partly as a consequence of activation-induced cell death in the LDA cultures (7–10). Thus, the long culturing of lymphocytes in LDA for 7 to 10 days could significantly influence the measurement of frequencies. Therefore, new tests should be developed that can verify LDA estimates of frequencies of alloreactive T cells in transplant recipients. These should avoid long culturing times and allow a more direct ex vivo measurement of frequencies of alloreactive T cells.

New approaches to analyze T-cell frequencies, with potential application in transplantation medicine, can be based on flow cytometric analysis. Examples of this are the use of HLA tetramer-peptide complexes (7, 8), 5-6-carboxyfluorescein diacetate succinimidyl ester (CSFE) marker to trace cell division (11), and intracellular cytokine detection on the single cell level (12–14). The use of HLA tetramers in a transplantation setting is not attractive because of the heterogeneity of allopeptides and allogeneic HLA molecules in (direct) allore cognition by T cells (15). Assays using the CSFE marker again need long culturing times, which should be avoided. We chose a relatively easy, direct, and specific method to detect alloreactive T cells, using a flow cytometry test for detection of intracellular cytokine production by T cells upon allostimulation. More specifically, we assessed alloreactive T-cell frequencies by short overnight allosimulation setting. We first set up a flow cytometry test to detect alloreactive T cells in PBL samples. For this we stimulated responder PBL from healthy controls with different types of stimulator cells and stained for intracellular IFN-γ production in activated (CD69bright) CD3+ CD4 and CD8 T cells. In some cases, alloreactive T cells in PBL were expanded in mixed lymphocyte culture (MLC) before frequency analysis by flow cytometry. Subsequently, we compared alloreactive T-cell frequencies detected by flow cytometry analysis and LDA in PBL from lung transplant recipients, before and 1 year after lung transplantation.

Recipient Cells

PBL from healthy individuals or allograft recipients were isolated from peripheral blood using Lymphoprep density gradients (Nycomed, Oslo, Norway). Cells were frozen in RPMI/10% human serum/10% dimethyl sulfoxide using an automated Cryo-10 series III freezing-apparatus (Planer Products, Middlesex, UK) and cryopreserved in liquid nitrogen. Before use, PBL were thawed by rapid thawing of the vials to 0°C followed by slowly diluting the dimethyl sulfoxide in the samples by dropping 10 mL of ice-cold RPMI/2% human serum albumin into the thawed samples. Cells were then resuspended in RPMI supplemented with 10% heat-inactivated fetal calf serum (RPMI/FCS) or RPMI supplemented with 15% heat-inactivated pooled human AB serum (RPMI/AB serum). Only samples with good cell viability (more than 85% viability, as judged by trypan blue dye exclusion) were analyzed.

Allospecific T cells were expanded in MLC by culturing 10^6 spleen cells with 10^6 irradiated (50 Gy) allogeneic LCL for 6 days. Cells were then washed and incubated for another 3 days in RPMI/FCS containing 2.5 U of interleukin (IL)-2/mL (Cetus, Emeryville, CA) before (re)stimulation and measurement of frequencies by the flow cytometry test as indicated below.

Stimulator Cells

LCL and T-cell-depleted spleen cells were tested as allogeneic stimulator cells. LCL and spleen cells were tissue typed using standard procedures. LCL were grown in RPMI/FCS and irradiated (50 Gy) before use as stimulator cells. Spleen cells were thawed and resuspended in ice-cold RPMI/FCS. After irradiation (50 Gy unless stated otherwise), T cells were removed from the spleen cell preparations. For this, 20×10⁶ spleen cells were incubated with anti-CD3 monoclonal antibody (hybridoma supernatant; clone RIV-9) for 30 min at 4°C on a head-over-head rotor. Then, cells were washed three times with ice-cold RPMI, and goat anti-mouse antibody-coated Dynabeads (Dynal, Oslo, Norway) were added at concentrations according to the manufacturer’s instructions. After a 20-min incubation at 4°C, beads and T cells were separated using a magnet and the remaining cells were washed once. T cell–depleted spleen cells contained on average less than 3% CD3 T cells.
Detection of Alloreactive T Cells by the Flow Cytometry Test

Alloreactive T cells were identified by measuring intracellular IFN-γ in activated (CD69 bright) CD4 or CD8 cells upon short allo-stimulation in vitro. For stimulation, 2 × 10^6 viable responder cells were added to 4-mL polystyrene tubes (Greiner, Solingen, Germany) containing RPMI/FCS only (unstimulated samples), 2 × 10^6 irradiated stimulator cells in RPMI/FCS (allostimulated samples), or RPMI/FCS containing 25 ng/mL phorbol myristate acetate (PMA; Sigma, St. Louis, MO) and 1 μg/mL ionomycin (Sigma; positive control sample). Unstimulated and allostimulated samples were incubated overnight at a 5° slant at 37° C and 5% CO₂. Brefeldin A (Sigma; 10 μg/mL) was used as a blocker of cytokine secretion and added to these cultures only after the initial 6 hr of culture. PMA/ionomycin-stimulated samples were incubated for 4 hr at a 5° slant at 37° C and 5% CO₂ in the immediate presence of Brefeldin A (10 μg/mL). After stimulation, samples were washed with PBS containing 1% bovine serum albumin, and resuspended in a small volume of PBS containing 1% bovine serum albumin and 5% human AB serum. Then, surface marker–specific antibodies were added (CyQ-labeled anti-CD3, IQ Products, The Netherlands; and APC-labeled anti-CD8, PharMingen, San Diego, CA) and cells were incubated for 15 sec at room temperature. Next, FACS lysing solution (Becton Dickinson, San Jose, CA) was added, and cells were incubated for 10 min at room temperature. After washing, CD69-specific phycoerythrin (PE)-labeled antibody (Coulter, Buffalo, NY) and IFN-γ-specific fluorescein isothiocyanate-labeled antibody or a fluorescein isothiocyanate-labeled isotype-matched control antibody (Becton Dickinson) was added and cells were incubated for 30 min. After washing, stained cells were analyzed on a flow cytometer (Coulter EPICS Elite) by gating on lymphocytes and CD3+ cells with subsequent analysis of CD69+ IFN-γ+ cells of total CD3+ CD4 or CD8 T cells. Ab, antibody.

Detection of Alloreactive T-Cell Precursors by Limiting Dilution Assay

A combined LDA was used to assess cytotoxic T lymphocyte precursor (CTLp) and helper T lymphocyte precursor (HTLp) frequencies. The protocol was adapted from the protocol described by Bouma et al. (17). Although longer culturing times are used in a combined LDA assay (10 days vs. 7 days in LDA determining CTLp only), CTLp frequencies are equal to frequencies found with an LDA determining CTLp only (17). The LDA was set up as described previously (9). Briefly, graded numbers of viable responder PBL (5 × 10⁴ to 1.25 × 10⁵ cells per well; 24 replicate cultures) were cultured with 5 × 10⁴ irradiated (50 Gy) stimulator cells (donor or third-party...
CD8 T cells, respectively. The latter data was available because the readings ([3H]thymidine incorporation in CTLL-D cells or 51Cr release from target cells) were higher than the mean counts plus 3 SD of the control wells containing stimulator cells only. Frequencies of donor or third-party allotype-specific T cells were determined by the chi-square minimization procedure and considered reliable only if probability values were indicative of single hit kinetics (P > 0.05). To exclude changes in the HTLp and CTLp frequencies caused by non-specific factors such as lymphopenia, frequencies were corrected for percentages of CD4 and CD8 lymphocytes present in the PBL samples and were expressed as HTLp and CTLp per 10^6 total CD4 or CD8 cells, respectively. The latter data was available because the same samples were also analyzed in the flow cytometry test (see above).

### Statistical Analysis

Differences in frequencies (either LDA estimates or frequencies of IFN-γ+ T cells) were analyzed by the Mann-Whitney U test (unpaired testing) or Wilcoxon paired testing. P values less than 0.05 were considered to represent statistically significant differences.

### RESULTS

#### Detection of Alloreactive T Cells by Intracellular IFN-γ Staining

For detection of alloreactive cells by flow cytometry, we used intracellular staining for IFN-γ in activated (CD69 bright) CD3+ CD4 and CD8 T cells. IFN-γ production was hardly seen in T cells of unstimulated samples (Fig. 1A; 0.02% of CD4 and of CD8 cells). We then stimulated the responder PBL overnight with irradiated, fully mismatched LCL and found that 0.17% and 0.39% of activated CD4 and CD8 T cells produced IFN-γ (Fig. 1B). Cells stained with isotype-matched control antibody did not show positive cells (Fig. 1C). PMA/ionomycin stimulation, as a positive control, gave 34% and 65% IFN-γ+ CD4 and CD8 cells (Fig. 1D). To optimize the assay we used different responder to stimulator ratio’s and incubation times: a responder-stimulator ratio of 1:1 and overnight stimulation yielded highest numbers of IFN-γ+ cells in both CD4 and CD8 T cells as compared to other responder-stimulator ratio’s or incubation times (data not shown). In testing 12 different, fully MHC-mismatched responder-stimulator combinations at these conditions we found alloreactive IFN-γ-producing T cells in a range of 0.1–0.58% (mean %, SD: 0.27 ± 0.17) and 0.1–0.66% (mean %, SD: 0.34 ± 0.17) of total CD4 and CD8 cells, respectively (Table 1).

#### Table 1. Numbers of alloreactive IFN-γ-producing CD4 and CD8 T cells in 12 different, fully MHC-mismatched, responder-stimulator combinations of healthy individuals

<table>
<thead>
<tr>
<th>Responder-stimulator combination</th>
<th>% IFN-γ+ CD4 cells</th>
<th>% IFN-γ+ CD8 cells</th>
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<tbody>
<tr>
<td>1</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
<td>0.66</td>
</tr>
<tr>
<td>5</td>
<td>0.11</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>0.12</td>
<td>0.42</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>0.29</td>
</tr>
<tr>
<td>8</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td>9</td>
<td>0.43</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>0.4</td>
<td>0.18</td>
</tr>
<tr>
<td>11</td>
<td>0.55</td>
<td>0.58</td>
</tr>
<tr>
<td>12</td>
<td>0.55</td>
<td>0.49</td>
</tr>
<tr>
<td>Mean, SD</td>
<td>0.27±0.17</td>
<td>0.34±0.17</td>
</tr>
<tr>
<td>Range</td>
<td>(0.1–0.58)</td>
<td>(0.1–0.66)</td>
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</table>

* Shown is percentage of IFN-γ+ positive cells of the total CD4 and CD8 cells, respectively.

Alloreactive T Cells in Mixed Lymphocyte Culture-Expanded Peripheral Blood Lymphocytes

Next, we tested whether T lymphocytes expanded in primary MLC showed an increase in numbers of IFN-γ+ cells. Indeed, high numbers of IFN-γ+ cells were seen upon overnight restimulation with LCL: numbers of T cells responding with intracellular IFN-γ production increased 25-fold or more to 4% and 12% for CD4 and CD8 T cells, respectively (Fig. 2B). MLC samples that were not restimulated showed low numbers of IFN-γ+ cells (Fig. 2A; 0.45% and 0.26% for CD4 and CD8, respectively).

To investigate whether T cells were specifically responding to the alloantigens, we restimulated T cells obtained from primary MLC (n = 9) with the same LCL that was used in the primary MLC (primary “donor” LCL) or a “third-party” LCL. Up to 23% of the T cells were found to be IFN-γ+ upon restimulation with donor LCL, in all cases much higher than the numbers seen after restimulation with a third-party LCL (Fig. 3; showing data only for CD8). Thus the IFN-producing T cells are allo-specific cells activated by the overnight stimulation. Taken together, these data show that T cells can be identified by flow cytometry as IFN-γ+ cells after overnight stimulation with allogeneic cells.

T Cell–Depleted Spleen Cells as an Alternative Source of Allogeneic Stimulator Cells

As shown above, LCL efficiently stimulate IFN-γ production in alloreactive T cells, but these cells may stimulate EBV-specific T cells as well (14). In that way, the use of these cells to detect alloreactive T cells in organ transplant recipients would be limited to EBV-negative responders or to fully mismatched recipient-donor combinations. We therefore exploited alternative sources of allostimulator cells, such as spleen cells. To ensure that the stimulator spleen T cells did not contribute to the IFN-producing population (for example as a result of backward stimulation), they were irradiated. Irradiation over 50 Gy decreased the frequency of IFN-producing spleen CD8 T cells after PMA/ionomycin stimulation from 76% to below 0.16% (Fig. 4A). To further minimize contribution of stimulator T cells to the IFN-producing T cells, the irradiated spleen cells were additionally depleted for T cells as outlined in the Material and Methods section. These irradiated and T cell–depleted spleen cells induced IFN production in alloreactive T cells to similar levels as seen after LCL stimulation (Fig. 4B; 0.12% and 0.42% IFN+ CD4 and CD8 cells, respectively). Again, positive cells were hardly seen in T cells of unstimulated samples or control antibody-stained samples (Fig. 4B). Thus, spleen cells that are irradiated and T cell depleted are good alternative stimulator cells that induce
IFN production in alloreactive responder T cells without interference with the measurement.

Comparison of Allospecific T-Cell Frequencies in Lung Transplant Recipients Detected by Flow Cytometry and Limiting Dilution Assay

We next compared the direct measurement of alloreactive T-cell precursor frequencies by flow cytometry with measurement of frequencies by LDA that uses long in vitro culturing time. For this, we used PBL samples obtained from patients before and 1 year after lung transplantation. The mean percentages of T cells responding with IFN production after PMA/ionomycin stimulation were not different before or after transplantation, showing that immunosuppression did not affect intracellular IFN-γ production in posttransplantation T cells (Table 2). For allostimulation, samples were stimulated with T cell-depleted and irradiated donor spleen cells. In these samples, we analyzed alloreactive T-cell frequencies, expressed as IFN-γ⁺ CD4 or CD8 T cells per 10⁶ total CD4 or CD8 cells, respectively (flow cytometry test), and as HTLp or CTLp per 10⁶ total CD4 or CD8 cells (LDA). Frequencies of donor-specific and third-party–specific IFN⁺ CD4 cells were compared with HTLp frequencies (Fig. 5) and frequencies of donor-specific and third-party–specific IFN⁺ CD8 cells were compared with LDA CTLp frequencies (Fig. 6). We found that mean donor-specific and third-party–specific IFN⁺ CD4 cell frequencies detected by flow cytometry and LDA HTLp frequencies largely overlapped (Fig. 5) for both time points. When comparing all flow cytometry IFN⁺ CD4 T-cell frequencies with LDA HTLp frequencies (all donor-specific and third-party–specific frequencies, for both time points), we found no correlation between these frequencies (r = 0.134, P = 0.552). It should be noted, however, that IFN-γ and IL-2 production (which is measured in the HTLp LDA) are not always coordinate in CD4 T cells. We found that, when isolated from PBL and stimulated with PMA/ionomycin, approximately 40% of IL-2⁺ producing CD4 T cells also produced IFN-γ, while of (PBL derived) CD4 T cells that had been allostimulated a number of times in vitro, approximately 60% of the IL-2⁺ producing CD4 T cells also produced IFN-γ (data not shown).

Also for CD8 T cells, pretransplantation mean flow cytometry IFN⁺ CD8 T-cell frequencies and mean LDA CD8 CTLp frequencies of donor-specific and third-party–specific cells overlapped (Fig. 6). After transplantation, however, mean donor-specific IFN⁺ CD8 T-cell frequencies were significantly higher than mean LDA donor-specific CD8 CTLp frequencies (P = 0.04, Fig. 6). This difference was not seen between third-party–specific IFN⁺ CD8 T cell and LDA CD8 CTLp frequencies (Fig. 6). In these patients, posttransplantation LDA donor-specific CTLp frequencies were significantly lower than LDA third-party–specific CTLp frequen-
FIGURE 4. T-cell–depleted spleen cells as alternative stimulator cells for induction of intracellular IFN production in alloreactive T cells. Irradiation of undepleted spleen cells strongly reduces their capability to produce intracellular IFN-γ upon PMA/ionomycin stimulation (A; data shown only for CD8 cells). Combined T-cell depletion and irradiation yields stimulator cells that induce intracellular IFN production in alloreactive T cells upon overnight stimulation (B). Frequencies are percentages of total CD3+ CD4 or CD8 T cells.

**Allostimulated/ control ab for IFN**

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specific and third-party T-cell frequencies with LDA CTLp frequencies (all donor-specific CD69 expression from the test because IFN cells). To simplify the test, it is possible to exclude analysis of T-cell specificity with phenotype (e.g., CD4-, CD8-positive edition, it allows a combination of the detection of alloreactive MLC or LDA because it avoids long culturing times. In addition, it offers clear advantages compared with classic tests such as flow cytometry test allowed us to compare frequencies of cytokine-producing donor-specific CD8 T cells 1 year after lung transplantation. This is a surprising result because donor-specific LDA CTLp frequencies were found to be significantly decreased at this time point (Fig. 6B). These data may point to either the existence of subsets of alloreactive CD8 T cells that do not display cytotoxic effector functions or, alternatively, to an increased sensitivity for activation-induced cell death of posttransplantation CD8 T cells in LDA cultures, as will be discussed below.

The flow cytometry test allowed us to compare frequencies of cytokine-producing donor-specific T cells with LDA frequencies of donor-specific T cells that display a specific effector function, for example, cytotoxicity. In our study, frequencies of IFN-producing alloreactive CD8 T cells (or CD4 T cells) did not correlate with LDA estimates of CTLp (or HTLp) frequencies. This is in contrast with a previous study by Tan et al. (18), who demonstrated a positive correlation between EBV-specific IFN-producing CD8 cells and LDA CTLp. The lack of correlation in our study cannot be attributed to the donor-specific decrease in CTLp frequencies after transplantation because pretransplantation frequencies alone also did not correlate. To explain the lack of correlation, it can be assumed that alloreactive T cells display a “functional diversity.” This functional diversity has previously been illustrated for EBV-specific T-cell lines, which showed an inverse relationship between IFN-γ production and cytotoxic potential (19, 20). On the other hand, effector CD8 T cells usually display both perforin/granzymes and IFN-γ (21). It would be of interest to determine whether the allospecific IFN-producing donor-reactive CD8 T cells detected in lung transplant recipients also contain perforin/granzymes. Cytotoxic or not, the detected cells may significantly contribute to

### DISCUSSION

In this study we have shown that alloreactive T cells in peripheral blood samples can be detected by flow cytometry using short allostimulation in vitro and detection of intracellular IFN production in activated T cells. This approach offers clear advantages compared with classic tests such as MLC or LDA because it avoids long culturing times. In addition, it allows a combination of the detection of alloreactive T-cell specificity with phenotype (e.g., CD4-, CD8-positive cells). To simplify the test, it is possible to exclude analysis of CD69 expression from the test because IFN+ cells were always CD69 bright (Figs. 1, 2, and 4). This would allow for analyzing the expression of more cytokines and activation and migration markers in addition to the currently used markers. This makes the test a versatile tool in analyzing alloreactive responses after allograft transplantation.

An important finding from this study is that the flow cytometry test detects persisting frequencies of donor-specific IFN-γ CD8 T cells 1 year after lung transplantation. This is a surprising result because donor-specific LDA CTLp frequencies were found to be significantly decreased at this time point (Fig. 6B). These data may point to either the existence of subsets of alloreactive CD8 T cells that do not display cytotoxic effector functions or, alternatively, to an increased sensitivity for activation-induced cell death of posttransplantation CD8 T cells in LDA cultures, as will be discussed below.

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### Table 2. Intracellular IFN-production in pretransplantation and posttransplantation samples upon PMA/ionomycin stimulation

<table>
<thead>
<tr>
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<th>Before transplantation (n=5)</th>
<th>After transplantation (1 year; n=6)</th>
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<tbody>
<tr>
<td>% CD69+/IFN+ CD4 T cells</td>
<td>16±7</td>
<td>22±6</td>
</tr>
<tr>
<td>% CD69+/IFN+ CD8 T cells</td>
<td>53±23</td>
<td>56±14</td>
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### Figure 5. Comparison of donor-specific and third-party-specific CD4 T-cell frequencies in lung transplant recipients (n=6) measured before (A) and after (B) transplantation by flow cytometry or LDA. For one patient, a pretransplantation sample was not available. Mean frequencies against donor or third party detected by flow cytometry or LDA did not differ significantly for both time points (P=0.42 and P=0.84, for donor-specific and third-party-specific frequencies before transplantation, respectively; P=0.48 and P=0.82, for donor-specific and third-party-specific frequencies after transplantation, respectively). Tx, transplantation.
A. Before Tx

B. After Tx

**FIGURE 6.** Comparison of donor-specific and third-party-specific CD8 T-cell frequencies in lung transplant recipients (n=6) measured before (A) and after (B) transplantation by flow cytometry or LDA. For one patient, a pretransplantation sample was not available. Mean frequencies against donor or third party detected by flow cytometry or LDA did not differ significantly before transplantation (P=0.43 and P=0.22, for donor-specific and third-party-specific frequencies, respectively). After transplantation, the mean donor-specific CTLp frequency was significantly lower than the mean IFN-γ CD8 T-cell frequency detected by flow cytometry (P=0.04). Also, the mean donor-specific CTLp frequency was significantly lower than the mean third-party-specific CTLp frequency at this time point (P=0.04). Mean third-party-specific CTLp frequencies after transplantation were not different from pretransplantation frequencies (P=0.4), whereas mean donor-specific CTLp frequencies after transplantation were lower than before transplantation (P=0.003).

graft inflammation by producing IFN-γ, and as a consequence, up-regulating alloantigen expression and inducing chemokine production (e.g., IP-10, MIG), which will further chemoattract activated T lymphocytes (22, 23). The clinical relevance of the persistence of donor-reactive IFN-γ-producing CD8 cells in lung transplant patients remains unknown. Although the investigated patients all had acute rejection episodes, none developed chronic rejection in the studied time interval. However, to investigate this issue in a meaningful way, frequencies of donor-reactive IFN-γ-producing CD8 cells in patients with and without chronic rejection should be compared.

The observed discrepancy in IFN-producing CD8 T-cell frequencies detected by flow cytometry and donor-specific CTLp frequencies detected by LDA may be a result of an increased sensitivity for activation-induced cell death of CD8 T cells in the long LDA culture, as suggested previously (7–10). The IFN-producing cells detected by flow cytometry could be precursors of cytotoxic effector T cells that up-regulate Fas ligand upon activation. Interaction of Fas ligand with Fas would lead to activation-induced cell death upon prolonged culture in vitro, particularly when cultured in IL-2 (which is added to LDA cultures), which is known to enhance apoptosis (24, 25). Consequently, strongly reduced frequencies will be detected by LDA. This process may particularly occur after transplantation where immunosuppressive agents, in particular steroids, may augment apoptosis of activated T cells in vitro (26). The fact that donor-specific CTLp T cells and not third-party–specific CTLp T cells show decreased frequencies may be a result of in vivo stimulation of T cells by donor alloantigens expressed by the graft, before their (re)stimulation in vitro in LDA cultures. Thus, an increased sensitivity toward activation-induced cell death of posttransplantation donor-specific CD8 T cells in LDA culture may have contributed to the discrepancy in donor-specific IFN-γ CD8 T cells and LDA CTLp frequencies.

The detection of alloreactive T cells by the flow cytometry test requires a short stimulation by allogeneic cells before detection of intracellular IFN-γ in responding cells. In the current study we used allogeneic LCL and T cell–depleted spleen cells for stimulation. Because the specificity of alloreactive T cells is not well defined and is complex in nature, stimulation and detection of T cells that respond through “direct” allorrecognition is not easily accomplished by methods using stimulation with immunodominant peptides or peptide-loaded HLA tetramers, as used for example in detection of virus-specific T cells in a “self-restricted” setting (10, 13, 18). Stimulation with whole cells carrying the alloantigens therefore seems by far the most efficient way because they carry all possible combinations of allogeneic MHC-peptide complexes. Their use, however, may be restricted when using EBV-transformed LCL. Clearly, these cells may evoke EBV-specific T-cell responses (14) and should be avoided when analyzing allospecific T cells in EBV− responders and (partly) matched recipient/donor combinations. In our experiments, responder and donor LCL were always fully mismatched to exclude detection of EBV-specific T cells. Yet, we saw low but significant numbers of IFN-γ CD8 cells upon restimulation with third-party LCL (Fig. 3). This may have resulted from the extensive activation and proliferation in the primary MLC. Alternatively, it may be a result of the fact that responder and third-party LCL were partly matched in a number of combinations, which may lead to EBV-specific responses upon restimulation with third-party LCL in case of EBV− responders (Fig. 3; EBV− responder in combinations 1, 2, 4, and 8; EBV-negative responders in combination 7; and unknown EBV status in combinations 3, 5, 6, and 9). Apart from the above considerations, LCL-derived IL-10 may influence intracellular IFN production in alloreactive T cells (27). In a similar experiment we found significant levels of IL-10 in short-term cultures of LCL and coculture of LCL with PBL, which may have influenced IFN production in alloreactive cells. To avoid a potential EBV-specific stimulation, donor-derived spleen cells seemed to be a good alternative source of allostimulators. They possess good immunostimulatory capacity in MLC and intracellular IFN production in the flow cytometry test. Their availability and the require-
ment for pretreatment (T cell depletion and irradiation) may, however, restrict their use to some extent.

**Conclusion**

Alloreactive T cells can be identified by flow cytometry showing IFN-γ production after short alloantigen stimulation. Using this method, we found a persistence of donor-specific IFN-γ-producing CD8 T-cell frequencies 1 year after lung transplantation, while LDA analysis demonstrated reduced donor-specific CTLp frequencies. We believe that the discrepancy between frequencies may be a result of activation-induced cell death of donor-specific CD8 T cells in LDA culture, which is enhanced by their prior activation in vivo, and exposure to immunosuppressive drugs. If this is true, flow cytometric analysis of alloreactive CD8 T-cell frequencies better reflects true frequencies than those measured by LDA, particularly when measured after transplantation.

**REFERENCES**