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# Donor-Reactive Regulatory T-Cell Frequency Increases During Acute Cellular Rejection of Lung Allografts

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**Background.** Acute cellular rejection is a major cause of morbidity after lung transplantation. Because regulatory T (Treg) cells limit rejection of solid organs, we hypothesized that donor-reactive Treg increase after transplantation with development of partial tolerance and decrease relative to conventional CD4<sup>+</sup> (Tconv) and CD8<sup>+</sup> T cells during acute cellular rejection. **Methods.** To test these hypotheses, we prospectively collected 177 peripheral blood mononuclear cell specimens from 39 lung transplant recipients at the time of transplantation and during bronchoscopic assessments for acute cellular rejection. We quantified the proportion of Treg, CD4<sup>+</sup> Tconv, and CD8<sup>+</sup> T cells proliferating in response to donor-derived, stimulated B cells. We used generalized estimating equation-adjusted regression to compare donor-reactive T-cell frequencies with acute cellular rejection pathology. **Results.** An average of 16.5 ± 9.0% of pretransplantation peripheral blood mononuclear cell Treg cell were donor-reactive, compared with 3.8% ± 2.9% of CD4<sup>+</sup> Tconv and 3.4 ± 2.6% of CD8<sup>+</sup> T cells. These values were largely unchanged after transplantation. Donor-reactive CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T-cell frequencies both increased 1.5-fold (95% confidence interval [95% CI], 1.3-1.6;  $P < 0.001$  and 95% CI, 1.2-1.6;  $P = 0.007$ , respectively) during grade A2 rejection compared with no rejection. Surprisingly, donor-reactive Treg frequencies increased by 1.7-fold (95% CI, 1.4-1.8;  $P < 0.001$ ). **Conclusions.** Contrary to prediction, overall proportions of donor-reactive Treg cells are similar before and after transplantation and increase during grade A2 rejection. This suggests how A2 rejection can be self-limiting. The observed increases over high baseline proportions in donor-reactive Treg were insufficient to prevent acute lung allograft rejection.

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Although lung transplantation may be lifesaving for some patients with end-stage lung disease, median survival after lung transplantation is only 5 to 6 years.<sup>1</sup> Chronic rejection is considered to be the major barrier to long-term survival after transplantation. Acute cellular rejection, in addition to causing morbidity directly, can contribute to development of chronic rejection pathology.<sup>2</sup> To identify subclinical acute cellular rejection, some transplantation centers, including ours, have allograft surveillance protocols

that include bronchoscopy with transbronchial biopsies.<sup>3</sup> Improved understanding of donor-reactive immune responses during acute cellular rejection may lead to better treatments to improve survival after lung transplantation.

Regulatory T (Treg) cells are a subpopulation of lymphocytes that maintain tolerance to self-antigens and diminish inflammation by suppressing conventional nonregulatory CD4<sup>+</sup> (Tconv) and CD8<sup>+</sup> T cells. In a murine model of lung transplantation, Treg depletion led to a three-fold increase

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Q.T. is listed as an inventor on 2 patents on regulatory T cell therapy. The authors have no other conflicts of interest to disclose. Immunosuppressant medications are used "off label" in lung transplantation.

J.R.G., G.H.C., Q.T. conceived the project, designed, and developed assays. R.R. analyzed data on HLA mismatching and DSAs. J.R.G., C.M.W., R.A., A.S.W., C.U., and P.J.W. collected samples and performed the assays. All authors contributed to reviewing and editing the article.

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in rejection pathology.<sup>4</sup> Moreover, Treg cell supplementation prevented rejection of heart and skin allografts in mixed chimerism models.<sup>5</sup> In a mouse lung transplantation model, CD40 ligand blockade increased the Treg-Tconv ratio and prevented acute cellular rejection.<sup>6</sup> Finally, depletion of donor-reactive T cells followed by infusion of donor-specific Treg can induce tolerance of mouse pancreatic islet allografts.<sup>7</sup> Although these data suggest that Treg deficits enhance acute cellular rejection in mice, the data from humans are less clear. A study of 18 lung transplant recipients reported a trend toward decreased peripheral blood mononuclear cell (PBMC)-derived Treg frequency at 3 months in subjects with diminished lung function.<sup>8</sup> However, a study of 27 subjects found no correlation between PBMC Treg frequency and risk of acute lung allograft rejection.<sup>9</sup> Increased Treg frequencies in bronchoalveolar lavage (BAL) fluid and transbronchial biopsy tissue have been associated with acute cellular rejection<sup>10,11</sup> and increased Treg cell frequencies have been found in endomyocardial biopsies before the development of acute cardiac allograft rejection.<sup>12</sup> Further, studies to date have evaluated total rather than donor-reactive subsets of Treg, leaving the association between acute rejection and systemic donor-reactive Treg undefined.

The recent development of therapeutic methods to manipulate Treg cell frequencies in humans further motivates the quantification of T-cell subsets, because the dose of donor-reactive Treg cell required to alter an immune response will depend on Treg cell frequency at baseline and during rejection. Donor-reactive Treg harvested from an allograft recipient can be expanded *ex vivo* and returned to the patient, as in multiple trials aimed at achieving solid organ transplant tolerance.<sup>13</sup>

Extrapolating from a model where a balance between Treg and non-Treg cells determines acceptance of the lung allograft, we hypothesized that donor-reactive CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T cell proportions diminish over time after transplantation whereas donor-reactive Treg proportions increase, as might be expected with development of partial allograft tolerance. Conversely, we hypothesized that CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T cell proportions increase but Treg proportions decrease during acute cellular rejection.

## MATERIALS AND METHODS

### Study Participants

The University of California, San Francisco Committee (UCSF) on Human Research approved this study under protocols 12-08767 and 13-10738. Adults older than 18 years who underwent lung transplantation between July 2012 and July 2014 at UCSF were evaluated for inclusion in this study. Subjects were excluded who had undergone previous organ transplantation or in whom donor PBMC or spleen tissue could not be obtained at the time of transplantation. Participant data were managed using RedCAP.<sup>14</sup>

### Clinical Procedures

As part of routine clinical care, surveillance bronchoscopy procedures, including BAL and transbronchial and endobronchial biopsies, were performed 0.5, 1, 2, 3, 6, and 12 months after transplantation as well as for clinical indications, such as symptoms of rejection or a decline in forced expiratory volume in 1 second. Transbronchial biopsies generally were

obtained from lower lobe segments, and histopathology was interpreted and graded primarily by 1 pathologist using International Society for Heart and Lung Transplantation (ISHLT) criteria,<sup>15</sup> as previously described.<sup>16</sup> The institutional practice is to treat grade A2 or higher acute cellular rejection detected within the first 6 months with 500 mg of intravenous methylprednisolone for 3 days, followed by a tapering course of oral prednisone. In the absence of symptoms or other clinical findings, high-dose corticosteroids are not prescribed for grades A1 or A2 rejection detected more than 6 months after transplantation. However, the doses of chronic immunosuppressive medications (typically prednisone, tacrolimus, and mycophenolate mofetil) may be increased and the biopsy repeated in 4 weeks.

Donor-specific antibodies (DSA) were measured by single HLA antigen bead-based Luminex assay. A subject was considered positive for DSA based on the presence of antibody of mean fluorescence intensity greater than 1000, even if the antibody became undetectable in subsequent measurements.

Infection was considered present when clinical laboratories cultured at least a moderate quantity of pathogenic fungi or bacteria from BAL fluid or identified a viral infection via polymerase chain reactions. Microbiologic culture results demonstrating oropharyngeal flora or *Penicillium* species were not considered to be pathogenic.

### Allogenic Stimulated B Cells

Stimulated B cells were generated by 13 days or longer culture of splenocytes or PBMC with IL-4 as a B-cell growth factor, and irradiated, CD40 ligand-expressing K562 cells to activate B cells by engaging CD40. Cyclosporine A was added to the culture from day 0 to day 4 to inhibit T-cell proliferation.<sup>17</sup> Third-party sBc were derived from a single subject who did not undergo transplantation. Stimulated B cells were irradiated at 3900 rads before use in mixed lymphocyte assays.

### Alloreactive T-Cell Frequency Assay

Before induction immunosuppression, blood was collected in acid-citrate dextrose tubes (364606; BD Biosciences, San Jose, CA) from study participants from an arterial line placed for the lung transplant procedure. After transplantation, blood was collected during placement of a peripheral intravenous catheter before bronchoscopy procedures where transbronchial biopsies were anticipated. Peripheral blood mononuclear cell isolated by Ficoll gradient (17-1440-03; GE Healthcare, Pittsburg, PA) were stored in 10% dimethylsulfoxide in fetal calf serum in liquid N<sub>2</sub>.

Assays were performed on specimens from 1 to 2 subjects at a time once samples from all time points had been obtained. Previously frozen PBMC were thawed, rested overnight, and then labeled by incubating with 1.25  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE, V12883; Life Technologies, Grand Island, NY) in cold phosphate-buffered saline for 5 minutes before quenching with fetal calf serum. For each sBc/responder combination, 150 000 irradiated sBc were added to each of 3 wells of a 96-well round-bottom plate (Nunc 163320; Thermo Scientific, Waltham, MA) containing 75 000 CFSE-labeled PBMC in a total volume of 250  $\mu$ L of assay medium, which contained Roswell Park Memorial Institute-1640 media with GlutaMAX (Gibco 61870-127; Life Technologies), nonessential amino acids, sodium pyruvate, penicillin/streptomycin and 10% human AB serum. Anti-CD3 (555336;

BD Biosciences) and anti-CD28 (555725; BD Biosciences) at 2.5 µg/mL were used as positive controls.<sup>17</sup>

After 96 hours of culture, cells were harvested and treated with human IgG and eFluor 506 viability dye (65-0866; eBioscience, San Diego, CA) before staining with peridinin chlorophyll protein complex (PerCP) anti-CD3 (347344, BD Biosciences), R-phycoerythrin (PE)-Cy7 anti-CD4, allophycocyanin (APC)-Cy7 anti-CD8 (348793, BD Biosciences), and v450 anti-CD25 (561257, BD Biosciences). After permeabilizing with forkhead box P3 (Foxp3) Staining Buffer Set (00-5523; eBioscience), cells were stained with APC anti-FOXP3 (7-4776-42; eBioscience) and PE anti-HELIOS (137216; BioLegend, San Diego, CA). CFSE intensity was determined using a 10-color Navios flow cytometer (Beckman Coulter, Brea, CA), using the gating strategy shown in **Figure S1** (SDC, <http://links.lww.com/TP/B259>). To quantify fluorescence intensity, cells were counted across 7 two-fold dilution gates using cutoff values spaced at  $2^8 \ln 2 / \ln 10$  relative fluorescence intensity units (because the Navios cytometer encodes each 10-fold change in intensity with 8-bits). To control for variability in CFSE labeling, these dilution cutoffs were set such that for the media alone controls, just less than 1% of cells were in the first dilution gate, which is labeled C2 in **Figure S1E** (SDC, <http://links.lww.com/TP/B259>). For a given distribution of cell counts  $C$  in  $n$  sequential peaks of increasing CFSE dilution, the alloreactive T-cell frequency was calculated as  $\sum_{n=1}^7 \frac{C_n}{2^n} / \sum_{n=0}^7 \frac{C_n}{2^n}$ , as previously described.<sup>18</sup>

### Treg Cell Depletion

Peripheral blood mononuclear cell were labeled with PE anti-CD127 (557938; BD Biosciences), PE-Cy7 anti-CD4 (557852; BD Biosciences), and APC anti-CD25 (561399; BD Biosciences) and sorted using a BD Biosciences FACSAria IIIu into CD4<sup>+</sup>CD127<sup>low/-</sup>CD25<sup>+</sup> Treg, non-Treg lymphocytes, and monocytes. The sorted cell populations were recombined to preserve original proportions, except that medium was substituted for Treg cell in the Treg-depleted group. Cells rested overnight before labeling and stimulation with sBc as described above.

### Statistical Analyses

Statistical analyses were performed in R (version 2.14.1, using the “gee” library) and in GraphPad Prism (version 6.0f). Correlations between assay results were determined using Pearson product-moment correlation. For the primary analysis, we tested a generalized estimating equation (GEE)-adjusted linear regression model comparing alloreactive T-cell frequencies according to the stage of rejection. Adjusted analysis included the presence of concurrent infection, the number of HLA mismatches, and weeks posttransplantation. GEEs were used to account for multiple observations within a single subject.<sup>16</sup> Robust variance estimates were used for inference. Data are presented as mean values ± standard deviation.

## RESULTS

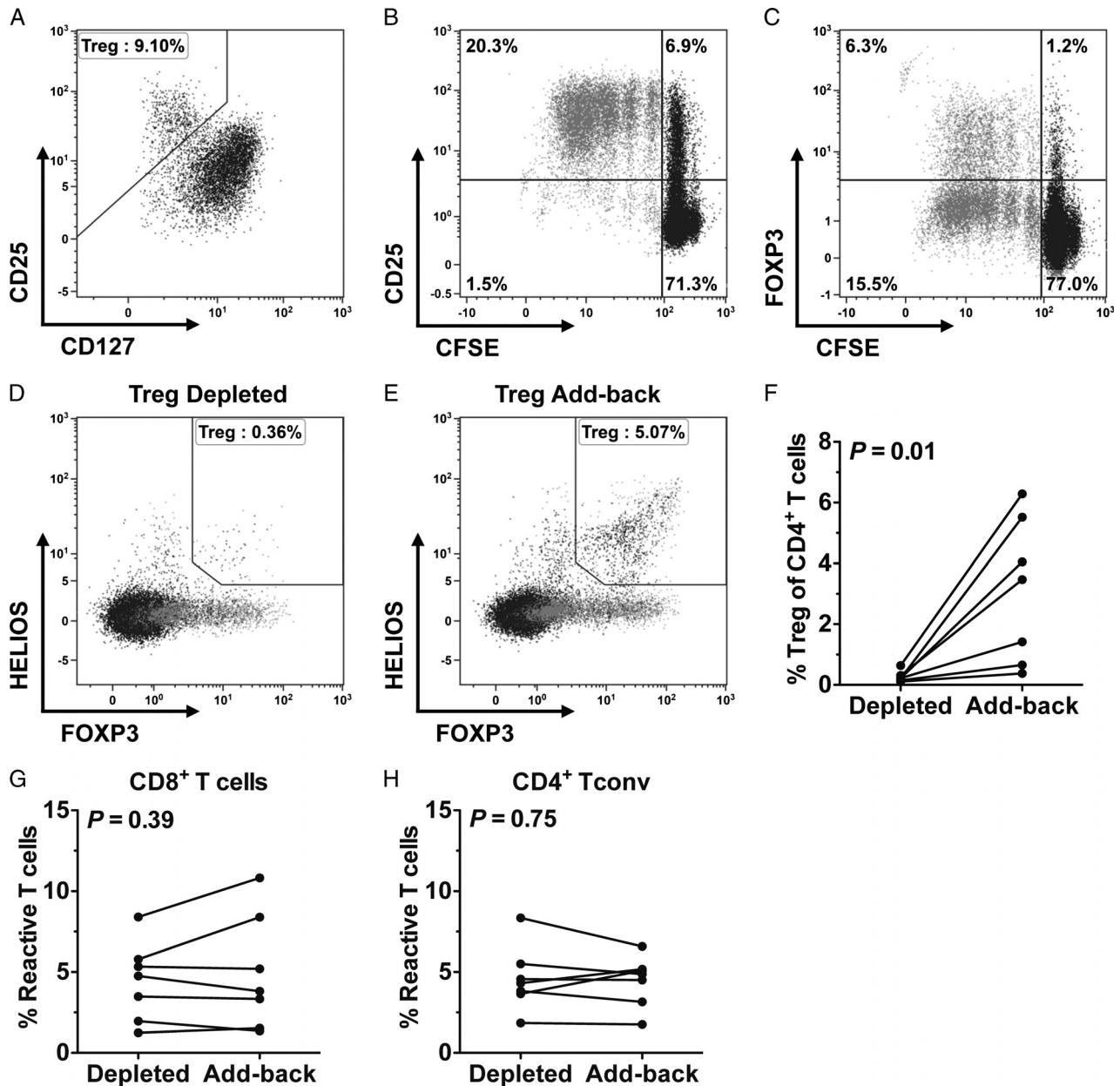
Treg in unstimulated PBMC can be identified within the CD4<sup>+</sup> T-cell population by the expression of CD25 and the low expression of CD127, as shown in Figure 1A, or by the expression of FOXP3. However, quantification of Treg cell after T-cell activation is complicated by the observation that CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T cells upregulate CD25 and

FOXP3 expression after stimulation, as can be seen with the sBc-stimulated, Treg-depleted cells in Figures 1B and C. We found that the combination of FOXP3 and HELIOS markers more specifically distinguish Treg cell from activated CD4<sup>+</sup> Tconv. Depletion of CD127<sup>low/-</sup>CD25<sup>+</sup> Treg cell from unstimulated PBMC before coculture with sBc removed FOXP3 and HELIOS double-positive cells after coculture (Figure 1D), and this population returned when CD25<sup>+</sup>CD127<sup>low/-</sup> Treg were added back proportionally before coculture (Figure 1E). Regulatory T cell depletion before sBc stimulation reduced the FOXP3 and HELIOS double-positive population from a median of 3.5% in added-back groups to 0.2% in depleted groups ( $P = 0.01$  by paired  $t$  test). This demonstrates that FOXP3 and HELIOS together can be used to identify Treg cell after T-cell stimulation. Importantly, Treg cell presence did not affect observed frequency of reactive CD4<sup>+</sup> Tconv (Figure 1G) or CD8<sup>+</sup> T cells (Figure 1H). This finding suggests that the presence of Treg cell in coculture does not falsely lower the estimation of non-Treg alloreactive T cell frequencies.

To assess the specificity of this method of determining donor-reactive T-cell frequencies, we compared the proportion of cells that proliferate after stimulation with recipient, donor, or third-party sBc, and with a combination of anti-CD3 and anti-CD28 antibodies. Third-party sBcs came from a single subject that was mismatched at a minimum of 3 class I and 3 class II loci (median, 5 and 4, respectively) from both donors and recipients. As expected, lymphocytes did not proliferate in response to self-antigens, consistent with clonal deletion of autoreactive T cells (Figure 2A). However, unmatched PBMC proliferated in response to recipient sBc (data not shown). Less than 100% proliferation was also seen for control cells derived from healthy subjects. Thus, the finding of precursor frequencies less than 100% after 96-hour stimulation with anti-CD3 and anti-CD28 antibodies may reflect cell toxicity from overstimulation, an analytic artifact from downregulation of CD3, or reduced responsiveness of transplant recipient cells. T-cell responses to donor and third-party alloantigens were strongly correlated (Figure 2B), with Pearson correlation coefficients of 0.80 (95% confidence interval [95% CI], 0.75-0.84) for CD8 T cells, 0.77 (0.71-0.82) for CD4<sup>+</sup> Tconv, and 0.72 (0.65-0.78) for Treg cell. The association between responses to donor and third-party alloantigens remained significant for all 3 cell types after adjusting for the number of MHC class mismatches between recipient cells and the third-party sBc ( $P < 0.001$ ).

We previously reported that alloreactive T-cell frequencies increase with greater numbers of cognate HLA-locus mismatches using this assay.<sup>17</sup> As shown in Table 1, lung donors and recipients had a high degree of mismatching across HLA-A, -B, -C, -DR, -DQ, and -DP. We observed a weak association between MHC class II HLA mismatches and CD4<sup>+</sup> Tconv alloreactive T-cell frequencies (Figure 2D,  $P = 0.04$ ), but not for MHC class I HLA mismatches and CD8 or MHC class II HLA mismatches and Treg (Figures 2C and E). Similar associations were observed between alloreactive T-cell frequencies and total MHC mismatches (see **Figure S2**, SDC, <http://links.lww.com/TP/B259>).

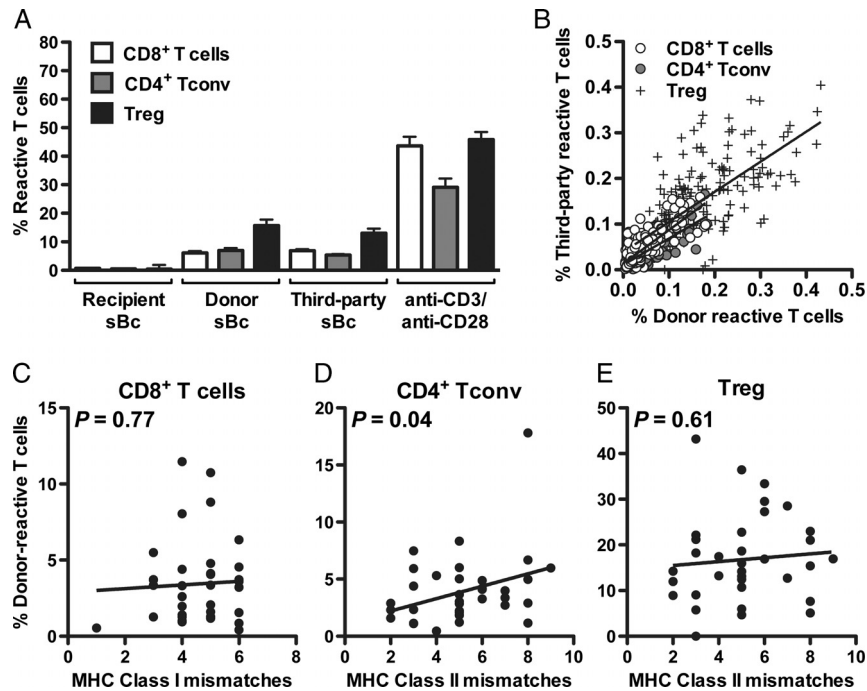
We used this assay to examine the natural history of donor-specific T cells posttransplant and as a correlate of acute cellular rejection in a single-center cohort of lung



**FIGURE 1.** Quantification of alloreactive Treg with FOXP3 and HELIOS after sBc co-culture. A, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> Treg were separated from unstimulated PBMC by fluorescence-activated cell sorting. CD25 (B) and FOXP3 (C) expression is upregulated in Treg cell-depleted T cells after coculture with sBc. Alloreactive T cells also proliferate in response to sBc stimulation, resulting in decreased CFSE concentration. CFSE<sup>low</sup> cells are in grey. D-F, Treg depletion by fluorescence-activated cell sorting before coculture resulted in a 10-fold (interquartile range, 5-15) median decrease in FOXP3 and HELIOS double-positive T cells after sBc stimulation, as compared with control samples, which Treg cells were added back proportionally. T regulatory cell depletion did not affect alloreactive T-cell frequency for CD8<sup>+</sup> T cells (G) or CD4<sup>+</sup> Tconv (H), when compared by paired *t* tests. Data shown were pooled from 3 experiments.

transplant recipients. As shown in Table 1, the indications for lung transplantation differed slightly from those in published ISHLT registry data<sup>19</sup> in that our study population contained fewer subjects transplanted for cystic fibrosis or chronic obstructive pulmonary disease (COPD) and more transplanted for pulmonary fibrosis, including 3 with hypersensitivity pneumonitis.<sup>20</sup> These differences reflect that UCSF is a referral center for interstitial lung diseases. Bilateral lung transplantation was also more common in the study population compared with ISHLT registry populations as a whole, although the frequency of double-lung transplantation has been increasing among ISHLT registry participants.

As shown in Figure 3, the proportion of T cells proliferating in response to donor antigens before transplantation (week 0) was substantial. On average, 16.5 ± 9.0% of Treg, 3.8 ± 2.9% of CD4<sup>+</sup> Tconv, and 3.4 ± 2.6% of CD8<sup>+</sup> T cells proliferated in response to donor sBc. To assess the hypothesis that donor-reactive CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T-cell frequency decreases after exposure to the donor lung, we assessed donor-reactive lymphocyte frequency as a function of weeks after transplantation (Figure 3). A negative correlation would suggest immunosuppression or tolerance, whereas positive correlation would suggest sensitization. However, we did not observe a significant relationship between donor-reactive



**FIGURE 2.** Alloreactive T-cell frequencies as a function of donor-recipient HLA mismatching. A, The percentage of CD8<sup>+</sup>, CD4<sup>+</sup> Tconv, or Treg cells that proliferate in response to stimulation with recipient sBc, donor sBc, third-party sBc, or anti-CD3 and anti-CD28 antibody stimulation are shown after subtracting response to medium alone. Data represent 45 time points from the 6 subjects for whom recipient sBc were generated. B, The percentage of T cells proliferating in response to third-party sBc is shown versus the percentage responding to donor sBc. Pretransplant donor-reactive CD8<sup>+</sup> T cell versus the number of HLA-A, B, and C donor-recipient mismatches are shown in (C). CD4<sup>+</sup> Tconv (D), and Treg (E) frequencies are plotted against the sum of donor and recipient mismatches at the HLA-DR, DQ, and DP loci. The *P* value for a slope of zero by linear regression (F test) is shown.

Treg, CD4<sup>+</sup> Tconv, or CD8<sup>+</sup> T-cell frequencies and the time after transplantation, as assessed by GEE-adjusted regression (*P* ≥ 0.28). Rather, the alloreactive T-cell population remained relatively constant after transplantation. There was a 20% decrease in donor-reactive Treg frequency between the pretransplant sample and the first sample within 1-month posttransplant as assessed by paired *t* test (*N* = 39, *P* = 0.02).

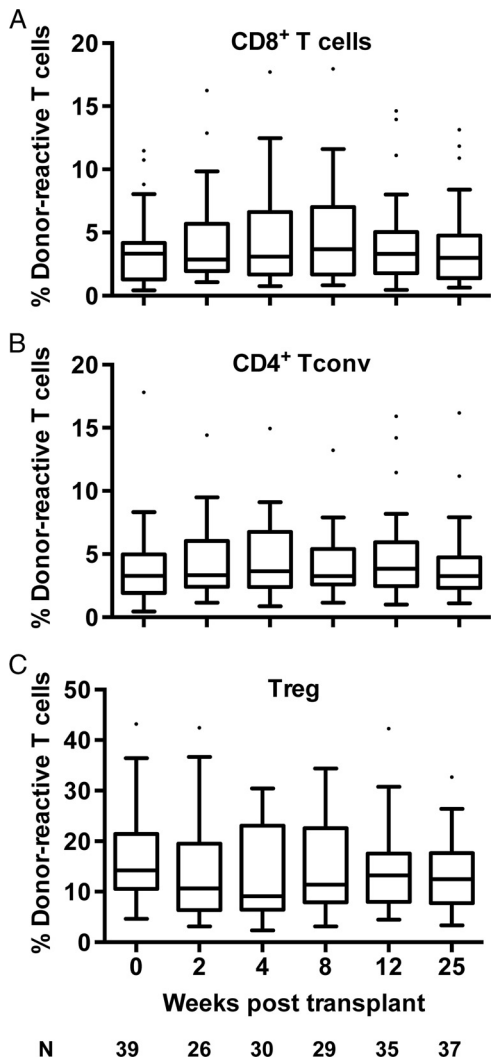
When limited to subjects receiving basiliximab induction, this decrease was only 16% (*N* = 35, *P* = 0.05).<sup>21</sup>

An association between peripheral blood cellular immune responses and antibody responses might reflect shared susceptibility to sensitization. Accordingly, we tested for an association between peripheral blood donor-reactive T cells and development of DSA before or after transplantation (Figure 4). We did

**TABLE 1.**  
**Subject characteristics**

	N	%		N	%
No. subjects	39				
Age at transplant (median, IQR)	61	[53–66]	MHC mismatches (median, IQR)		
Male sex	22	56%	Class I	5	[4–5]
			Class II	5	[3–6.25]
			Total	9	[8–11]
Indication			Immunosuppression		
Idiopathic pulmonary fibrosis	13	33.3%	Prednisone	39	100%
Other fibrosis	9	23.1%	Tacrolimus	33	85%
COPD	6	15.4%	Sirolimus ± low-dose tacrolimus	6	15%
Cystic fibrosis	3	7.7%	Mycophenolic acid	26	67%
Other	8	20.5%			
Induction			Bronchoscopic evaluations		
Basiliximab	35	90%	Per subject (median, IQR)	5	[4,5]
Antithymocyte globulin	4	10%	A0	139	
Transplant type			A1	22	
Single lung	4	10%	A2	16	
Double lung	35	90%			

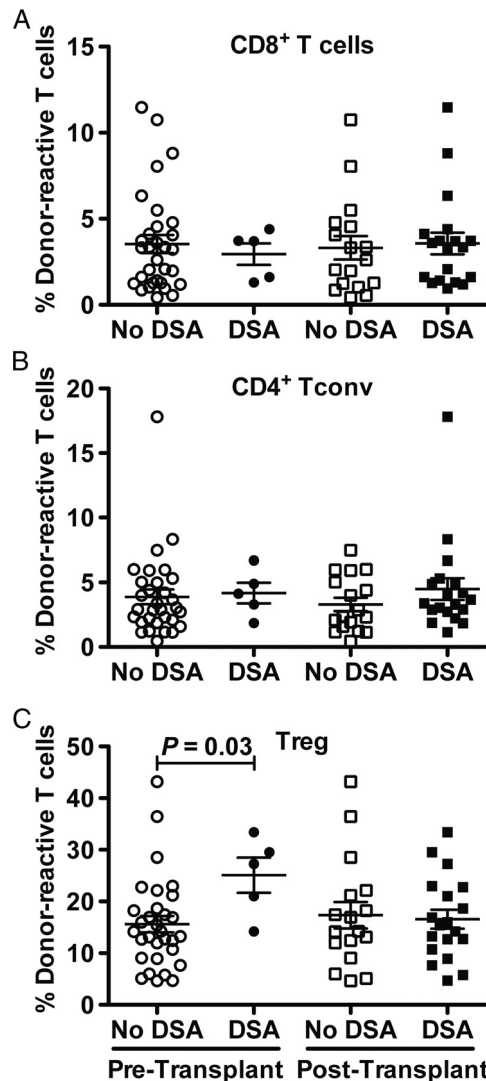
IQR, interquartile range.



**FIGURE 3.** Donor-reactive T-cell frequency over time. Proportions of (A) CD8<sup>+</sup>, (B) CD4<sup>+</sup> Tconv, and (C) Treg cells reactive to donor sBc are shown by week posttransplantation using Tukey box and whiskers plots. The number of subjects included per time point (N) is shown below.

not observe a difference between pretransplant donor-reactive T-cell frequencies and the development of de novo DSA. Interestingly, we did observe an increase in the frequency of Treg cell at the time of transplantation in subjects who had had DSA before transplantation compared with those who did not (25% vs 16%, unadjusted  $P = 0.03$ ).

We also tested the association between donor-reactive T-cell frequencies and acute cellular rejection across the 38 rejection episodes observed during the study period (Table 2). Consistent with our hypothesis, donor-reactive CD4<sup>+</sup> Tconv increased 1.5-fold (95% CI, 1.3-fold to 1.6-fold;  $P < 0.001$ ) and CD8<sup>+</sup> T cells 1.5-fold (95% CI, 1.2-fold to 1.6-fold;  $P = 0.007$ ) during A2 rejection (Figure 5). Interestingly, we found that Treg frequency actually increased 1.7-fold (95% CI 1.4-fold to 1.8-fold,  $P < 0.001$ ) during A2 rejection. At the time of A1 rejection, there was a trend toward increased Treg cell frequency ( $P = 0.09$ ) but no significant change in donor-reactive CD4<sup>+</sup> Tconv or CD8<sup>+</sup> T-cell frequency. We also observed a trend toward increased donor-reactive Treg in PBMC samples obtained at the time of the bronchoscopy procedure before the development of A2 rejection, consistent

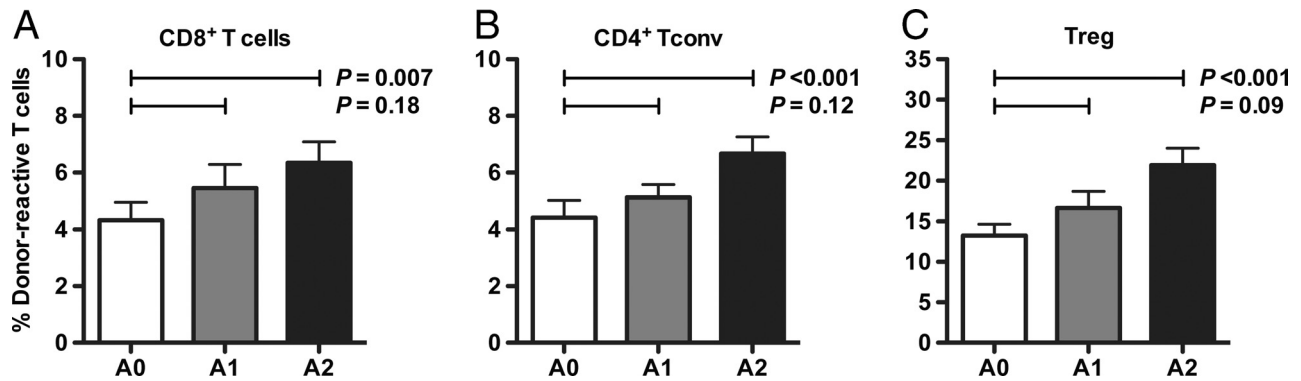


**FIGURE 4.** Pretransplant donor-reactive T-cell frequencies in recipients with and without donor-specific antibody (DSA) responses. The frequency of (A) CD8<sup>+</sup>, (B) CD4<sup>+</sup> Tconv and (C) Treg cells reactive to donor sBc before transplantation are shown stratified by the presence of at least 1 DSA (of mean fluorescence intensity > 1000) pretransplant (circles) and by the development of de novo DSA after transplantation (squares). Student *t* tests were used to compare DSA and no DSA groups.

with findings in cardiac allograft rejection.<sup>12</sup> Before A2 rejection, we observed an increase in donor-reactive Treg cell of 1.5-fold (95% CI, 1.0-fold to 2.1-fold;  $P = 0.06$ ). There was

**TABLE 2.** Acute cellular rejection episodes and treatment

	A1	A2
	N (%)	N (%)
Total occurrences	22 (100)	16 (100)
Observed during surveillance	19 (86)	13 (81)
Concurrent symptoms of rejection	2 (9)	4 (25)
Treated with high-dose corticosteroids	0 (0)	11 (69)
Augmented immunosuppression only	0 (0)	3 (19)
Decreased rejection grade on follow up	17 (77)	15 (94)
Weeks posttransplant (median, IQR)	10 [5-12]	12 [4-26]



**FIGURE 5.** Association between donor-reactive T-cell frequency and acute cellular rejection grade. Average frequencies of recipient (A) CD8<sup>+</sup>, (B) CD4<sup>+</sup> Tconv, and (C) Treg reactive to donor sBc are shown, stratified by concurrent transbronchial biopsy A-score for 139 A0, 22 A1, and 16 A2 samples. Error bars representing standard deviations and *P* values were adjusted using generalized estimating equations.

no association between acute lymphocytic bronchiolitis, as assessed by B-score, and donor-reactive T-cell frequencies ( $P \geq 0.51$ ).

Donor-reactive T cell frequencies over time for 4 subjects with grade A2 acute cellular rejection are shown in Figure S3 (SDC, <http://links.lww.com/TP/B259>). These plots show that donor-reactive Treg and CD4<sup>+</sup> Tconv frequencies increased over subject baselines for most but not all episodes of acute cellular rejection. A high CD4<sup>+</sup> Tconv relative to Treg response was seen in 1 subject with recurrent acute cellular rejection.

Potential confounders include (1) the number of cognate donor-recipient HLA mismatches, (2) effects of concurrent infection on heterologous immune responses, and (3) reduction of doses of immunosuppressive medications over time after transplantation per clinical protocols. To assess for contributions of these potential confounders, we performed an adjusted analysis that included the presence of HLA mismatching, concurrent infection, and time posttransplantation as covariates in the regression model. After adjustment, donor-reactive CD4<sup>+</sup> Tconv increased 2.1-fold (95% CI, 1.2-fold to 3.0-fold;  $P = 0.02$ ), CD8<sup>+</sup> T cells increased by 1.5-fold (95% CI, 0.8-fold to 2.3-fold;  $P = 0.15$ ) and Treg cell increased 2.5-fold (95% CI, 1.7-fold to 3.3-fold,  $P < 0.001$ ) during A2 rejection. Therefore, adjustment for HLA mismatching, infections, and time from transplantation did not substantially affect the results.

## DISCUSSION

The findings reported here are the first to document the natural history of donor-reactive T cell subtypes after lung transplantation. Consistent with our hypothesis, donor-reactive CD4<sup>+</sup> Tconv and CD8<sup>+</sup> T-cell frequency increased in peripheral blood during grade A2 acute cellular rejection. Surprisingly, donor-reactive Treg cell frequency also increased during grade A2 rejection proportionate to the increase in non-Treg cell frequency, yet was either insufficiently robust or too late to prevent the observed pathology.

Consistent with results of studies in mice, alloreactive T-cell frequencies before lung transplantation were high relative to the magnitude of immune responses to infections.<sup>22</sup> Our observation that alloreactive T-cell frequencies were higher for Treg cell than that for CD4<sup>+</sup> Tconv or CD8<sup>+</sup> T cells is consistent with in vitro allogeneic and xenogeneic responses

of PBMC populations derived from untransplanted donors.<sup>23</sup> A substantial decrease in the total CD25<sup>+</sup> Treg cell population has been reported after basiliximab induction,<sup>24</sup> but much of this apparent decrease may reflect downregulation of CD25 expression.<sup>25,26</sup> Here, we observed only a small decrease in the donor-reactive Treg population after basiliximab induction, consistent with the theory that this medication minimally affects Treg function. After liver transplantation, donor-reactive lymphocytes are clonally deleted, resulting in tolerance,<sup>27</sup> but we found no evidence of donor-reactive lymphocyte deletion after lung transplantation. This discrepancy may help explain the dramatic differences in chronic rejection observed after these 2 procedures.

The concordance between donor- and third party-reactive T-cell frequencies may represent overlap between HLA antigens between donor and third-party populations or reflect cross-reactivity of alloreactive T-cell populations, as has been reported.<sup>28</sup> Alloreactivity may also depend on the activation state of the responder cells. The finding that third-party sBc can be used as stimulatory cells is fortuitous, as it suggests that we may forego the labor-intensive process of creating donor-specific sBc and use third-party cells to assay alloreactive T-cell population frequency. Such a strategy has been used in kidney transplant recipients to develop a panel of reactive T cells assay, analogous to panel-reactive antibody testing.<sup>29</sup>

The optimal management of low-grade acute cellular rejection detected on surveillance bronchoscopy is controversial. Some practitioners advocate for treatment of asymptomatic low-grade acute cellular rejection with high-dose corticosteroids,<sup>30</sup> based on the observation that a single episode of grade A1 rejection confers increased risk for chronic lung allograft dysfunction<sup>31</sup> and that grade A1 rejection may progress to more severe rejection if untreated.<sup>32</sup> On the other hand, many transplant centers do not treat grade A1 or even grade A2 rejection with high doses of corticosteroids in the absence of symptoms or other clinical findings,<sup>33</sup> and 1 study found no benefit of performing surveillance bronchoscopic biopsies in individuals lacking symptoms to suggest rejection or infection.<sup>34</sup> High Treg cell frequencies during acute cellular rejection are consistent with a self-limiting process. However, because rejection improved in 90% of the cases, this study was not powered to assess whether low Treg cell frequencies predict persistence of rejection.

These data demonstrate the utility of HELIOS in combination with FOXP3 for distinguishing Treg cell from activated



effector T cells after in vitro stimulation. Although CD4<sup>+</sup>Tconv cells upregulate HELIOS or FOXP3 after stimulation,<sup>35</sup> the double-positive population derived from cells that were CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> before stimulation. However, the extent that HELIOS<sup>-</sup> Treg cells are classified as CD4<sup>+</sup>Tconv by this assay is unknown. Although HELIOS was proposed as a marker of thymic-derived natural Treg cell, induced and natural Treg cells lacking HELIOS expression have been reported,<sup>36</sup> and more recent data suggest that HELIOS is a marker for Treg stability.<sup>37</sup>

This study has limitations. The observed high frequencies of alloreactive T cells partially result from an assay that depends on cell proliferation in mixed culture rather than on effector function. It is likely that lower frequencies would be seen with Elispot or limiting dilution assays. At the same time, this assay may not measure the immune response to all relevant graft-specific antigens. Although Treg cells that are specific for alloantigens presented directly by allogenic dendritic cells predominate over those specific for antigens presented indirectly by self-dendritic cells,<sup>38</sup> assays using HLA-mismatched sBcs do not assay potential roles or importance of indirect allorecognition and presentation of self-antigens. The diagnosis of acute cellular rejection by transbronchial biopsy is hampered by sampling error and low interobserver reliability.<sup>39</sup> However, these sources of variation would bias our findings toward the null. Because none of the subjects enrolled in this study experienced grade A3 or grade A4 rejection, it is unclear what changes in donor-reactive T cells would occur in the setting of these much less common higher grades of rejection. In the present form, the overlap between alloreactive T-cell frequencies at the time of rejection, and no rejection is large enough that this assay would likely not add much certainty to biopsy-based methods of diagnosing acute cellular rejection. Also, although a high frequency of Treg cell may be the most sensitive predictor of acute cellular rejection, it is possible that rare lung transplant recipients with low Treg cell frequencies in the setting of acute cellular rejection could be at highest risk of refractory acute cellular rejection and chronic lung allograft dysfunction. In support of this theory, potent Treg cell responses during acute rejection of renal allografts predicted resolution.<sup>40</sup> Long-term follow-up will be needed to assess outcomes, such as chronic rejection and survival.

In summary, we found that frequencies of donor-reactive T cells, including Treg cell, increase during acute cellular rejection. In contrast to results reported after liver transplantation, our study did not detect clonal deletion of donor-reactive T cells after lung transplantation.<sup>27</sup> Prospective studies will be needed to determine whether innate or induced increases in the ratio of donor-reactive Treg cell to non-Treg cell can reduce the risk of chronic lung allograft dysfunction.

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