CD28 Down-Regulation on CD4 T Cells Is a Marker for Graft Dysfunction in Lung Transplant Recipients

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Rationale: Repeated antigen-driven proliferations cause CD28 on T cells to down-regulate. We hypothesized that alloantigen-induced proliferations could cause CD28 down-regulation in lung transplant recipients.

Objectives: To ascertain if CD28 down-regulation on CD4 T cells associated with manifestations of allograft dysfunction in lung transplant recipients.

Methods: Peripheral blood CD4 T cells from 65 recipients were analyzed by flow cytometry, cytokine multiplex and proliferative assays, and correlated with clinical events.

Measurements and Main Results: Findings that CD28 was present on less than 90% of total CD4 T cells were predominantly seen among the recipients with bronchiolitis obliterans syndrome (specificity = 88%). Perforin and granzyme B were produced by >50% of the CD4⁺CD28^{null} cells, but less than 6% of autologous CD4⁺CD28⁺ cells ($P < 0.006$). CD4⁺CD28^{null} cells also had increased productions of proinflammatory cytokines, but less frequently expressed regulatory T-cell marker FoxP3 ($2.1 \pm 1.3\%$), compared with autologous CD4⁺CD28⁺ (9.5 ± 1.4 ; $P = 0.01$). Cyclosporine A (100 ng/ml) inhibited proliferation of CD4⁺CD28^{null} cells by $33 \pm 11\%$ versus $68 \pm 12\%$ inhibition of CD4⁺CD28⁺ ($P = 0.025$). FEV₁ fell 6 months later (0.35 ± 0.04 L) in recipients with CD4⁺CD28⁺/CD4_{total} less than 90% (CD28^{Low}) compared with 0.08 ± 0.08 L among CD4⁺CD28⁺/CD4_{total} (CD28^{High}) greater than 90% (CD28^{High}) recipients ($P = 0.013$). Two-year freedom from death or retransplantation in CD28^{Low} recipients was $32 \pm 10\%$ versus $78 \pm 6\%$ among the CD28^{High} subjects ($P < 0.0001$).

Conclusions: CD28 down-regulation on CD4 cells is associated with bronchiolitis obliterans syndrome and poor outcomes in lung transplantation recipients. CD4⁺CD28^{null} cells have unusual, potentially pathogenic characteristics, and could be important in the progression of allograft dysfunction. These findings may illuminate a novel paradigm of transplantation immunopathogenesis, and suggest that CD28 measurements could identify recipients at risk for clinical deteriorations.

Keywords: bronchiolitis obliterans syndrome; obliterative bronchiolitis; chronic allograft rejection; regulatory T cells; cyclosporine

Chronic allograft rejection (CR) is the major limitation of success following transplantations of lungs and other solid organs (1). The prevalence of CR has not seemingly diminished

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

It is known that some lung transplant recipients develop severe, progressive chronic allograft rejection, but many of the pathogenetic details remain unknown.

What This Study Adds to the Field

CD28 down-regulation on CD4 cells is associated with bronchiolitis obliterans syndrome and poor outcomes in lung transplantation recipients. CD4⁺CD28^{null} cells have unusual, potentially pathogenic characteristics, and could be important in the progression of allograft dysfunction.

over several decades, despite interval implementations of various immunosuppressants and other treatments (1–3). Fulminate CR in lung allograft recipients manifests with expiratory airflow obstruction, defined as bronchiolitis obliterans syndrome (BOS), as well as increased mortality (2, 3). The natural history of this disorder is highly variable, however, and relatively quiescent courses that do not require or likely benefit from aggressive treatment are not uncommon (4).

Current therapies, with agents having nonspecific immunosuppressive effects, have uncertain efficacies and considerable toxicities (1–3). Better understandings of allograft pathogenesis could illustrate new approaches for clinical management of lung transplantation recipients. As an example, the ability to predict the likely progressiveness of CR in individual recipients could more productively direct surveillance and treatment to those at greatest risk, while limiting needless, and often substantial, side effects of immunosuppression augmentation among recipients destined for benign courses. In addition, the identification of a disproportionately pathogenic cell type could focus efforts to more specifically target or modify these particular cells.

We have previously shown that circulating CD4 T cells of lung allograft recipients with CR undergo abnormal oligoclonal expansions, as distinct from those cells from recipients with no evidence of chronic rejection (5). We have since focused further study on CD4 T-cell processes in lung allograft recipients, given the singular role of these lymphocytes in orchestrating adaptive immune responses, including allograft rejection (6–8). CD28, a costimulatory molecule, is often down-regulated on T cells of patients with chronic adaptive immune diseases, and the unusual CD4 T cells that do not express CD28 (CD4⁺CD28^{null}) have also been implicated in the immunopathogenesis of these disorders (9–21). We hypothesized that alloantigen-induced proliferations could cause CD28 down-regulation in lung transplant recipients.

We tested our hypothesis by determining the proportion of circulating CD4 T cells that lacked expression of CD28 by flow cytometry in individual lung transplant recipients, and by correlating these results with the subjects' diagnoses, pulmonary

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function tests (PFTs), and clinical outcome. In addition, expression of cell activation markers, intracellular FoxP3, cytotoxic mediator and cytokine production, and resistance to cyclosporine of the CD4⁺CD28^{null} cells from recipients were compared to those of autologous, and more typical, CD4 T cells that express CD28, through flow cytometry and functional assays.

Some of the results of these studies have been previously reported in the form of abstracts (22, 23).

METHODS

See the online supplement for additional details.

Subjects

Lung allograft recipients were recruited during routine outpatient evaluations. Two successive cohorts of transplant patients were examined: The initial subject population (cohort A), recruited during the first 2 years of this study, consisted of consecutive recipients seen in clinic who fulfilled inclusion criteria (*see METHODS* in the online supplement). Based on findings of these interval analyses, and in order to more productively characterize CD4⁺CD28^{null} cells *per se*, which are predominantly found in recipients with BOS (*see below*), subsequent recruitments (cohort B) consisted of recipients with preexisting diagnoses of BOS (again, otherwise consecutively selected as they appeared in clinic). Some recipients consented to replicate assays, but, unless noted otherwise, only results from first determinations were analyzed here. Routine clinical protocols are described elsewhere (24; *see also* the online supplement). Healthy volunteers were recruited by solicitation.

Diagnoses of BOS were established by physiologic and clinical consensus criteria (25). Cytomegalovirus (CMV) or other viral infections at any time after transplantation were determined by positive viral cultures from bronchoalveolar or other specimens, diagnostic histopathology, the presence of pp65 antigenemia, or, more recently, by quantitative polymerase chain reaction.

Cell Preparations and Flow Cytometry

Peripheral blood mononuclear cells were isolated by density gradient centrifugation from peripheral blood for use in flow cytometry or cell assays.

Phenotypic characterizations were ascertained using fluorochrome-conjugated monoclonal antibodies, including isotype controls, and flow cytometry quantitations performed on more than 10,000 live cells. CD4^{bright} T cells were gated, and CD28 and other phenotypic markers were quantitated within respective CD4⁺CD28⁺ and CD4⁺CD28^{null} subpopulations (Figures 1A and 1B).

CD4 T cells for functional assays were isolated from peripheral blood mononuclear cell by negative selection with immunomagnetic beads (Miltenyi Biotech, Auburn, CA). CD28^{null} cells and CD28⁺ cells were individually segregated from this CD4 T-cell population by staining with anti-CD28-phycoerythrin, followed by positive selection with anti-phycoerythrin immunomagnetic beads (Miltenyi Biotech). Purity of the respective cell populations was 95% or greater.

Cytokine Assays

A total of 1×10^5 CD4 T cells (autologous CD28⁺ and CD28^{null}) were separately cultured in 96-well plates in both stimulated (10 μ g/ml plate-bound anti-CD3 antibody) and unstimulated (basal) conditions. Cul-

Figure 1. Characteristics of CD4 T-cell subpopulations in lung allograft recipients. (A and B) Flow cytometry methodology. (A) T cells that stained brightly with anti-CD4 monoclonal antibody conjugated to allophycocyanin and CD3 conjugated to Cy-Chrome were gated for subsequent determinations of cellular expressions. (B) Expressions of other phenotypic markers, in this example denoted by anti-CD25 phycoerythrin (PE) antibody staining, were individually determined in autologous CD4⁺CD28⁺ and CD4⁺CD28^{null} subpopulations. In the example here, these respective subpopulation are denoted by the presence or absence, respectively, of costaining with anti-CD28 antibody conjugated to fluorescein isothiocyanate (FITC). (C) The percentages of circulating CD4 T cells that also expressed CD28 (CD28%) were reduced in lung transplant recipients with bronchiolitis obliterans syndrome (BOS) in comparisons with healthy normal (nontransplanted) control subjects. Horizontal lines denote population means. The No-BOS recipient with a CD28% of 67.4 had obliterative bronchiolitis on lung biopsy, but normal expiratory flow at the time of this CD4

assay. (D) In contrast to autologous CD4⁺CD28⁺, the CD4⁺CD28^{null} T cells from lung transplant recipients with BOS less often express activation marker CD25 ($n = 16$). Assays in later cohorts of consecutive, randomly selected subjects also show that the CD4⁺CD28^{null} cells from recipients with BOS much more frequently produce the cytotoxic mediators, perforin and granzyme B (both $n = 10$), but less often express FoxP3⁺ ($n = 6$), in comparisons to autologous CD4⁺CD28⁺ cells.

ture supernatants were analyzed for cytokine productions using a protein-suspended bead array platform (Bio-Plex) multiplex kit (Bio-Rad, Hercules, CA), following the manufacturer's protocols.

Inhibition of Proliferation by Cyclosporine A

Segregated CD28⁺ and CD28^{null} CD4 T-cell subpopulations were individually cultured in duplicate wells coated with anti-CD3 monoclonal antibody (OKT3) (*see above*). Autologous nurse cells, positively selected during the initial CD4 T-cell isolations (*see above*), were added in a 3:1 nurse cell:CD4 T-cell ratio. In order to eliminate potential confounding, all CD4⁺ cells were depleted from the nurse cells (<<1% residual), prior to their use in cultures, by positive selection with anti-CD4-coated magnetic beads (Invitrogen, Carlsbad, CA). Cyclosporine (Novartis, East Hanover, NJ) (100 ng/ml) was added to half the wells.

Proliferation within the respective CD4 subpopulations were measured by bromodeoxyuridine incorporation, using reagents and methods supplied in a kit (BD Pharmingen). The proportion of proliferating CD4⁺ T cells (bromodeoxyuridine⁺) among the viable cells ($\geq$ diploid DNA content) was determined by flow cytometry.

RESULTS

CD28 Down-Regulation on CD4 T Cells Is Associated with BOS

We initially determined the proportion of circulating CD4 T cells that expressed CD28 (CD28%) among 35 consecutive recipients, seen in routine clinic visits, who fulfilled inclusion criteria (cohort A) (*see also the online supplement*). Laboratory investigators were blinded to the identity and characteristics of these subjects. The CD28% among the 25 lung transplant recipients with no evidence of BOS (No-BOS) (25) was 94.6 (SEM, ± 1.5), and similar to that of 16 healthy, normal volunteers (98.2 ± 0.5). Three of these recipients (including one with a CD28% of 67.4) had prior lung biopsies showing obliterative bronchiolitis (OB) (26), but had normal expiratory flow at the time of their CD4 assays, and are classified here as No-BOS. All three subsequently had deteriorations of expiratory flow and formally met BOS criteria (25) between 2 and 10 months later. Ten (10) of the randomly recruited lung transplant recipients in cohort A had established diagnoses of BOS prior to these CD4 studies (unbeknownst to the laboratory investigators). CD28% values among these BOS recipients were comparatively diminished (Figure 1C). Cutoff values for CD28% of 0.9 seemed to provide the best compromise of specificity (the higher consideration) and sensitivity for concurrent BOS, as confirmed by receiver operator characteristic curve analysis (*see Figure E1 in the online supplement*). A total of 5 (50%) of the 10 recipients with BOS had values for CD28% less than 90, whereas only 3 (12%) of the 25 No-BOS subjects had CD28% values less than 90 (Table 1).

Aside from BOS prevalence, there were no apparent intergroup differences of demographic or other clinical parameters that could easily account for these CD28 variances (Table 1). CD4⁺CD28^{null} cells can reportedly accumulate with aging, but this typically occurs in subjects who are much older than these recipients (9, 10). Furthermore, both subject groups had equivalent ages (Table 1), and there was also no correlation between age and extent of CD28 expression in these subjects ($r_s = 0.04$; $P = 0.8$). In addition, CD28 down-regulation was also not seen in our healthy (nontransplanted) control subjects, carefully screened to exclude subjects with underlying immunologic and other "normal" disorders of senescence, despite their greater ages (58.3 ± 2.6 yr).

CD28 down-regulation (albeit to an apparently lesser degree) has also been described among renal transplant recipients after CMV infections (11), but we could not discern a confounding effect of these infections here. The proportion of previously

TABLE 1. CHARACTERISTICS OF COHORT A: RANDOM, CONSECUTIVE, RECIPIENT RECRUITMENTS

	CD4 ⁺ CD28% $\geq$ 90	CD4 ⁺ CD28% < 90
	(CD28% High)	(CD28% Low)
n	27	8
Age, yr	50.3 $\pm$ 1.9	51.4 $\pm$ 2.8
Male, n (%)	12 (44)	5 (63)
Recipients with BOS at time of T-cell assays, n (%)	5 (19)	5 (63)*
Months since transplant	39 $\pm$ 9	53 $\pm$ 13
CD28%	96.8 $\pm$ 0.5	78.6 $\pm$ 2.6 [†]
Transplantation type, n (%)		
SL	13 (48)	4 (50)
DL	12 (44)	4 (50)
HL	2 (7)	0 (0)
Pretransplant diagnoses, n (%)		
COPD	10 (37)	6 (75)
Pulmonary fibrosis	8 (30)	1 (13)
Cystic fibrosis	3 (11)	0
PA hypertension	3 (11)	0
Sarcoidosis/scleroderma	3 (11)	1 (13)
Prior CMV, n (%)	7 (26)	4 (50)
Interval since CMV, mo	23 $\pm$ 7	16 $\pm$ 9
ACR, n (%)	8 (30)	2 (25)
ACR grade	1.8 $\pm$ 0.4	2.0 $\pm$ 0.0
Induction regimens, n (%)		
Glucocorticosteroid/azathioprine	14 (51)	7 (88)
Daclizumab	4 (15)	0
RATG	6 (22)	1 (13)
Alemtuzumab	3 (11)	0
Donor:recipient MHC mismatches		
Class I	3.5 $\pm$ 0.2	3.9 $\pm$ 0.4
Class II	3.0 $\pm$ 0.2	2.7 $\pm$ 0.4

Definition of abbreviations: ACR = acute cellular allograft rejection; BOS = bronchiolitis obliterans syndrome; CMV = cytomegalovirus; COPD = chronic obstructive pulmonary disease; DL = double-lung transplantation; HL = heart/lung transplantation; MHC = major histocompatibility complex; PA = pulmonary artery; RATG = rabbit antithymocyte globulin; SL = single-lung transplantation.

CD4⁺CD28% $\geq$ 90 (CD28% High) denotes those recipients in whom 90% or more of their circulating CD4 T cells also expressed CD28, whereas recipients in whom CD28 was expressed on fewer than 90% of their circulating CD4 T cells are denoted as CD4⁺CD28% < 0.9 (CD28% Low). "Previous CMV" describes subjects who had CMV infections prior to these CD4 T-cell assays, and "Interval since CMV" describes the time interval between these infections and the CD4 assays. For ACR, grade was established by histologic criteria (26). One of these subjects (a CD28% High subject) was taking rapamycin at the time of their CD4 assay.

* $P < 0.016$ (χ^2).

[†] $P < 0.0001$ (Mann-Whitney). None of the other comparisons was statistically significant.

infected recipients tended to be greater in the CD28% Low group, although the intergroup difference in prevalence of prior CMV infections (Table 1) was not statistically significant by Chi-square test ($P = 0.39$). Similarly, bivariate logistic regression analyses confirmed a significant independent association between CD28% Low and BOS (odds ratio = 6.4; 95% confidence interval = 1.1–37.4; $P = 0.04$), whereas CD28 expression did not seem to associate with prior CMV (odds ratio = 2.0; confidence interval = 0.3–11.8; $P = 0.46$). Furthermore, of the 11 recipients with prior CMV, 7 (64%) were CD28% High, and half of the CD28% Low recipients had no history of these viral infections. No subject here had CMV diagnosed at the time of their CD4 assays or for at least 2 months thereafter.

Because acute cellular allograft rejection (ACR) is nearly ubiquitous among lung allograft recipients within the first year after transplantation (2, 26), potential confounding by this complication could also diminish the significance of CD28

down-regulation as an indicator of CR. Every lung transplant subject studied here had one or more ACR episodes prior to their CD28 measurements. In addition, a small number of subjects unexpectedly had acute rejection on lung biopsies that were performed concurrently with their T-cell assays, and these events occurred equally frequently in both groups (Table 1). For the most part, these incidental (and cryptic) cases were relatively mild (grade I–II) (26). However, three recipients had unsuspected grade III acute rejection at the time of their CD4 assays, and all of these were in the CD28% High group.

CD28 down-regulation also does not appear to be a simple function of cellular accumulation over time since transplantation, given the considerable overlap of elapsed intervals among the two populations (Table 1) and the absence of a meaningful correlation between time since transplantation and CD28% ($r_s = -0.18$; $P = 0.3$).

Similarly, there were no simple differences among the type of transplantation, induction regimens, or pretransplant diagnoses that could account for the differences of CD28 expression (Table 1). There was a relative excess of subjects with pretransplant diagnoses of chronic obstructive pulmonary disease, and fewer proportions of patients with pulmonary fibrosis among the CD28% Low group; however, these values were not significantly different than those of the CD28% High group ($P = 0.35$). These minor disparities are likely anomalies arising from the small numbers of subjects, particularly as no similar patterns were evident in either cohort B or the aggregate study population (see Tables E1 and E2 in the online supplement).

CD4⁺CD28^{null} T Cells from Recipients Have Pathogenic Characteristics

We subsequently performed a series of investigations to begin characterizations of the unusual CD4⁺CD28^{null} T cells. Because few of the No-BOS recipients had appreciable proportions of these cells, we limited subsequent subject enrollments to sequential, outpatient lung transplant patients seen in clinic who had preexistent BOS (cohort B). All of these recipients fulfilled previously described inclusion criteria (see METHODS in the online supplement), were otherwise consecutively recruited, and laboratory tests were interpreted by investigators blinded to identities, demographic details, treatments, clinical courses, or other subject characteristics (see Table E2).

Based on analogous studies in autoimmune and other patients with chronic inflammatory diseases (9–21), we expected the CD4⁺CD28^{null} cells to be highly activated. A greater proportion of the CD4⁺CD28^{null} (36.5 ± 6.9%) did express major histocompatibility complex antigen class II than the corresponding autologous CD4⁺CD28⁺ cells (11.4 ± 3.7%) ($n = 16$; $P = 0.006$), but the latter more frequently expressed CD25, another marker of T-cell activation (Figure 1D).

This latter finding led us to speculate that the proportion of regulatory CD4 T cells (T_{regs}), defined by expression of FoxP3 (in turn, associated with CD25 expression [27]), may also be diminished among CD4⁺CD28^{null} cells. Measures within a recent, randomly selected, consecutive subpopulation of cohort B subjects showed that FoxP3⁺ cells among the CD4⁺CD28^{null} are significantly less frequent than in the autologous CD4⁺CD28⁺ lymphocytes (Figure 1D) or CD4⁺CD28⁺ T cells of recipients with No-BOS (13.7 ± 2.5%; $n = 7$; $P < 0.008$).

CD4⁺CD28^{null} cells derived from patients with autoimmune and other chronic immunologic diseases also frequently produce the cytotoxic mediators, perforin and granzyme B, in striking contrast to normal CD4 T cells that do not elaborate these substances (11, 13). The assays here confirm that CD4⁺CD28^{null} cells of lung transplant recipients with BOS also produce these potentially pathogenic mediators (Figure 1D).

CD4⁺CD28^{null} T Cells of Recipients with BOS Produce Proinflammatory Cytokines

Differential cytokine production of autologous CD4⁺CD28⁺ and CD4⁺CD28^{null} cells were apparent in multiplex analyses. CD4⁺CD28^{null} cells generally elaborated much greater amounts of proinflammatory and Th1 mediators under basal conditions, and, in many cases, this production was strikingly increased by activation after T-cell antigen receptor (TCR) cross-linking with anti-CD3 antibody (Figure 2). Conversely, and with the exception of IL-4, CD4⁺CD28^{null} cell elaborations of Th2 cytokines, notably including putatively immunosuppressive IL-10 (28), were reduced compared with production by autologous CD4⁺CD28⁺ cells.

CD4⁺CD28^{null} T Cells from Recipients Are Relatively Resistant to Antiproliferative Effects of Cyclosporine A

CR typically develops and often progresses despite intense immunosuppression (1–3). We hypothesized that, if CD4⁺CD28^{null} lymphocytes are indeed disproportionately pathogenic, as suggested by their occurrence primarily among recipients with BOS, as well as their seemingly pathogenic phenotype (Figures 1D and 2), these cells might also be relatively resistant to immunosuppressant medications. Using cyclosporine A as a prototype calcineurin inhibitor, and an agent that is a novel exposure for these specimens, we examined the ability of the respective CD4 subpopulations to proliferate after TCR stimulation with plate-bound anti-CD3 monoclonal antibody (thereby replicating T-cell engagements with antigens/alloantigens) while in the presence of therapeutic drug concentrations. Although freshly isolated autologous CD4⁺CD28⁺ and CD4⁺CD28^{null} lymphocytes proliferated comparably with *in vitro* stimulation in control wells (no cyclosporine), proliferation of the CD4⁺CD28^{null} cells was comparatively less inhibited by cyclosporine in every subject (Figure 3). Moreover, these results are not confounded by pharmacologic effects of the small amount of cyclosporine carrier (solvent) in these cultures, as addition of pure carrier, in concentrations of more than 100-fold higher than that used in these drug resistance studies, had no effect on T-cell proliferation (data not shown). Preliminary findings in small numbers of subjects ($n = 2$) also indicate that CD4⁺CD28^{null} T cells are similarly relatively resistant to tacrolimus (unpublished observations).

CD28 Down-Regulation on CD4 T Cells of Recipients Is Associated with Pulmonary Function

If the unusual CD4⁺CD28^{null} cells are pathogenic, we hypothesized that the extent of CD28 down-regulation among particular recipients could possibly be associated with clinical manifestations of allograft injury. We performed these analyses in the initial, consecutively recruited subject population (cohort A) to avoid cryptic ascertainment biases.

As single cross-sectional “snapshots,” values of CD28% had little correlation with concurrent pulmonary function, given that baseline values of FEV₁, performed at the time of the initial CD4 assays, were similar among CD28% High and CD28% Low populations (see Tables E3 and E4). It seems likely that these simple assessments may be prone to confounding by the heterogeneity within the populations (e.g., double- vs. single-lung transplantations, obstruction vs. restriction in native single lungs), as well as differences of subject body sizes, ages, genders, and so on. In addition, variances of pulmonary function values within groups (“noise”) were overtly increased by the presence of BOS subjects (with diminished FEV₁) among the CD28% High subjects and, conversely, No-BOS recipients (with normal expiratory flow) among the CD28% Low subjects (see Table E3).

However, CD28% values did associate with future changes of pulmonary function among these subjects, as decrements of

Figure 2. Cytokine elaborations by autologous CD4 subpopulations of recipients with chronic rejection. The initial (left) data point in each series represents control unstimulated (US) condition, whereas the second (right) data point delineates production of cells after stimulation with plate-bound anti-CD3 antibody (Stim). These paired specimens (US and Stim) are also connected by lines. CD4⁺CD28^{null} cells from transplant recipients with BOS (open circles) tend to elaborate greater amounts of proinflammatory and Th1 cytokines (top two rows), whereas CD4⁺CD28⁺ (open squares with paired specimens connected by solid lines) have an apparent Th2 bias (bottom row) (n = 5 randomly selected, consecutive specimens in each). G-CSF = granulocyte colony-stimulating factor; MIP-1b = macrophage inflammatory protein-1b.

FEV₁ at subsequent routine 6-month (surveillance) spirometry were more than fourfold greater among the CD28% Low recipients (Figure 4A). Because an increased proportion of the CD28% Low subjects had BOS, it could be postulated that the greater pulmonary function deterioration in this population

could possibly be attributable to confounding by the expected progression of their graft dysfunction. Accordingly, *post hoc* analyses limited to the recipients with BOS were performed. One of the five original CD28% Low subjects with BOS rapidly deteriorated after her CD4 assay, and did not have further

Figure 3. Effects of cyclosporine A on proliferation of CD4 T-cell subpopulations isolated from lung transplant recipients with chronic rejection. (Left panel) Illustrative example of proliferation quantitation as assessed by bromodeoxyuridine (BrdU) incorporation and flow cytometry. Viable cells were gated and respective populations of CD4 T cells (in this case CD4⁺CD28^{null}) that incorporated BrdU were determined. In this example, ~83% of the CD4⁺CD28^{null} incorporated BrdU. All CD4⁺ cells had been previously depleted from these cultures, except the particular CD4 T-cell subpopulation being evaluated (either CD28⁺ or CD28^{null}) (see METHODS). (Middle panel) Proliferation of CD4 T-cell subpopulations in the presence of cyclosporine A (100 ng/ml) compared to control (no cyclosporine) values. (Right panel) The aggregate decrement of proliferation in the presence of cyclosporine, relative to control (no cyclosporine) values, was significantly greater among the CD4⁺CD28⁺ cells. APC = allophycocyanin; CsA = cyclosporine A.

tions, ascertained by the percentages of respective autologous CD4 T-cell subpopulations that incorporated BrdU after stimulation by plate-bound anti-CD3 antibody, were approximately equivalent in both CD4 T-cell groups in the absence of cyclosporine (control). In every recipient with BOS tested (n = 6), however, proliferation among their CD4⁺CD28⁺ cells were decreased more by cyclosporine (100 ng/ml) than were the proliferations of their autologous CD4⁺CD28^{null} cells. (Right panel) The aggregate decrement of proliferation in the presence of cyclosporine, relative to control (no cyclosporine) values, was significantly greater among the CD4⁺CD28⁺ cells. APC = allophycocyanin; CsA = cyclosporine A.

Figure 4. Associations of CD28 expression with clinical outcomes. (A) Decrements of FEV₁ were significantly greater among cohort A recipients with CD4⁺CD28⁺/CD4_{total} values less than 0.9 (CD28% Low; n = 7) compared with recipients with CD4⁺CD28⁺/CD4_{total} values of 0.9 or greater (CD28% High; n = 27). Routine surveillance pulmonary function tests (PFTs) were performed 6.0 (SEM, ±0.5) months and 6.6 (SEM, ±1.3) months after CD28 determinations (CD28% Low and CD28% High, respectively). One CD28% Low subject did not have pulmonary function measured after her initial CD4 assay due to severe allograft dysfunction (BOS) and later demise. (B) Association of changes in FEV₁ (as percentages of initial values)

versus changes in CD28% among those cohort A subjects who were available and consented to replicate studies. *Open squares* denote those recipients who were non-BOS at the time of their first T-cell assay, but had progressed to BOS by the time of their second determination; *closed squares* denote those recipients who had BOS at both CD28% determinations; and *open circles* represent recipients who were non-BOS throughout. (C) Survival curves showing cumulative freedom from major adverse events of CD28% High (n = 46) and CD28% Low (n = 19) among all recipients in the aggregate subject populations (both cohorts A and B). *Tick marks* denote interval-censored events, and numbers in *parentheses* at end of the survival curves denote remaining, unaffected subjects who were censored at 24 months of observation. (D) Survival curves showing cumulative freedom from major adverse events of CD28% High (n = 24) and CD28% Low (n = 16) among all BOS recipients in the aggregate subject populations (both cohorts A and B).

pulmonary function testing. Deteriorations of expiratory airflow among the four (4) BOS CD28% Low subjects were much greater than in the five (5) BOS CD28% High subjects, and intergroup differences remained significant, despite the small population sizes (see Figure E2A).

Intergroup differences in the extent of FEV₁ changes between CD28% High and CD28% Low groups also seemed evident 1 year after the CD28 assays (Figure E2B). However, interval drop-outs from deaths, retransplantations, or severe allograft dysfunction that precluded PFTs resulted in smaller numbers of evaluable subjects, particularly among the CD28% Low group.

We were fortuitously able to perform replicate CD28 assays in 14 of the cohort A recipients during repeated, routine clinic visits (Figure E3). These recruitments too were essentially random selections resulting from recipient return appearances in outpatient clinic, continued absence of exclusion criteria, and willingness to participate by providing another blood sample. Changes of CD28% over time in these serial, replicate measurements correlated with corresponding pulmonary function alterations among the individual subjects (Figure 4B).

CD28 Down-Regulation on CD4 T Cells of Recipients Is Associated with Adverse Clinical Outcomes

Inexorable allograft injury in lung transplantation recipients eventually leads to pulmonary retransplantation and/or death

(1–3). Given the apparent associations between the extent of CD28 expression and pulmonary function (Figures 4A and 4B), we evaluated the possibility that CD4 T-cell phenotype abnormalities could also be linked with overall clinical outcome. Accordingly, we performed survival analyses of the subjects, again dichotomously stratified by their CD28 expressions (i.e., CD28% High vs. CD28% Low).

Product-limit analyses demonstrated that major events (death or retransplantation) during the 2 years after their first CD4 T-cell assays occurred much more frequently among CD28% Low subjects compared with CD28% High recipients (Figure 4C).

These differences in outcome seem unlikely to be simply attributable to the higher prevalence of BOS, with inherent increased morbidity and mortality (1–3), among the CD28% Low subjects (Table 1). To begin with, irrespective of the diagnoses at the time of their CD28% assays here, every subject who suffered a major adverse event had progressed to severe BOS before the occurrences of their serious complications (see Tables E5 and E6). Moreover, survival analyses limited to recipients with BOS already present at the time of their initial CD28% assays show even greater intergroup differences, with still considerable statistical significance, despite smaller numbers of subjects (Figure 4D). The event-free survival advantage of CD28% High was also evident in subpopulation analyses of individual subject cohorts (Figures E4A–E4C).

DISCUSSION

These data demonstrate that CD28 down-regulation on peripheral CD4 T cells of lung allograft recipients is associated with subsequent allograft dysfunction. The CD4⁺CD28^{null} cells seen in greater proportions among recipients with BOS exhibit unusual phenotypes, including discordant expression of activation markers, frequent production of cytotoxic mediators, and elaboration of large amounts of proinflammatory mediators, but decreased T_{reg} marker FoxP3 expression and relative cyclosporine resistance. Altogether, these findings suggest that CD4⁺CD28^{null} cells are a pathogenic T-cell subpopulation involved in allograft injury.

Nearly all normal human CD4 T lymphocytes express CD28 on their cell surfaces (29), and findings that proportions of circulating CD4⁺CD28^{null} T cells are increased are distinctly abnormal (9–21). Cognate interactions of T-cell CD28 with ligands CD80 and CD86 provide a “second signal” for lymphocyte activation, in conjunction with the “primary signals” mediated by TCR engagements with complexes of antigen-major histocompatibility complex. Nonetheless, neither previously activated (“memory”) CD4 nor naïve CD8 T cells require CD28 for activation, and other costimulatory molecules can also provide the necessary second signal for initial activations of naïve CD4 cells (29, 30). CD28 down-regulation on CD4 T cells in humans is a hallmark of ongoing, chronic adaptive immune responses, and has been frequently noted in patients with autoimmune and other chronic inflammatory diseases (9–21).

Previous studies have indicated that CD4⁺CD28^{null} cells derived from patients with autoimmune diseases are highly pathogenic. These particular cells autonomously elaborate IFN- γ (16) (*see also* Figure 2), express natural killer cell killer immunoglobulin-like receptors (17), and frequently produce cytolytic mediators (11, 13) (*see also* Figure 1D). The CD4⁺CD28^{null} cells of autoimmune patients are markedly oligoclonal, thus demonstrating that they are daughter progeny of repeated antigen-driven proliferations (9, 10, 18–20). In addition, the CD4⁺CD28^{null} clones bear idiotypic TCR sequences that are also present within autologous “normal” CD4⁺CD28⁺ cells, showing that the former are phenotypic variants of the latter, and that both populations share common progenitors (19, 31). CD4⁺CD28^{null} cell specificity for autoantigens has also been demonstrated (14), and CD28^{null} quantitation may correlate with clinical progression of autoimmune disease (12).

The cognate recognition of peptide alloantigens (notably including, but not limited to polymorphic major histocompatibility antigens) by recipient lymphocytes is widely recognized as an early and critical step in the cascade of responses leading to CR (5–7). CD4 T cells have pleotropic effector capabilities that can account for the allograft injuries associated with chronic rejection, either directly or by mediator elaborations that activate and/or recruit secondary tiers of downstream effector cells and other processes (8). In the particular case of human lung transplantation, nonimmunologic injuries (e.g., graft ischemia and various infections) have also been implicated in allograft injury (1–3, 32). Although multiple distinct injury pathways may converge to produce OB, it is perhaps also possible that many of these injuries ultimately enhance immunologic recognition of the allograft by causing increased production or presentation of alloantigens and/or proinflammatory mediators, and thereby facilitate and/or promote the injurious adaptive immune responses that lead to dysfunction of the transplanted lung(s) (33–37).

The pathogenic potential of the CD4⁺CD28^{null} cells appears to be considerable, and, in many respects, is highly comparable

to that reported for analogous cells from patients with autoimmune conditions and other patients with chronic inflammatory diseases (9, 10). Perforin and granzyme B production by the CD4⁺CD28^{null} cells (Figure 1D) may explain previous observations of CD4 T-cell cytotoxicity among lung transplant recipients (38). In what we believe is the most extensive characterization of cytokine production by CD4⁺CD28^{null} cells to date, it is evident that mediators that generally initiate and amplify immune responses were typically produced in much greater quantities by these particular lymphocytes than in their autologous CD4⁺CD28⁺ counterparts (Figure 2). These assays also show that most Th2 cytokine production by the CD4⁺CD28^{null} subpopulation are, typically, relatively little increased upon TCR stimulation (notably excepting IL-4). The comparatively lesser production of IL-10 by the CD4⁺CD28^{null} cells (relative to autologous CD4⁺CD28⁺ cells) may have singular biologic importance, given the possible role of this cytokine in suppression of injurious immune responses (27, 28, 39).

Further evidence of dysfunctional regulation among the CD4⁺CD28^{null} cell subpopulation is indicated by their relatively diminished expression of T_{reg} marker, FoxP3 (27), which, to the best of our knowledge, is another novel finding of the present study (Figure 1D). CD28 signaling has been implicated in the generation of CD4⁺CD25⁺ T_{regs} (40), and lack of this particular costimulatory function could possibly account for the comparative paucity of FoxP3 induction in the CD4⁺CD28^{null} cells. The net effects of these altered regulatory processes could result in relatively imbalanced (and, thus, more proinflammatory and injurious) responses to engagements with antigens (including alloantigens) by T-lymphocyte populations that have increased proportions of CD4⁺CD28^{null} cells.

Unlike reports based on studies of CD4⁺CD28^{null} cell lines derived by extensive propagation *ex vivo* after initial procurements from patients with autoimmune conditions (9–21), the freshly isolated CD4⁺CD28^{null} cells from recipients are clearly able to proliferate (Figure 3). We doubt that this seeming discrepancy is attributable to a fundamental biologic difference, or an epiphenomena of the immunosuppressant environment in which these cells arose in the transplantation recipients, because we have seen essentially similar results among CD4⁺CD28^{null} cells freshly isolated from patients who have not undergone transplantation (and are medication free) with other immunologic lung disease (unpublished observations). Instead, it seems far more likely that the replicative senescence of CD4⁺CD28^{null} cell lines described in previous reports (9–21) is a consequence of their protracted *in vitro* propagation. The proportion of CD4⁺CD28^{null} among transplant recipients with BOS is comparatively large (a possible effect of the intense antigenicity of alloantigens [6]), enabling us to procure adequate numbers of freshly isolated cells for studies, and thereby avoid potential introduction of functional changes induced by repeated *in vitro* propagations.

The finding that a T-cell subpopulation with unusual pathogenic potential is comparatively resistant to a class of medications that is a mainstay of transplantation could also have practical relevance (Figure 3). A clinically frustrating and perplexing problem of allograft transplantation centers on the mechanism by which allograft injuries continue to occur and progress despite treatment with intense immunosuppression (1–3). If a disease-causing T-cell subpopulation developed relative drug resistance, a consequence perhaps of phenotypic alterations induced by repetitive antigen-driven proliferations, it could be expected that chronic therapy with the agent(s) would result in considerable selection pressure for accumulation of the resistant cell subpopulation(s), and a likely predilection for treatment failures. Moreover, these drug treatments could exert other deleterious

effects if the more susceptible T cells also had useful specificities (e.g., avidities for microbial antigens), and increased frequencies of infections are seen in transplant recipients with CR (1–3). Alternatively, or in addition, the more susceptible T cells could be rejection-inhibiting regulatory T cells, possibly including IL-10-producing regulatory T cell, type 1 and/or CD4⁺CD25⁺ FoxP3⁺ T_{reg} (28), and globally decreased frequencies of putative T_{reg} cells have been reported in recipients with BOS (41). In addition to specific antigen-driven proliferations, expansions of the residual drug-resistant (and potentially pathogenic) lymphocytes could also be enhanced by their homeostatic proliferations in the periphery to fill the “void” created by proliferative blockade of other (more susceptible) T-cell populations (42). This hypothesis may be further supported by demonstrations that drug resistant lymphocyte clones may emerge in autoimmune diseases (43), and the extent of T-cell oligoclonality in transplant recipients with CR can become extreme (analogous to T-cell leukemia) in the presence of chronic immunosuppression and multiple-interval treatments (5, 44).

The present findings also raise several questions and identify potential areas for additional study. Demonstrations that CD4⁺CD28^{null} have specific avidities to alloantigens would compellingly implicate their role in immunologic allograft recognition and rejection, and functional assays that can directly ascertain the relative T_{reg} activity (if any) of these cells are also amenable to experimentation. The mechanism of drug resistance in the CD4⁺CD28^{null} lymphocytes is not yet known. CD8 T cells also down-regulate CD28 with chronic antigen stimulations (9, 10), and this process too could have relevance for lung transplantation, but only initial characterizations of these particular cells have so far been performed.

We do not believe that the present data adequately justify use of CD28 assays as a clinical tool in lung transplantation recipients, pending additional and confirmatory investigation(s). This initial, exploratory study was focused on investigating immunopathogenic phenomena *per se*, and, as such, we intentionally excluded from recruitment any recipient with potentially confounding recent infections, changes in immunosuppression, or known ACR. Thus, these data may have incorporated cryptic ascertainment biases that could confound use of the peripheral CD4 T cell measures in actual patient management. Furthermore, subject recruitment here was limited to recipients seen in outpatient clinics, and it is possible that analyses of more gravely ill, hospitalized patients could have other, poorly defined confounders. Preliminary results accrued to date in an ongoing prospective, longitudinal cohort trial, however, suggest that acute bacterial infections do not alter CD28% in lung allograft recipients (unpublished data), and concurrent (albeit occult) ACR did not seemingly affect these measures in either the present study (Table 1), nor in a prior investigation of renal allograft recipients (11). In any event, serial observations in a recipient cohort (irrespective of their other, ongoing transplant complications) will more accurately define the specific operating characteristics of these CD4 T-cell assays for clinical diagnostic and prognostic purposes.

Based on results of this initial study, it seems very unlikely that CD4 assays will supplant spirometry as a diagnostic tool to detect CR, as PFTs are already the widely accepted standard, are even less invasive (and probably less expensive) than phlebotomies and flow cytometry, and appear to have much greater sensitivity for BOS, *per se*, than CD28 determinations.

However, in addition to possibly providing novel insights into the immunopathogenesis of BOS, the present findings do suggest that characterizations of circulating CD4 T cells may have utility as a means of predicting impending deterioration of allograft function in individual lung transplant recipients. If so, and given the apparent correlation between interval changes of

CD28 expression with corresponding lung function (Figure 4B), it may also be likely that serial CD28 determinations in individual recipients could have greater diagnostic and prognostic accuracies than the random cross-sectional measurements that were performed in this initial study. This hypothesis too will be tested in an ongoing longitudinal trial.

In summary, the present data show that the extent of CD28 expression on circulating CD4 T cells among lung transplant recipients is associated with later allograft function, and it seems probable that similar considerations may apply to recipients of other allogeneic organ transplantations (21). These findings may provide insights relevant to the immunobiology of CR, and potentially illuminate novel approaches that could eventually lead to more effective counters for this transplantation complication. If further substantiated, the practical value of simple, minimally invasive T-cell assays that identify recipients destined for progression of allograft dysfunction could also be considerable. Among other considerations, heightened surveillance and/or interventions could be directed to those recipients at greatest risk, while possibly obviating toxic treatments among those destined for more indolent courses.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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