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T cell Co-Stimulatory molecules ICOS and CD28 stratify idiopathic pulmonary fibrosis survival

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a devastating disease that kills as many Americans as breast cancer each year. This study investigated whether lung function decline and survival associates with adaptive immunity in patients with IPF, specifically the expression of checkpoint molecules ICOS, CD28 and PD-1 on circulating CD4 T cells. Clinical data, blood samples and pulmonary function tests were collected prospectively and longitudinally from 59 patients with IPF over a study period of 5 years. Patients were followed until death, lung transplantation, or study end, and cell surface expression of CD45RO, CD28, ICOS, and PD-1 was measured on CD4 T cells via flow cytometry. Repeated measures of ICOS and CD28 on CD4 T cells revealed significant associations between declining ICOS and CD28 expression, and declining lung function parameters FVC and DLCO, independent of age, sex, race, smoking history, or immunosuppressant use. Strikingly, patients in the highest quintile of ICOS at study entry had markedly improved survival, while those with low CD28 fared poorly. No change in PD-1 expression was found. Analysis of ICOS and CD28 from the first blood draw identified three populations of IPF patients; those at high risk for early death, those with intermediate risk, and those at low risk. These results highlight the role of T cell mediated immunity in IPF survival, finding the assessment of two T cell stimulatory checkpoint molecules, CD28 and ICOS, was sufficient to discriminate three distinct survival trajectories over 5 years of patient follow up.

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Author contributions

Conception and design: CAB, MES, IN, AIS. Data collection: CAB, RV, STM, JMO, KMB, MES, IN. Data analysis and interpretation: CAB, KMB, MMC, AIS. Drafting the manuscript for important intellectual content: CAB, CLH, RV, STM, JMO, KMB, MMC, MES, IN, AIS. Dr. Sperling is the guarantor of this paper and takes responsibility for the integrity of the work, from inception to published article.

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Appendix A. Supplementary data

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Keywords

Idiopathic pulmonary fibrosis; T cells; ICOS; CD28; Immune checkpoint

1. Introduction

IPF is an interstitial lung disease of unknown etiology with median survival of 3–5 years after diagnosis [1]. The pathology of IPF includes activation of pulmonary fibroblasts and exuberant collagen deposition resulting in progressive, irreversible pulmonary fibrosis [2,3]. Recent landmark studies [4–7] demonstrate the efficacy of pirfenidone and nintedanib, anti-fibrotic drugs that slow the progression of IPF via mechanisms that include TGFβ [8,9] and tyrosine kinase inhibition [10,11]. However, the interplay of fibrosis generation, IPF survival, and the immune system remains controversial partly due to failure of high-profile clinical trials testing immune modifying therapies [12,13].

Data from several groups associates suppression of T cell-mediated immunity with poor outcomes in IPF [14–17]. Gene expression profiles of IPF patients revealed a 52 gene signature differentiating transplant-free survival [15,18]. Decreased expression of ICOS, CD28, and T cell specific tyrosine kinases, ITK and LCK, were most associated with shortened transplant-free survival, and a stronger predictor of outcome than clinical data alone. While mRNA from the co-inhibitory molecule, CTLA-4 was also associated with outcomes, the co-inhibitory molecule PD-1 had no association with outcomes.

ICOS and CD28 are co-stimulatory molecules found on the surface of T cells, while PD-1 and CTLA-4 are co-inhibitory molecules. Co-stimulatory molecules are key mediators of T cell activation, proliferation, and cytokine production during generation of innate and adaptive immune responses. ICOS promotes Th2 mediated responses and to a lesser extent Th1, and induces IL-10 production for the maintenance of suppressive T regulatory cells [19]. CD28 is a major activator of naïve T cells, and is crucial for IL-2 production and T cell survival [20]. Co-inhibitory molecules such as PD-1 and CTLA-4 are known to dampen the activation of T cells and can induce T cell exhaustion [21]. Inhibition of these checkpoint molecules has become an important new therapeutic target for many types of cancer. Together, expression of molecules in the CD28 family fine-tunes the T cell response.

In this study, we addressed whether expression of T cell co-stimulatory and co-inhibitory molecules on the surface of CD4 T cells would correlate with lung function and/or predict survival. Because CTLA-4 must be measured intracellularly and is thus less practical for clinical testing, we focused on cell surface expression of ICOS, CD28, and PD-1, the molecules most likely to translate into clinical application. Our findings demonstrate that IPF patients with low ICOS and low CD28 on peripheral blood T cells at the time of first clinical presentation have remarkably shortened survival. When both ICOS and CD28 are considered, three distinct survival trajectories become apparent. Together, our data identify a novel, T cell mediated pathway impacting IPF progression, and have practical application towards identifying patients most likely to experience early mortality.

Some of the results of these studies have been previously reported in the form of scientific abstracts [22,23].

2. Methods

2.1. Study enrollment and sample collection

Peripheral blood samples were collected prospectively from 59 IPF patients and 22 age and gender matched control individuals at the University of Chicago from February 1, 2009 to September 1, 2012. All patients were recruited from outpatient clinic. IPF diagnosis was established by a multidisciplinary group of pulmonologists, pathologists, and radiologists in concordance with the 2001 American Thoracic Society/European Respiratory Society criteria [24], and in agreement with 2011 guidelines [1]. IPF patients were excluded at study entry if they had connective tissue disease, malignancy, active infection, drug exposures or occupational exposures known to cause pulmonary fibrosis. Informed consent was obtained from all patients (https://clinicaltrials.gov/, clinical trial ID NCT00470327), under University of Chicago institutional review board study number 14163. Clinical characteristics were collected at study enrollment (Table 1). Full PFTs including measurement of forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO) were performed at study enrollment and within 3 months of each later blood draw. Patients were followed until death, transplant or study end on April 10, 2014.

We followed this IPF cohort longitudinally in our outpatient clinic at a median interval of 6 months, for total median follow up time of 26 months until death, lung transplantation, or study end. Twenty-three patients underwent more than one blood draw. Six patients underwent a third blood draw, and two patients contributed a fourth blood draw for flow cytometry analysis. Thus a total of 90 peripheral blood mononuclear cell (PBMC) samples and pulmonary function tests were included in the mixed effect regression model examining pulmonary function decline, T cell surface molecule expression, and demographic variables.

2.2. Flow cytometry protocol

Fresh live PBMCs were isolated over a Ficoll-Hypaque gradient and stained with anti-CD4 allophycocyanin cyanine (APC-Cy7), anti-ICOS allophycocyanin (APC), anti-CD28 peridinin chlorophyll protein cyanine (PerCP-Cy5.5), anti-CD45RO fluorescein isothiocyanate (FITC), and anti-PD1 phycoerythrin (PE) conjugated antibodies (Biolegend, eBiosciences) to determine the expression of co-stimulatory molecules on the CD4 T cell surface. Cells were analyzed on a LSR-II cytometer (Becton-Dickinson). Prior to each run, laser voltages were adjusted with Ultra Rainbow Calibration Particles (Spherotech) to ensure run-to-run consistency of the cytometer over the duration of this study. Data was analyzed using FlowJo software.

2.3. Statistical analysis

Continuous variables are reported as means with standard deviation and compared using a two-sample *t*-test. Categorical variables are reported as counts and percentages and compared using Fisher's exact test. Repeated measures of T cell surface markers and

PFT values were analyzed using mixed effect regression models controlling for the fixed effects of age, sex, race, smoking history, and steroid use over time. Survival analysis was performed with unadjusted log rank test and plotted using the Kaplan-Meier survival estimator. Survival time was defined as the time from first blood draw until death, lung transplantation, or end of study period, and patients were censored at time of transplant, or study end. All patients' mortality status was confirmed by either direct contact, phone follow up, or Social Security Death Index listing at the study end. Univariate and multivariate Cox regression was performed. Data was analyzed using STATA, version 13 (StataCorp; College Station, Texas) and GraphPad Prism 7.04.

3. Results

3.1. Demographics

Study demographics were compatible with a classic IPF cohort, with a predominance of male, Caucasian patients who were former smokers (Table 1). Mean age was 68 years. Early recruitment into the study predated results of the PANTHER trial [25], thus two patients were taking low-dose systemic corticosteroids at the time of enrollment (one patient on 5 mg oral prednisone daily, and a second patient on 10 mg oral prednisone daily), though neither carried a diagnosis of autoimmune disease. The IPF cohort had moderate to severe pulmonary function impairment at study entry, with mean FVC 66% of predicted, and mean DLCO 47% of predicted. IPF patients were matched for age, gender, and race with control individuals who had no known lung disease or immune-mediated diseases. Pulmonary function tests, smoking history and medication use history were not collected in the control group.

3.2. IPF and control subjects show no difference in co-stimulatory molecule expression on CD4 T cells

Given the known effects of increasing age on diminished T cell function, we first sought to compare IPF patients with control individuals. Comparison of PBMCs showed no significant difference in the numbers of CD4 T cells, which were normalized as a percentage of the total circulating lymphocyte population (Fig. 1A). Likewise, IPF patients demonstrated a spectrum of co-stimulatory molecule expression and T cell maturity markers on CD4 T cells similar to control individuals (Fig. 1B–E). These results show that levels of co-stimulatory molecules are not simply reflective of overall changes in percentage of CD4 T cells in IPF. Neither are IPF patients as a group defective in T cell activation or T cell maturity in comparison to their peers without IPF.

3.3. Decreasing co-stimulatory molecule expression associates with pulmonary function decline

We found declining ICOS levels on the surface of CD4 T cells correlated significantly with FVC ($p = 0.038$) and DLCO ($p = 0.003$) over time in patients with IPF (Fig. 2). CD28-null CD4 T cells are found at higher levels in older individuals, and have previously been suggested as a potential IPF biomarker [26]. While ICOS cell surface expression is best characterized as a continuous variable, CD28 expression occurs in a bimodal distribution, and thus we quantified CD28 expression as a percentage of total CD4 cells

with CD28 present or absent. Similar to ICOS, decreasing percentage of $CD4^+CD28^+$ T cells independently associated with declining DLCO ($p = 0.001$) and a trend with declining FVC ($p = 0.092$) over time (Fig. 3). No effect was observed for differing levels of PD-1 expression (Supplemental Fig. 1).

3.4. ICOS and CD28 surface expression are independent of T cell maturity

ICOS surface expression decreased in association with declining PFT values in both mature $CD4^+CD45RO^+$ and immature $CD4^+CD45RO^-$ T cells (Supplemental Fig. 2), suggesting that the effect of ICOS was independent of previous T cell antigenic experience. ICOS expression trended down, but did not decrease significantly on $CD4^+CD28^+$ T cells ($p = 0.059$) or $CD4^+CD28^-$ T cells ($p = 0.067$) (Supplemental Fig. 3). This demonstrates that ICOS down regulation in IPF patients with worsening pulmonary function is not driven by an increase in previously reported CD28-null $CD4^+$ T cell populations [26] and is consistent with the complementary but independent functions of these molecules.

3.5. ICOS high patients have extended survival

We sought to determine if a single measurement of T cell co-stimulatory molecule surface expression associated with patient survival outcomes. Due to rolling enrollment into the study as well as survival outcomes, patient time in the study was variable, but was at minimum 24 months (unless earlier death or lung transplantation) and at maximum 63 months. Two patients underwent transplant, at 22 and 24 months, and were censored at the time of transplant. None were lost to follow up.

Measurement of ICOS and CD28 from the first blood draw in clinic yielded striking dichotomies in survival outcomes. IPF patients who ranked in the highest quintile of ICOS expression showed significantly improved survival by unadjusted log rank analysis ($p = 0.0231$) in comparison to IPF patients in the lowest 4 quintiles of ICOS expression (Fig. 4 and Supplemental Fig. 4). There were no deaths in the ICOS high group during the predetermined study period. In contrast, 21 of 48 patients in the low to medium ICOS expression group died during follow up with a mean survival time of 2.2 years.

Examination of ICOS levels in the 23 patients who underwent repeat blood draws revealed stability of ICOS categorization in most patients (Supplemental Fig. 5). Four patients who fell in the ICOS high group underwent a repeat blood draw, shown in red. One was transplanted 20 months after the second draw, in which the patient's ICOS status decreased from high to low. The others maintained their ICOS high status at the second blood draw and remained enrolled for an additional mean time of 24 months, surviving until the study end. Six patients had an increase in ICOS at the second or third visit, enough to exceed the threshold for ICOS high classification. Four of the six patients who made this transition from ICOS low to high survived until study end, with a mean additional survival time of 22 months after the second draw. Two patients with the most dramatic decrease in ICOS level between draw 1 and draw 2 (42% mean decrease, shown in blue, Supplemental Fig. 5), survived a mean of 2 months after their second draw.

3.6. CD28 low patients have poor survival

In contrast to our finding that ICOS high expression was associated with extended survival time, IPF patients within the lowest quintile of $CD28^+$ $CD4^+$ T cells at study enrollment ($p = 0.0047$) were most likely to die earlier (Fig. 5 and Supplemental Fig. 6). Eight of 12 patients (67%) in the CD28 low group died, with a mean survival time of 1.6 years. Only 14 of 47 patients (30%) in the CD28 high group died during study follow up.

Examination of the data from the 23 patients who underwent a repeat blood draw showed stability of CD28 categorization in most patients over time, with only two patients demonstrating a transition between CD28 categories; one patient transitioned from CD28 high to low, while another patient transitioned from CD28 low to high (Supplemental Fig. 7).

PD-1 expression on circulating CD4T cells did not predict survival (Supplemental Fig. 8).

3.7. High ICOS surface expression on CD4 T cells associates with improved survival independently of CD28, gender, age and lung function

To investigate the extent to which ICOS and CD28 affect survival and whether these effects are independent for each surface molecule and other patient characteristics, we performed univariate and multivariate Cox regression analysis. The estimated hazard ratio for ICOS MFI in multivariate Cox regression analysis was 0.74, indicating a 26% lower risk of death for each unit increase in ICOS MFI (adjusted HR 0.74, 95% CI 0.56–0.97, $p = 0.031$) (Table 2). Interestingly, while CD28 low status was significantly associated with survival when considered apart from other variables (unadjusted HR 3.34, 95% CI 1.38–8.09, $p = 0.008$), the addition of ICOS along with gender, age, physiology, and other demographic variables revealed loss of independent significance for CD28 status on mortality (adjusted HR 1.42, 95% CI 0.36–5.61, $p = 0.61$). As we expected, increasing age and lower DLCO showed significant independent association with mortality. Corticosteroid use was also significantly associated with mortality (adjusted HR 66.21, 95% CI 1.42–3091, $p = 0.033$), however of the patients taking low-dose corticosteroids at the time of first blood draw, one did not survive the duration of the study, while the other did. To ensure that the inclusion of the 2 patients who were taking low dose prednisone did not distort the analysis, repeat univariate and multivariate Cox regression analysis excluding these patients was performed (Supplemental Table 1). This analysis showed that ICOS continued to associate independently with mortality (HR 0.71, 95% CI 0.53–0.94, $p = 0.018$), with similar associations in the other variables as presented in Table 2, notably that CD28 expression did not significantly associate with mortality when considered together with ICOS.

3.8. Assessment of both ICOS and CD28 yields 3 separate survival trajectories

In contrast to ICOS, where survival differences were driven by the highest quintile, CD28 survival differences were driven by excess mortality in the lowest quintile. Given these different effects, we assessed if combined analysis of ICOS and CD28 risk groups had further utility for stratifying patient survival. As shown in Fig. 6, similar to our multivariate Cox regression analysis, we found all ICOS high patients survived regardless of CD28

status, while patients who were low for both markers had the shortest survival. Patients who were ICOS low but CD28 high comprised the largest group, and experienced intermediate survival outcomes.

4. Discussion

We demonstrate a single assessment of two CD4 T cell co-stimulatory checkpoint molecules at the time of study entry stratifies patients into three risk categories for all-cause mortality over the next 5 years of follow up. Furthermore, we demonstrate an association between worsening pulmonary function and downregulation of CD4 T cell surface expression of ICOS and CD28, independent of gender, age, and other demographic co-factors. These results stress the impact of patients' immune status on their capacity to survive IPF.

Our matched cohorts demonstrated that IPF patients had a spectrum of ICOS, CD28, and PD-1 cell surface expression similar to the control cohort without IPF. The finding of no significant difference in co-stimulatory status or T cell maturity between IPF and controls is of interest. It supports the hypothesis that IPF is a disease intensified by immunologic changes universal to the aging process [27]. Aging and concomitant immune senescence has been proposed as one mechanism contributing to the accelerated propensity for fibrosis in many organ systems [28], and T cell co-stimulatory molecules are known to decrease over time in aging populations. A strength of this study is that IPF patients and controls were well matched for multiple demographic variables including age. Others have reported co-stimulatory molecule differences in cohorts with significant age differences [29], or who were age matched but without other demographic variables reported [26].

Our data on age and gender matched controls supports a model in which low co-stimulatory molecule status is not unique to IPF, but instead develops in many individuals as they age. This observation is key to contextualize our results in the IPF group, for whom low co-stimulatory T cell status associates with IPF progression and early mortality. We posit that low ICOS and CD28 on circulating CD4 T cells marks a change to an aged, immune senescent phenotype. Importantly, when immune senescence occurs in IPF patients, vulnerability to IPF progression and poor long-term survival outcomes may be exposed. In the absence of active T cell immunity, a "second hit" such as viral infection or fibroblast activation may become a non-survivable event.

The loss of ICOS on circulating CD4 T cells may also signal a more specific change in an individual's response to immune challenge. ICOS is known to mediate type 2 adaptive immune responses, commonly associated with asthma and allergy, and also activates type 2 innate lymphoid cells [30]. In support of this link between type 2 immunity and ICOS in IPF, we have recently demonstrated that ICOS deficiency is linked to defects in type 2 innate lymphoid cells in IPF lung tissue and in the bleomycin model of pulmonary fibrosis [31]. We propose that downregulation of type 2 immune responses allows opposing pathways such as the pro-fibrotic type 17 response to blossom. In support of this hypothesis, canonical type 17 cytokine IL-17 is highly expressed in epithelial cells, macrophages and T cells from zones of active disease within IPF lungs [32], and at transplant, IPF lung tissue from patients with the worst lung function demonstrates type 17 skewed CD4 T cells

[33]. At the bench, IL-17A induces synthesis and secretion of collagen and pro-fibrotic epithelial-to-mesenchymal transition [34], and recruits neutrophils to the airways [35]. A growing body of literature debates the role of IL-17 in the lung [36]. Our data supports that a shift away from ICOS-mediated type 2 responses and perhaps towards type 17 is detrimental to IPF survival.

Notably for IPF patients, co-stimulatory T cell status is modifiable using currently available treatments developed as immunotherapy for cancer [37,38]. Recently, immune checkpoint blockade via anti-CTLA4 therapy has been shown to increase ICOS expression on CD4 T cells [39]. Furthermore, ICOS upregulation can be induced directly via ICOS ligand binding, and augments the anti-tumor effects of anti-CTLA4 therapy [40]. Common pathologic mechanisms between IPF and malignancy have been proposed [41], and polymorphisms in genes encoding ICOS, CD28 and CTLA4 have been associated with increased susceptibility to malignancies [42–44]. Our data highlight that the T cell co-stimulatory pathway may be a commonality between IPF and cancer that is ripe for further investigation, particularly given the recent development of targeted pharmaceuticals.

We have illuminated ICOS and refreshed CD28 as molecular biomarkers for IPF mortality, while recognizing that several other potential biomarkers have come to the fore, notably SP-A [45–47] and MMP-7 [48,49] marking alveolar epithelial injury, and CCL18 marking macrophage activation [50,51]. We posit that a combined biomarker signature, as we have modeled here with ICOS and CD28, is most likely to be predictive of outcomes. We further note that ICOS and CD28 bear specific utility as prognostic biomarkers, and as such provide actionable information to clinicians and patients. Development of ICOS and CD28 as biomarkers will require validation in multiple centers. Fortunately, innovations such as commercially available automation in sample processing [52] and automated data analysis pipelines [53] have made flow cytometry a feasible methodology for multicenter, high-throughput studies, and significant research infrastructure for multicenter collaborations exists in the IPF community. Alternate methods of assessment of these biomarkers by plasma or RNA quantification would first require correlative studies.

No study has examined in detail the cell surface expression of ICOS and its relationship to survival outcomes in IPF patients. Recently, the role of ICOS was highlighted in an unbiased proteomics analysis of plasma searching for IPF progression biomarkers, in which ICOS ranked among the top 6 of 1129 analytes [16]. Interestingly, high levels of ICOS measured in plasma associated with poor IPF outcomes. ICOS cleavage from the cell surface may explain why high ICOS in plasma, yet low ICOS on cell surfaces is associated with worsening IPF. It is known that activation of murine T cells leads to ICOS shedding into supernatant [16], and ICOS ligand is shed upon ICOS ligation [54]. These results emphasize the need for further study on how altered ICOS expression and function contributes to IPF disease progression and patient survival.

Previous studies have proposed increased CD28 low or null cells as poor outcome predictors, with increased production of cytotoxic mediators and Th1 pro-inflammatory cytokines [26]. Increased numbers of CD28null T cells are observed in IPF lung tissue [55]. Another study, however, failed to find an association with CD28-null CD4 T cells and

IPF patient survival [56]. Our results link poor survival with low cell surface expression of CD28. We have found measurement of ICOS in addition to CD28 best stratifies outcomes, with quantification of ICOS splitting the CD28 low population into intermediate and high-risk mortality groups. In addition, others have hypothesized that ICOS downregulation could be a side effect of increasing numbers of CD28-null CD4 T cells [15]. Importantly, and in contrast to previously proposed hypotheses, we found ICOS downregulation with disease progression most apparent on $CD28^{+}$ CD4 T cells, not CD28-null CD4 T cells, suggesting that changes in ICOS expression were not driven by the CD28-null CD4 T cells. ICOS and CD28 have non-redundant functions for induction and maintenance of adaptive immunity, and our data suggest that they also have non-redundant roles in IPF pathogenesis [19].

A recent study demonstrated significant upregulation of co-inhibitory molecule PD-1 on CD4 T cells in patients with IPF compared to control subjects [57]. We did not find a difference in PD-1 expression between IPF and controls (Fig. 1), and did not find that PD-1 changed significantly in association with pulmonary function over time (Supplemental Fig. 1) or mortality (Supplemental Fig. 8). Several key methodologic differences may have resulted in this disparity. First, given that differences could be induced if IPF cells are more sensitive than controls to freezing and thawing, we used fresh live PBMCs prepared after each clinic visit, with calibration beads to ensure run-to-run consistency on the cytometer. Second, given the clinically observed effect of age and sex on IPF biology, we recruited control subjects that were matched in age and gender to our IPF cohort. Lastly, we analyzed our data as mean fluorescence intensity of PD-1 expression, while Celada et al. report the percentage of CD4 T cells with PD-1 absent or present. These differences in fresh versus frozen sample processing, control subject demographics, and data analysis may account for the difference in results between Celada et al. and our study.

Our study is limited in its small sample size and single center experience. However, we note that our hypotheses are derived from unbiased gene expression analyses performed in separate cohorts, and exhibit strong biological rationale and plausibility corroborated by studies in other centers. Our findings on the impact of CD28 down-regulation independently replicate those of Gilani et al. [26], and emphasize the need to assess other T cell checkpoints such as ICOS. Like current studies of immunotherapy for cancer that combine checkpoint inhibitors [58], treatment of pulmonary fibrosis may require modulation of multiple T cell checkpoints. Mechanistic studies are needed to determine whether co-stimulatory molecule downregulation is causal or reflective of IPF pathology.

In conclusion, we report that a single assessment of two co-stimulatory T cell checkpoint molecules can discriminate disparate survival outcomes among patients with IPF. Furthermore, longitudinal follow up of patients with IPF revealed that downregulation of ICOS and CD28 on the CD4 T cell surface associated with pulmonary function decline. These findings highlight molecular disease mechanisms in a novel T-cell mediated pathway impacting all-cause mortality for IPF patients. Further research is needed to demonstrate how the confluence of age, immunity, fibrotic injury and repair contributes to IPF disease progression and survival. Development of T cell focused therapies with personalized attention to those who exhibit lowered immunity may drive forward treatment for IPF patients who currently face a devastating and uncertain diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	allophycocyanin fluorescent dye
APC-Cy7	allophycocyanin cyanine fluorescent dye
DLCO	diffusing capacity of the lung for carbon monoxide
FITC	fluorescein isothiocyanate fluorescent dye
FVC	forced vital capacity
IPF	idiopathic pulmonary fibrosis
PBMC	peripheral blood mononuclear cell
PerCP-Cy5.5	peridinin chlorophyll protein cyanine fluorescent dye
PE	phycoerythrin fluorescent dye

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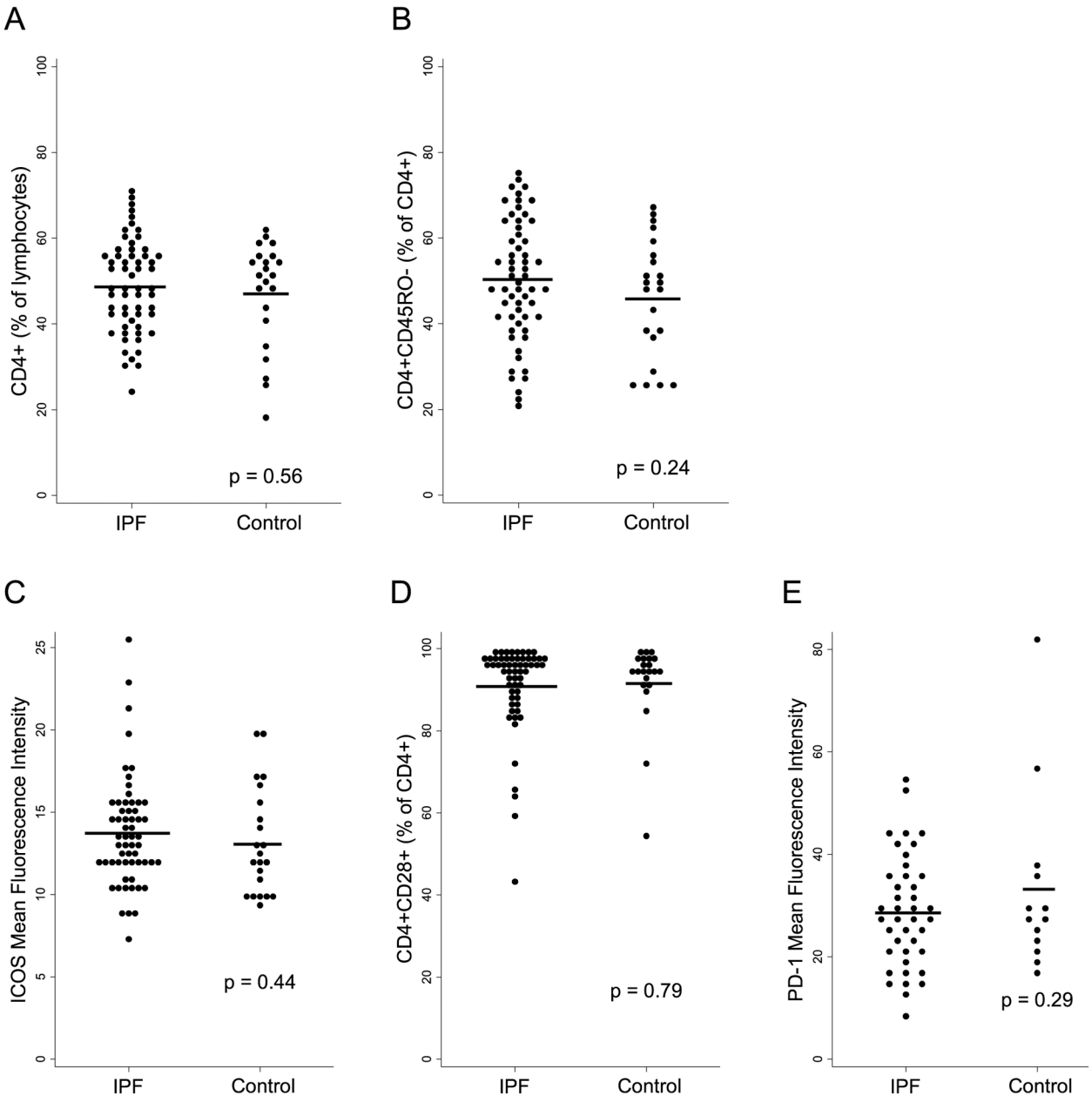


Fig. 1.

IPF and control groups at the time of study entry show no significant difference between A. Percentage of CD4 lymphocytes in peripheral blood samples, B. Cell surface expression of the maturity marker CD45RO as a percentage of CD4 lymphocytes, C. ICOS mean fluorescence intensity, D. Percentage of CD4 lymphocytes expressing CD28, and E. PD-1 mean fluorescence intensity. Panel E reports n = 41 patients with IPF, and n = 13 controls, as PD-1 staining was added to the experimental protocol in 2011. Statistical analysis by two-sample *t*-test.

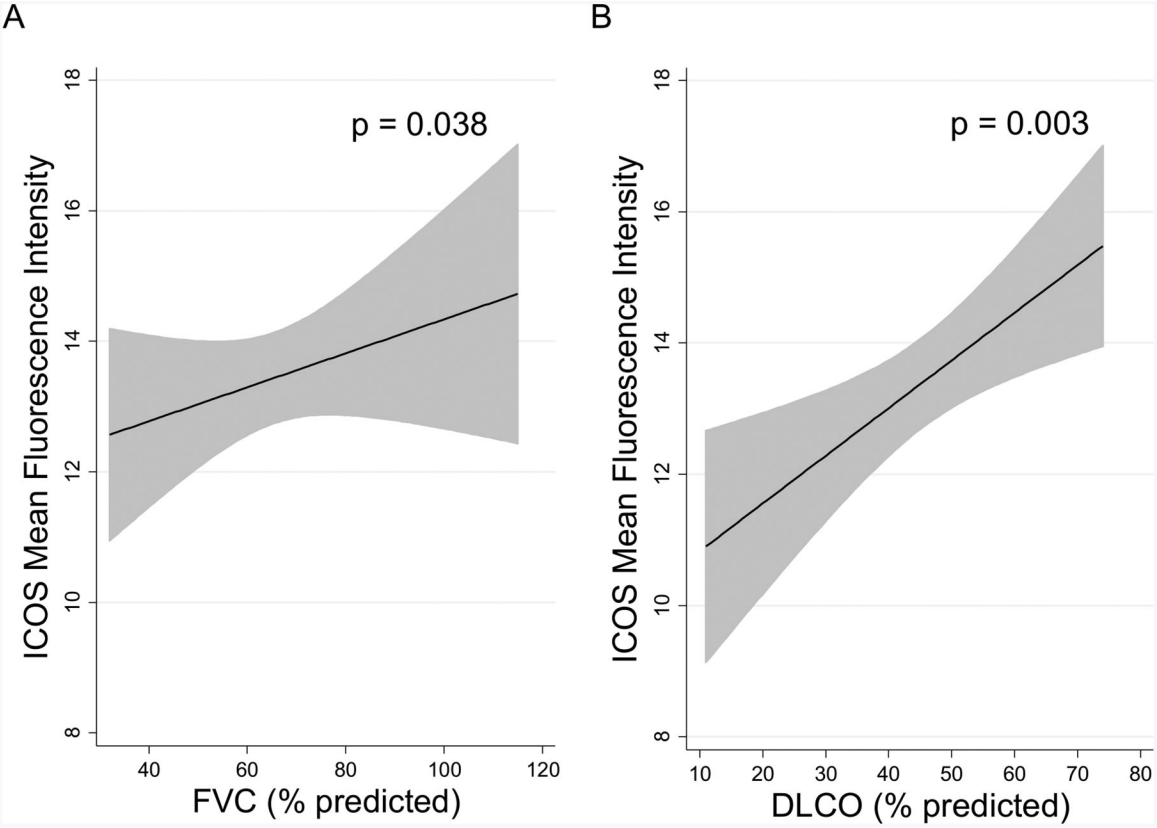


Fig. 2.

Decreased surface expression of ICOS on CD4 lymphocytes is significantly associated with decline in pulmonary function. Mixed effect regression models controlling for age, sex, race, steroid use, and smoking history reveal that decreased ICOS expression independently associates with IPF disease progression as measured by decline in A. forced vital capacity (FVC) and B. diffusing capacity of the lung for carbon monoxide (DLCO) over time. Mixed effect regression statistical analysis.

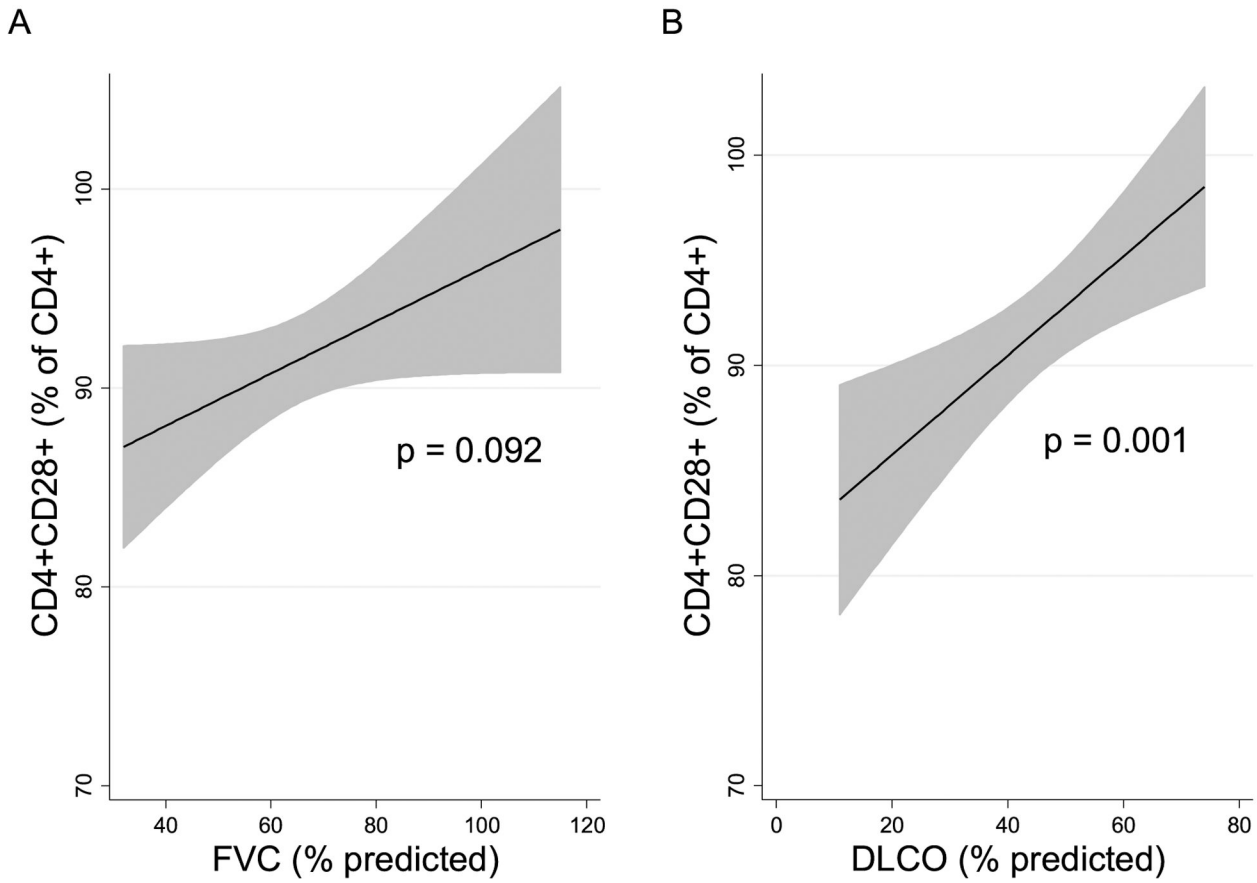


Fig. 3.

Analysis of $CD4^{+}CD28^{+}$ cells confirms an association of decreased percentage of circulating $CD4^{+}CD28^{+}$ cells with pulmonary function decline in IPF patients. Mixed effect regression models controlling for age, sex, race, steroid use, and smoking history reveal that a decreased percentage of $CD4^{+}CD28^{+}$ cells independently associate with IPF disease progression as measured by decline in A. forced vital capacity (FVC) and B. diffusing capacity of the lung for carbon monoxide (DLCO) over time. Mixed effect regression statistical analysis.

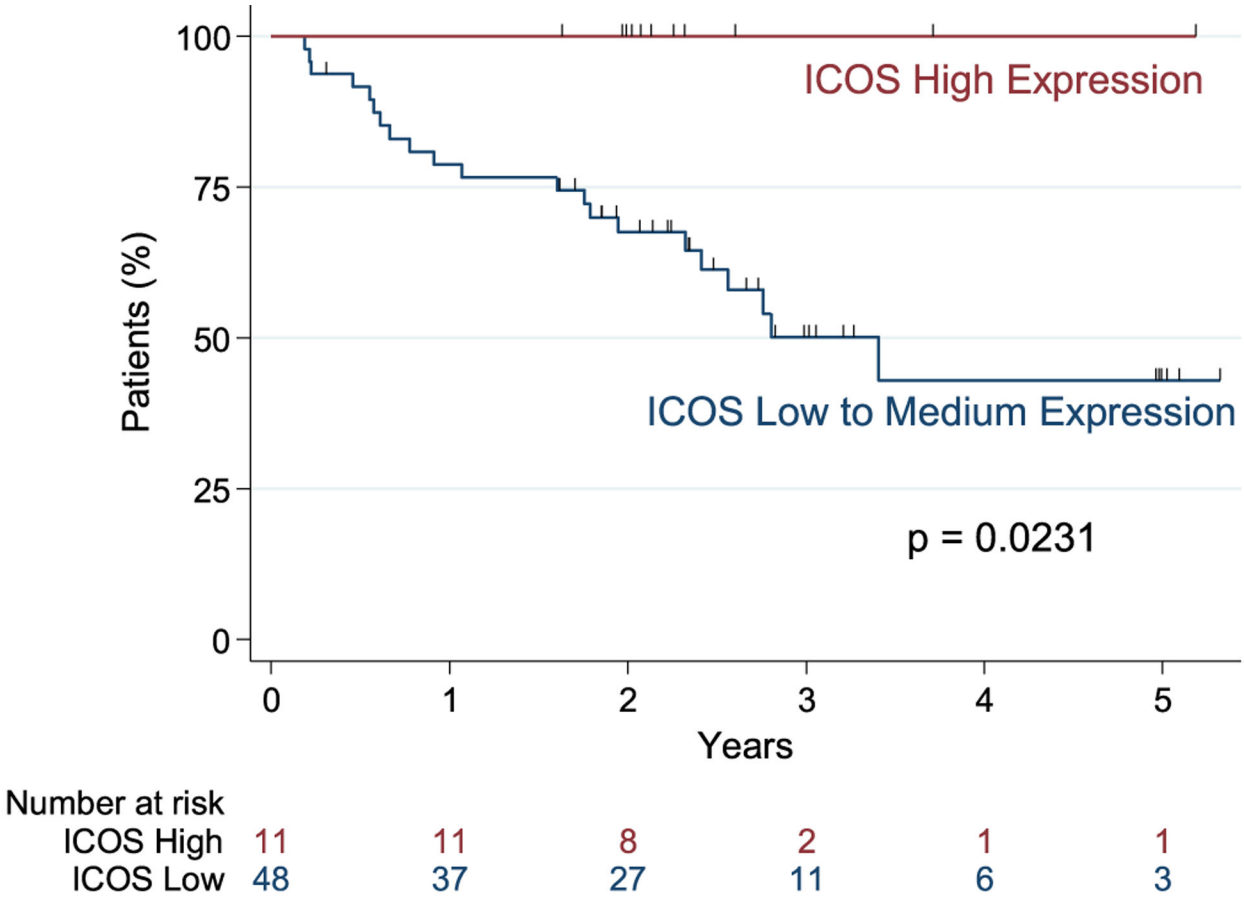


Fig. 4.

IPF patients with high ICOS have improved survival. No deaths occurred in the patient group who had the highest quintile of ICOS surface expression on CD4 lymphocytes at study entry. Patients within the lowest four quintiles of ICOS expression at study entry had a mean survival time of 2.2 years. Patients were censored at the time of study end, or transplant. Two patients were transplanted, at 22 and 24 months after study enrollment. Statistical analysis by log-rank with Kaplan Meier survival estimation.

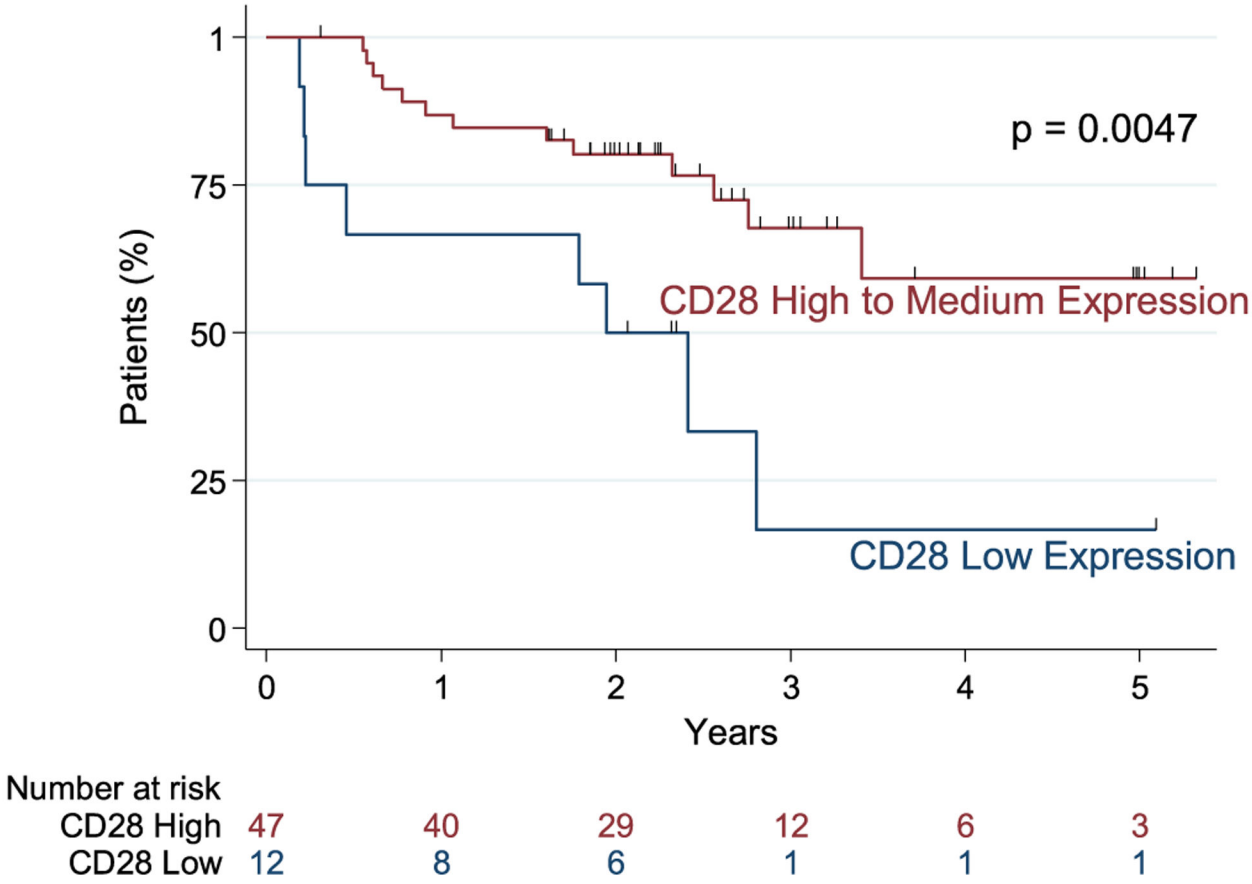
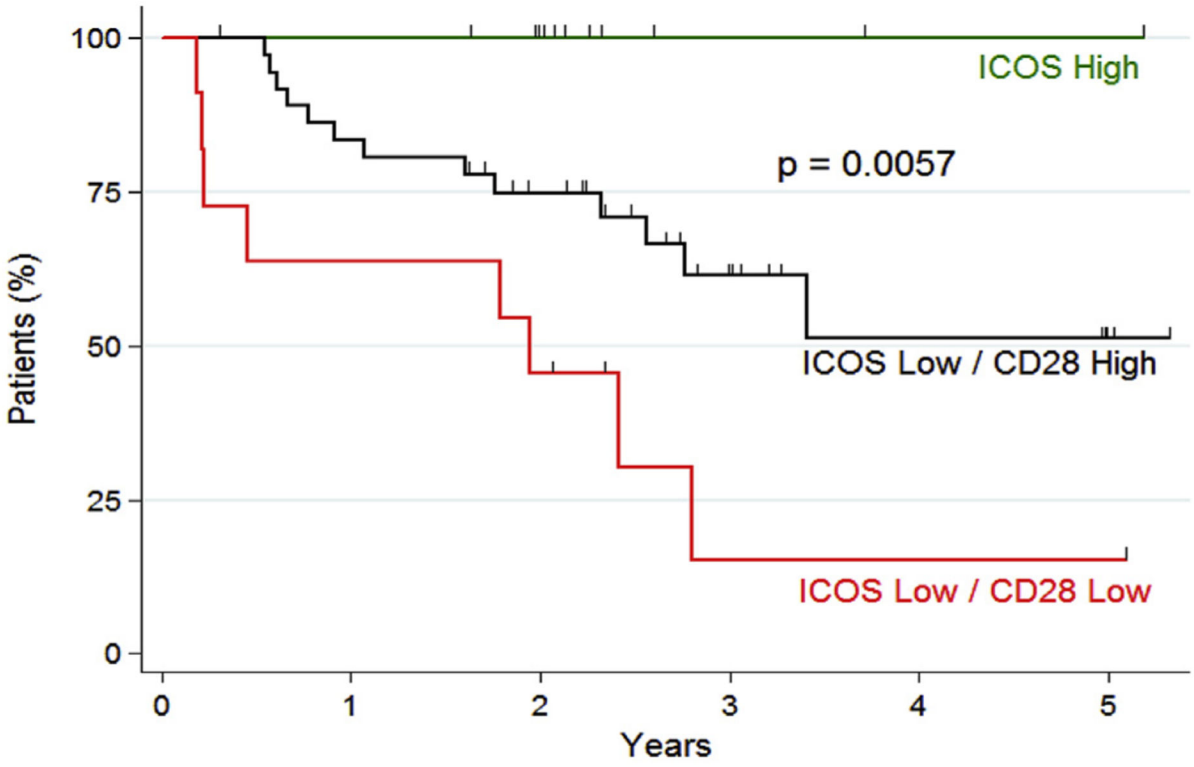


Fig. 5.

IPF patients with a high percentage of $CD4^{+}CD28^{+}$ lymphocytes have improved survival. Patients within the highest four quintiles of CD28 at study entry had a mean survival time of 2.4 years, compared to median survival time of 1.6 years for patients in the lowest CD28 quintile. Patients were censored at the time of last clinic visit, or transplant. Statistical analysis by log-rank with Kaplan Meier survival estimation.



Number at risk

ICOS High	11	11	8	2	1	1
ICOS Low / CD28 High	37	30	22	10	5	2
ICOS Low / CD28 Low	11	7	5	1	1	1

Fig. 6.

IPF patients who are ICOS high survive longest regardless of their CD28 status, while ICOS low/CD28 low patients have poor survival. No deaths occurred in the patient group who had the highest quintile of ICOS surface expression on CD4 lymphocytes at study entry. Among ICOS low patients, high numbers of CD28 cells conferred improved survival compared to patients who fell in the lowest 4 quintiles of ICOS and the lowest quintile of CD28. Patients were censored at the time of last clinic visit, or transplant. Statistical analysis by log-rank with Kaplan Meier survival estimation.

Table 1

Demographics.

	IPF n = 59	Control[a] n = 22
Male Sex	40 (68%)	16 (73%)
Age (years)	68 ± 7.9	65 ± 8.6
Caucasian Race	48 (81%)	19 (86%)
Baseline FVC (% predicted)	66 ± 15	
Baseline DLCO (% predicted)	47 ± 14 (n = 57)[b]	
Former Smoker	36 (61%)	
Corticosteroid Use[c]	2 (3%)	

Data are presented as mean ± standard deviation or number (%).

FVC = forced vital capacity; DLCO = diffusing capacity of the lung for carbon monoxide.

[a]Pulmonary function tests, smoking history and corticosteroid use history were not collected in the control group.

[b]Two patients in the IPF group were unable to provide DLCO data due to their inability to perform a breath holding maneuver.

[c]Includes 1 patient on 5 mg oral prednisone daily, and a second patient on 10 mg oral prednisone daily at the time of their first blood draw.

Table 2

Variables predicting survival in patients with IPF.

Characteristic	Log Rank	Unadjusted (n = 59)		Adjusted (n = 57)[a]			
	P value	HR	95% CI	*P* value	HR	95% CI	*P* value
ICOS (Mean Fluorescence Intensity)	-	0.87	0.74–1.03	0.102	0.74	0.56–0.97	0.031
CD28 Low Status	0.0047	3.34	1.38–8.09	0.008	1.42	0.36–5.61	0.61
Male Gender	0.3165	1.66	0.61–1.55	0.32	2.40	0.51–11.3	0.27
Age	-	1.09	1.02–1.16	0.012	1.08	0.99–1.17	0.055
FVC (% predicted)	-	0.99	0.96–1.02	0.45	1.03	0.99–1.06	0.12
DLCO (% predicted)	-	0.92	0.89–0.95	< 0.0001	0.91	0.86–0.96	0.001
Smoking	0.6181	1.26	0.51–3.13	0.62	1.44	0.35–6.05	0.61
Caucasian Race	0.0407	6.29	0.84–47	0.073	9.72	0.33–282	0.19
Corticosteroid Use	0.5619	1.80	0.24–13.6	0.57	66.21	1.42–3091	0.033

[a]Patients without DLCO measured were omitted.