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Plasma B Lymphocyte Stimulator and B Cell Differentiation in Idiopathic Pulmonary Fibrosis Patients

Jianmin Xue,*† Daniel J. Kass,*† Jessica Bon,* Louis Vuga,* Jiangning Tan,* Eva Csizmadia,† Leo Otterbein,† Makoto Soejima,* Marc C. Levesque,* Kevin F. Gibson,* Naftali Kaminski,* Joseph M. Pilewski,* Michael Donahoe,* Frank C. Sciurba,* and Steven R. Duncan*‡

We hypothesized B cells are involved in the pathogenesis of idiopathic pulmonary fibrosis (IPF), a progressive, restrictive lung disease that is refractory to glucocorticoids and other nonspecific therapies, and almost invariably lethal. Accordingly, we sought to identify clinically associated B cell–related abnormalities in these patients. Phenotypes of circulating B cells were characterized by flow cytometry. Intrapulmonary processes were evaluated by immunohistochemistry. Plasma B lymphocyte stimulating factor (BLyS) was assayed by ELISA. Circulating B cells of IPF subjects were more Ag differentiated, with greater plasmablast proportions (3.1 ± 0.8%) than in normal controls (1.3 ± 0.3%) (p < 0.03), and the extent of this differentiation correlated with IPF patient lung volumes (r = 0.44, p < 0.03). CD20+B cell aggregates, diffuse parenchymal and perivascular immune complexes, and complement depositions were all prevalent in IPF lungs, but much less prominent or absent in normal lungs. Plasma concentrations of BLyS, an obligate factor for B cell survival and differentiation, were significantly greater (p < 0.0001) in 110 IPF (2.05 ± 0.05 ng/ml) than among 53 normal (1.40 ± 0.3%) (hazard ratio = 4.0, 95% confidence interval = 1.8–8.7, p < 0.03). CD20+B cell aggregates, diffuse parenchymal and perivascular immune complexes, and complement depositions were all prevalent in IPF lungs, but much less prominent or absent in normal lungs. Plasma concentrations of BLyS, an obligate factor for B cell survival and differentiation, were significantly greater (p < 0.0001) in 110 IPF (2.05 ± 0.05 ng/ml) than among 53 normal (1.40 ± 0.04 ng/ml) and 90 chronic obstructive pulmonary disease subjects (1.59 ± 0.05 ng/ml). BLyS levels were uniquely correlated among IPF patients with pulmonary artery pressures (r = 0.58, p < 0.0001). The 25% of IPF subjects with the greatest BLyS values also had diminished 1-y survival (46 ± 11%), compared with those with lesser BLyS concentrations (81 ± 5%) (hazard ratio = 4.0, 95% confidence interval = 1.8–8.7, p = 0.0002). Abnormalities of B cells and BLyS are common in IPF patients, and highly associated with disease manifestations and patient outcomes. These findings have implications regarding IPF pathogenesis and illuminate the potential for novel treatment regiments that specifically target B cells in patients with this lung disease. The Journal of Immunology, 2013, 191: 2089–2095.

Idiopathic pulmonary fibrosis (IPF) is a morbid, fibroproliferative disorder characterized by progressive lung restriction and gas exchange abnormalities (1–3). The annual incidence of IPF in the United States is ∼40,000, and afflicted patients have a median survival of ∼3 y (1–3).

Although the pathogenesis of IPF is widely considered to be enigmatic (2), abnormalities of adaptive immunity are common among patients with this disease (Refs. 4–22). HLA allele frequency perturbations are a typifying feature of immunological disorders, and HLA-DRβ1*15 is overrepresented among IPF subjects (20), as well as being linked to dysregulated autoimmune responses in this population (13). Infiltrates of activated T cells are present in IPF lungs, and the magnitude of these abnormalities is proportionate to disease severity and patient mortality (19, 21, 22). Circulating T cells among IPF patients are also Ag activated, clonally expanded, dysregulated, and have augmented productions of myriad proinflammatory and profibrotic mediators, and many characteristics of these lymphocytes are associated with clinical manifestations (9, 18, 19). One or more Ags in IPF lungs stimulate autologous CD4 T cells (9), including heat shock protein 70, which induces lymphocyte proliferation and IL-4 production (13).

B cell studies in IPF are more limited, but examinations of diseased lungs from these patients have shown the presence of highly abnormal intrapulmonary B cell aggregates (4, 5) and overexpressions of Ig genes (6). Potentially pathogenic immune complexes have been found in the sera, bronchoalveolar lavage, and pulmonary parenchyma of IPF patients (7, 8, 13). Diverse circulating IgG autoantibodies are present in >80% of these subjects (8–16), and some particular Ig specificities have been linked to disease severity and/or poor prognoses (10–13).

We hypothesized that B cell characteristics and/or their associated mediators might be correlated with clinical features of IPF. If so, these findings could have considerable importance. To begin, the course of IPF is unpredictable among afflicted individuals (2, 3). Thus, facile, valid biomarkers could be very useful to identify patients who are destined for poor near-term outcomes, and thus optimize timings of lung transplantations, and/or aid in selection of high-risk subjects for experimental treatments.

Moreover, the absence of a definitive, mechanistic paradigm of IPF pathogenesis has precluded the rational selection of therapies that are directed specifically at the causal biological process(es) (2, 3). Medical treatments used for IPF to date, typically based on gluco-
Materials and Methods

Specimens for autoantibody studies

Peripheral blood specimens were obtained from consecutive IPF patients (9, 13, 19, 20), healthy volunteers, and subjects with cigarette smoking–attributable chronic obstructive pulmonary disease (COPD) and/or emphysema (30, 31), henceforth collectively denoted as COPD. Plasma was obtained from these specimens by centrifugation, aliquoted, and stored at −80°C prior to use in the B lymphocyte stimulating factor (BLyS) assays. Diagnoses of lung disease were established by expert clinicians, who analyzed all information and were blinded to the experimental laboratory tests. All IPF subjects fulfilled consensus diagnostic criteria (9, 19, 20) and had normal conventional autologous serological tests (13). COPD was diagnosed by spirometry (30), and emphysema was detected by chest computed tomography scans (31). Healthy controls were recruited from volunteers among hospital personnel or research registries. Those with tobacco smoking histories had normal spirometry and no radiographic evidence of emphysema.

Subpopulations of IPF and COPD subjects had pulmonary artery (PA) pressures (PAP) measured by right heart catheterizations during evaluations for possible lung transplantation, or other clinical indications. These catheterizations were performed by cardiologists who were independent and unaware of this study.

All subjects gave written informed consent. This study was approved by the University of Pittsburgh Institutional Review Board.

B cell phenotypes

PBMCs were isolated from venous phlebotomy specimens by density gradient centrifugation (9). B cells among the PBMCs were stained with panels of mAb and characterized by flow cytometry, as fully detailed previously (32). BLyS was quantified in plasma using BLyS ELISA kits (R&D Systems), according to manufacturer instructions. OD at 405 nm for replicate specimens were determined in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA), blanked against untreated wells.

Statistical analyses

Two- and three-group comparisons of continuous variables were made by Mann–Whitney or Kruskal-Wallis tests, respectively. Associations between continuous variables were established by linear regression. Logistic regression was used to perform multivariate analyses. Survival analyses were performed with product-limit estimation, with intergroup comparisons by log rank. Hazard ratios and 95% confidence interval were established by proportional hazard regression. The p values <0.05 were considered significant. Unless otherwise denoted, data are depicted as means ± SE.

Results

Subjects and plasma specimens

Plasma specimens for BLyS assays were collected between December 28, 2005 and December 16, 2011. Characteristics of the aggregate lung disease populations used in these studies are detailed in Table I. Normal controls (n = 53) were 63 ± 1 y old, 64% male, and 57% were former or current smokers.

B cell phenotypes

To conduct an initial evaluation for B cell abnormalities in the respective cohorts, the phenotypes of their circulating lymphocytes were prospectively determined by flow cytometry among recently recruited consecutive subjects. Characteristics of the subjects who provided these specimens are detailed in Supplemental Table I. B cell phenotype distributions among the IPF patients were abnormal compared with healthy subjects, and similar to those of the subjects with COPD (Fig. 1B), a clinically distinct lung disease in which pathogenic autoantibodies have been implicated (30).

Table I. Demographic and clinical characteristics of lung disease subjects in whom BLyS was quantified

<table>
<thead>
<tr>
<th></th>
<th>IPF</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>Age (y)</td>
<td>69 ± 1 (71, 51–87)</td>
<td>64 ± 1 (65, 47–83)*</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>73</td>
<td>44*</td>
</tr>
<tr>
<td>FVC % predicted</td>
<td>62 ± 2 (59, 25–113)</td>
<td>81 ± 2 (82, 35–136)*</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>75 ± 2 (73, 31–130)</td>
<td>53 ± 3 (54, 12–121)*</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.85 ± 0.01 (0.84, 0.71–1.22)</td>
<td>0.48 ± 0.02 (0.46, 0.15–0.81)*</td>
</tr>
<tr>
<td>DLCO % predicted</td>
<td>47 ± 2 (47, 14–110)</td>
<td>51 ± 2 (48, 12–122)</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>55</td>
<td>100*</td>
</tr>
</tbody>
</table>

Data are depicted as means ± SE, and in parentheses: (median, minimum–to–maximum values). FVC % predicted = FVC, as a percentage of predicted values; FEV1 % predicted = FEV in the first second of expiration, as a percentage of predicted values; FEV1/FVC = the ratio of FEV in the first second to FVC; DLCO % predicted = diffusing capacity for carbon monoxide, as a percentage of predicted values. Smoking history denotes subjects with ≥5 pack years of cigarette smoking.

*p < 0.0001 for nonparametric comparisons between the COPD and IPF cohorts.
The magnitude of plasmablast differentiation among the IPF subjects was inversely correlated with forced vital capacities (FVC), a measure of lung restriction (Fig. 1C). There was a trend in the COPD cohort for an analogous inverse correlation between the proportion of plasmablasts among B cells and the ratio of forced expiratory volume (FEV) in the first second of expiration to FVC (FEV1/FVC), a defining criterion of expiratory airflow obstruction ($r = 0.32$, $p = 0.09$).

Plasmablast differentiation among the COPD subjects was significantly correlated with diffusing capacities for carbon monoxide, a correlate of intrapulmonary gas exchange (Fig. 1D).

**In situ B cells**

Having found evidence of circulating B cell abnormalities in IPF patients, we performed additional studies to confirm these lymphocytes were also present within patient lungs. Focal aggregates of CD20+ B cells were present in all IPF lungs examined by immunohistochemistry ($n = 11$), typically proximate to small airways, and to a somewhat lesser extent near small blood vessels (Fig. 2A). Fewer and typically more scattered CD20+ cells were evident in normal lungs ($n = 9$) (Fig. 2B). Ki-67 was only infrequently seen among the IPF B cells (Fig. 2A).

Analogous B cell aggregates were also evident in COPD lung sections (data not shown), as previously described and illustrated (33).

**BLyS**

BLyS plasma concentrations were significantly greater among IPF subjects than in both normal and COPD controls (Fig. 3A). BLyS concentrations were highest among those IPF subjects who had PA hypertension, defined as PA mean pressure $>$ 25 mmHg with PA wedge pressure $<$ 15 mmHg, and also among those who died within 1 y of the specimen acquisitions (Fig. 3B). The BLyS levels were significantly higher among IPF subjects compared to COPD and normal controls (Fig. 3C).

**FIGURE 1.** Phenotypes of circulating B cells. (A) Cells were stained with panels of mAb and gated on CD19+, CD20+, CD3+, and IgD− B cells. Plasmablasts among these (CD2hi, CD38hi) are denoted in the upper right quadrant of this example. (B) Selected phenotypic characteristics of circulating B cells among COPD ($n = 29$), IPF ($n = 25$), and normal controls ($n = 20$). The lowest, second lowest, middle, second highest, and highest box points represent 10th, 25th, median, 75th, and 90th percentiles, respectively. Means are represented by squares. Trans refers to transitional (recent bone marrow emigrant B cell) and successively differentiated developmental stages: non-isotype switched (IgA-, IgG-) memory (nsMem), isotype (IgG) switched memory (sMEM), and efficient Ab-producing plasmablasts (PB). Absolute sMEM values are $10^3$ those depicted in this figure. The $p$ values denote Kruskal–Wallis comparisons of the three cohorts: * denotes the group that is significantly different from the other two. Note increased B cell differentiation among the disease cohorts (COPD and IPF) relative to healthy controls, and the general similarities between those two lung disease subpopulations. (C) The percentage of plasmablasts (PB) among circulating B cells was inversely correlated with FVC, as a percentage of predicted values (FVC%$p$) in IPF subjects. (D) The percentage of plasmablasts (PB) among circulating B cells was inversely correlated with DLCO%$p$ in COPD subjects.

**FIGURE 2.** Intrapulmonary CD20+ B cells. (A) Representative images show sections from normal lungs (left panel) and IPF lungs (middle panel) immunostained for CD20. These B cells are only infrequently positive for Ki-67 (right panel) (original magnification $\times 100$). (B) Direct counting confirmed CD20+ cells are more numerous in IPF compared to normal lung specimens.
values were significantly correlated with PAP among individuals with IPF (Fig. 3C). Pulmonary function tests were obtained 6.1 ± 0.3 mo in 64 of the IPF subjects (those still alive, not transplanted, or not too ill to perform these tests). BLyS concentrations in these patients tended to be inversely associated with subsequent changes in FVC ($r = 0.26$, $p = 0.04$).

To examine for possible associations of BLyS with subsequent outcomes, the IPF patients were stratified into the quartile with highest circulating concentrations of this mediator versus the 75% of subjects with lower BLyS levels. Actuarial analyses confirmed that subjects with the greatest concentrations of BLyS had worse 1-y outcomes than those subjects with lesser BLyS levels (Low). Cross-hatches and numbers in parentheses denote censored events (end of observation). Absolute mortality was also greater among IPF patients with the highest quartile plasma BLyS concentrations (High) after omission of the subpopulation that had lung transplantations during the observation interval.

There were no significant gender differences of BLyS levels among the IPF patients (2.1 ± 0.1 versus 1.9 ± 0.1 ng/ml, for males and females, respectively, $p = 0.66$), but there was a trend for greater proportions of males within the quartile of subjects with highest BLyS concentrations (Table II). Otherwise, there were no appreciable associations of the clinical/demographic parameters in Table II with levels of this mediator. None of these clinical/demographic characteristics were associated with survival independently of BLyS. PAP was also not an independent correlate of outcome in this study cohort.

To date, very few of the COPD cohort have died (precluding meaningful analyses of survival correlates). However, BLyS levels in COPD subjects did not significantly correlate with PAP ($r = 0.20$, $p = 0.21$) or measures of pulmonary function (data not shown). There were no differences of circulating BLyS concentrations between males (1.56 ± 0.08 ng/ml) and females (1.62 ± 0.06 ng/ml) among the COPD cohort. Proportions of males and females in the highest quartile of BLyS concentrations among COPD subjects were 25 and 24%, respectively.

Intrapulmonary immune complexes and complement
Given the association between BLyS and PAP, we examined lung sections for evidence of Ab-mediated processes involving pulmonary blood vessels (Fig. 4). Diffuse parenchymal immune complexes and complement depositions were much more extensive in IPF lungs compared with normals, as has been previously

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**FIGURE 3.** BLyS levels. (A) Plasma BLyS concentrations were significantly greater in IPF subjects ($n = 110$) than in either COPD ($n = 90$) or normals ($n = 53$). (B) Among the IPF cohort, BLyS levels were highest among those who died during the next year ($n = 25$), and in those who had pulmonary artery hypertension (PAH) ($n = 17$), compared to those with lesser pulmonary artery (PA) mean pressures (NI) ($n = 28$). PAH was defined as PA mean pressures >25 mmHg with PA wedge (PAW) pressures <15 mmHg. Subjects with PAW >15 were not tabulated here ($n = 5$). These five excluded subjects had PA mean pressures >25, and their BLyS concentrations were 1.92 ± 0.32 ng/ml. (C) BLyS concentrations were correlated with pulmonary artery pressures among IPF subjects.

(D) The quartile (25%) of IPF subjects with greatest BLyS levels (High) had worse outcomes than those subjects with lesser BLyS levels (Low). Cross-hatches and numbers in parentheses denote censored events (end of observation). Absolute mortality was also greater among IPF patients with the highest quartile plasma BLyS concentrations (High) after omission of the subpopulation that had lung transplantations during the observation interval.
Discussion
Ag-stimulated B cells undergo incremental maturations that result in highly differentiated lymphocytes with increased efficacy for the elaboration of avid, isotype-switched IgG Abs (34). In comparisons with healthy controls, B cell phenotypes among patients with recognized autoantibody diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE), are more differentiated, with lesser proportions of early B cells and greater relative proportions of mature lymphocytes, including autoantibody-producing plasmablasts (35–37). Moreover, the magnitude of this B cell differentiation is a biomarker for the clinical activities of these diseases (35–37). The present data show the extent of B lymphocyte differentiation among IPF subjects is similarly abnormal (Fig. 1B) and is also analogously correlated with the pulmonary function of these lung disease patients (Fig. 1C).

Other findings in this study confirm previous reports of abnormal B cell aggregates within IPF lungs (4, 5) (Fig. 2). The B cell accumulations in these specimens are predominately Ki-67 negative, and thus are almost certainly attributable to trafficking from extrapulmonary compartments rather than local proliferation. This particular finding is also congruent with a previous report (5).

BLyS, also known as B cell–activating factor, is a TNF ligand family cytokine that is produced by a variety of leukocytes, and provides essential, nonredundant signals necessary for B lymphocyte survival, maturation, and Ab production (29, 38). Circulating BLyS is increased in patients with many autoantibody-mediated diseases, including SLE and rheumatoid arthritis, and levels of this mediator among individuals with these diseases are associated with their clinical manifestations (29, 38, 39). The present data show that plasma concentrations of BLyS are similarly increased in IPF subjects, as well as also being correlated with important clinical features and prognoses of these patients (Fig. 3). PAP were measured in this study among patient subpopulations that had these determinations during evaluations for lung transplantations and/or other clinical indications (e.g., for disproportionate dyspnea). Accordingly, it is possible the cohorts with these measures were biased, which could have included an enrichment of patients with pulmonary artery hypertension. Thus, the association of BLyS with PAP may not be as rigorous in cross-sectional, unselected IPF populations. Nonetheless, the IPF subpopulation analyzed in this study also included many patients with normal PAP in whom the BLyS-PAP association was still evident (Fig. 3C). Most importantly, circulating BLyS concentrations were also highly associated with patient outcomes (Fig. 3D, 3E), and the latter analyses are not subject to selection bias.

The findings in this study are further evidence that B cell abnormalities are common in IPF subjects (4–16) and linked to clinical manifestations and mortality (10–13). Studies of B cells in IPF are ongoing, but there are several plausible mechanisms by which these lymphocytes could have pathogenic effects.

Tissue-bound Abs produced by B cells can cause cytotoxicities and promote neutrophil recruitment by the formation of Ab-Ag (immune) complexes and complement fixation (Fig. 4), and/or by activation of NK cells (17, 34, 40, 41). Observations in this study confirm previous findings of diffuse immune complex and complement depositions in IPF lungs (13), and additionally show these processes are proximally associated with intrapulmonary blood vessel pathology (Fig. 4).

Autoantibodies can also deleteriously alter target cell functions (13, 16, 42, 43) by cross-linking cell surface autoantigen-receptor complexes that transduce and enhance proinflammatory responses.
or after gaining access to intracellular autoantigens (44). Previous studies have shown that autoantibodies of IPF patients increase the production of profibrotic TGF-β by alveolar epithelia (16). Anti–heat shock protein 70 IgG isolated from these patients activates monocytes and increases their elaborations of IL-8 (13), a chemokine biomarker of IPF that has been implicated in the pathogenesis of this disease (45).

Although perhaps less widely appreciated, activated B cells per se also directly elaborate numerous cytokines and other mediators that have vasoactive, proinflammatory, and profibrotic effects (34). Intrapulmonary lymphoid aggregates are an abundant source of diverse, highly active mediators, essentially always an abnormal finding, and most likely have pathogenic consequences (5, 34, 46, 47). Lymphoid aggregates in proximity to pulmonary blood vessels are associated with anatomic and functional vascular abnormalities among IPF and other disease populations (5, 47).

Activated B cells are also efficient APCs for T lymphocytes (34). In turn, Ag (or autoantigen)-stimulated T cells produce myriad pathogenic mediators (48), and have been singularly implicated as the initiators of many disease-associated inflammatory cascades, including those that result in pathological fibrosis (41, 48, 49). Numerous T cell abnormalities have been described in IPF patients, including reactivity to autologous lung proteins and intrapulmonary autoantigens, and several characteristics and functions of these lymphocytes are correlated with clinical manifestations and/or patient outcomes (9, 13, 18, 19, 21, 22).

The present data, in conjunction with other independent evidences in IPF patients (4–22), illuminate several parallels between this lung disease and recognized autoimmune syndromes (34–39, 41, 42, 46, 49). Thus, these collective findings may be an impetus to consider innovative experimental trials for this inexorable and highly lethal syndrome. Severe autoantibody-mediated lung diseases in particular (e.g., Goodpasture’s disease, anti–Jo-1–associated interstitial lung disease, Wegener’s granulomatosis, anti–all-antigen–mediated rejection, etc.) are usually inadequately responsive or even refractory to corticosteroid-based therapy. However, these disorders are often more responsive to treatments with modalities that remove autoantibodies [e.g., plasma exchange (23)] and/or B cell–depleting agents, including rituximab (24–27), and a preliminary report (28) postulated that B cells could perhaps benefit patients afflicted with this morbid syndrome. Severe autoantibody-mediated lung diseases in particular are more responsive to treatments with modalities that remove autoimmunity (10) in sera from rapidly progressive idiopathic pulmonary fibrosis. J. Med. Invest. 48: 181–189.


Supplementary Fig 1. Optimal concentration of PKCI compounds. A. Downregulation of costimulatory molecules on human DCs treated with three PKCI compounds. PKCI-treated DCs were prepared by culturing iDCs with a maturation cocktail in the presence of 3 PKCI compounds, Bis I, Gö6983, or Ro32-0432 at various concentrations for 48 h. Surface phenotype of costimulatory molecules (CD40, CD80, and CD86) on each DCs was analyzed by flow cytometry. Values are the mean and SD of mean fluorescence intensity (MFI) of each phenotype on DCs from three experiments. *p<0.01. NS, not significant. B. Viability of human DCs treated with three PKCI compounds. Viability of each DCs was examined with trypan-blue staining. *p<0.01. NS, not significant. C. PKC kinase activity of human DCs treated with three PKCI compounds. iDCs, mDCs and PKCI-treated DCs (1 x 10^6) at various PKCI concentrations were lysed with lysis buffer and each supernatant was analyzed for PKC kinase activity. PKC kinase activity of iDCs was used as a control. Each PKC kinase activity was calculated by subtracting the PKC kinase activity of iDCs. The PKC kinase activity of PKCI-treated DCs was expressed relative to that of mDCs. *p<0.01. NS, not significant.

Supplementary Fig 2. Surface phenotype and chemotaxis of human iDCs, mDCs, and PKCI-treated DCs prepared in serum-free X-VIVO15 medium. A. Surface phenotype on iDCs, mDCs, and PKCI-treated DCs. B. CD14 expression on monocytes, iDCs, mDCs, and PKCI-treated DCs. PKCI-treated DCs were generated using X-VIVO15 medium by culturing iDCs with a maturation cocktail in the presence of PKCIs such as Bis I (10 μM), Gö6983 (10 μM), or Ro32-0432 (10 μM) for 48 h.
Surface phenotype on each DCs was analyzed by flow cytometry. Numbers in the corners indicate the mean fluorescence intensity (MFI) of expression level of each surface marker. Representative data from three experiments are shown. C. Chemotactic activity of iDCs, mDCs, and PKCI-treated DCs to CCL19. Each assay was performed in triplicate. *p<0.01. NS, not significant.

Supplementary Fig 3. Characterization of human iDCs, mDCs, and PKCI-treated DCs prepared in serum-free X-VIVO15 medium. A. Cytokine production from iDCs, mDCs, and PKCI-treated DCs. iDCs, mDCs, and PKCI-treated DCs were prepared in serum-free X-VIVO15 medium as described in Materials and Methods. These DCs (1 x 10^6/mL) were cultured in X-VIVO15 medium for assay of IL-6, IL-10, IL-12, IFN-γ, and TGF-β. The cell supernatants were harvested after 96 h and assayed. Values are the mean and SD of three experiments. *p<0.01. NS, not significant. B. Comparison of suppressive properties among iDCs, mDCs, and PKCI-treated DCs. In vitro T cell proliferation assay was performed as described in Materials and Methods. T cell proliferation by iDCs and PKCI-treated DCs was expressed relative to that by mDCs. Values are the mean and SD. Representative data from three experiments are shown. *p<0.05. **p<0.01. NS, not significant. C. Phagocytic ability of iDCs, mDCs, and PKCI-treated DCs. DCs were incubated with FITC-latex beads-rabbit IgG for 24 h, and then analyzed by flow cytometry.

Supplementary Fig 4. Comparison of downregulation of costimulatory molecules on DCs treated with the specific PKCIs. PKCI-treated DCs were prepared by culturing iDCs with a maturation cocktail in the presence of the specific PKCIs,
Gö6976 (a PKC α- and βI-specific inhibitor), CGP53353 (a PKC βII-specific inhibitor), and HBDDE (a PKC α- and γ-specific inhibitor) at various concentrations for 48 h. Bis I was used at a concentration of 10 μM. Surface phenotype of costimulatory molecules (CD40, CD80, and CD86) on each DCs was analyzed by flow cytometry. Values are the mean and SD of mean fluorescence intensity (MFI) of each phenotype on DCs from three experiments. *p<0.05 vs mDCs. **p<0.01 vs mDCs.