Regulation of PD-L1 expression is a novel facet of cyclic-AMP-mediated immunosuppression

Sasi et al.

Supplemental data

1) Materials and methods
2) Table
3) Supplemental figures legends
4) Supplemental figures

Supplemental materials and methods.

Cell lines and primary tumor samples. Human DLBCL cell lines SU-DHL2, SU-DHL6, SU-DHL10, OCI-Ly1, OCI-Ly3, RIVA and U2932 were cultured at 37°C, 5% CO2 in RPMI-1640 (Corning, Manassas, VA) medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesufonic acid (HEPES) buffer; OCI-Ly3 was cultured with 20% FBS. HEK-293T cells (Thermo-Fisher Scientific) were maintained in DMEM (Corning, Manassas, VA) with 10% FBS, supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 10 mM HEPES buffer. In a subset of experiments, the DLBCL cell lines were grown also in conditioned media culture. In these assays, after 1h exposure to forskolin (40uM), the media was removed, the cells extensively washed, and replenished with forskolin-free fresh media, which was collected 7h later and used to culture a fresh batch of DLBCL cell lines for 8h. Thus, these supernatants are forskolin-free, indicating that the induction of PD-L1 in DLBCL cell lines cultured with conditioned media are reflective of the effects of cAMP on the production and secretion of interleukins, and not from a fortuitous presence of forskolin on the conditioned media. The mouse B cell lymphoma cell line A20 was cultured in RPMI 1640 containing 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, and 50 µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). The identity of the cell lines was confirmed by STR profiling and the cell lines were regularly tested for mycoplasma contamination. All cell lines were pre-existent in the investigator's laboratory and/or were earlier obtained from ATCC or DSMZ cell banks. The PDXs were obtained from the public repository of xenografts (https://www.proxe.org/) and included one DLBCL not otherwise specified (NOS) of the ABC-like subtype (#18689), and two DLBCL with MYC and BCL2 translocation (double hit lymphomas, also known as high-grade B-cell lymphoma - HGBL) of the GCB-like molecular subtype (#20854 and # 69487). The PDXs were examined in multiple assays ex vivo and cultured in RPMI 20% FBS for 1 to 16h. Fresh de-identified primary human lymph node biopsies (n= 7, including DLBCL, MZL, MCL, SLL, LPL, FL) or reactive tonsils (n=3) were obtained from the Department of Pathology. The use of these anonymized samples

was approved by the Institutional Review Board of the University of Texas Health Science Center San Antonio. Informed consent was obtained from all subjects.

Reagents. Forskolin (Cat. no: F9929) and Roflumilast (Cat. no: sc-208313) were purchased from LC laboratories (Woburn, MA) and Santa Cruz Biotechnology (Dallas, TX), respectively. 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Cat. no: 1140) and the JAK inhibitor pyridone 6 (Cat. no: 6577) were purchased from Tocris (Minneapolis, MN). The PKA inhibitor H89 (Cat. no: B1427) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human cytokines IL-10 (Cat. No: 200-10), IL-6 (Cat. No: 200-06) and IL-8 (Cat. No: 200-08) were purchased from PeproTech (Rocky Hill, NJ). Recombinant human IFN-γ (Cat.no: 570202) was purchased from Biolegend (San Diego, CA). InVivo MAb anti-mouse PD-L1, Clone 10F.9G2, antibody (Cat. no: BE0101) and isotype control InVivo MAb rat IgG2b (Cat. no: BE0090) antibody were purchased from BioXCell (West Lebanon, NH).

Mice. The Pde4b knockout mouse (B6N;129P2-Pde4btm1Mct/Mmucd), herein referred to as Pde4b-/-, was obtained from the Mutant Mouse Regional Repository Center (MMRRC, University of California, Davis); the knockout alleles in this strain were previously transferred to a C57BL/6 background by backcrossing over 13 generations. A local colony was maintained by crossing Pde4b+/- mice; Pde4b-/- and Pde4+/+ littermate controls were sacrificed and spleen collected for downstream processing applications, including western blot, q-RT-PCR and FACS analyses. All mice were maintained in specific pathogen-free conditions. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center San Antonio.

Tumor Inoculation and Animal Studies. Six to 8-week-old female Balb/c were purchased from the Jackson Laboratory (Bar Harbor, ME). Mouse B-cell lymphoma A20 cells in exponential growth phase were washed and re-suspended in PBS and mice inoculated with 5×10^6 A20 cells by subcutaneous injection to the right flank of 60 mice, three independent cohorts of 20 mice. A conservative power calculation estimated that if a 30% decrease (with SD = 30) was detected in

the tumor size of mice receiving anti-PDL1 treatment, we would need approximately 53 mice to obtain a statistical significance, α=0.05 and (1-β)=0.80. Tumor growth was monitored with a digital caliper every day and expressed as volume (length X width^2). Mice were randomized into four treatment groups once the tumors reach approximately 100mm^2 - 5 to 8 days post inoculation. The investigators were not blind to group allocation. Roflumilast (5mg/kg body weight) or vehicle control (PBS/DMSO) were administered daily intra-peritoneally (IP). Five doses of anti-mouse PD-L1 (clone 10F.9G2) or rat IgG2b isotype control were administered IP (200µg per injection) at 2 to 3 days intervals (days 1, 3, 6, 8, 11). Mice were euthanized after 5 cycles of anti-PDL1 therapy or, as per animal protocol, when tumor volume reached 1000mm^2, whichever occur first.

FACS analysis. For human DLBCL models, APC-labeled mouse anti-human PD-L1 antibody (Clone 29E.2A3, Cat. no: 329707, Biolegend) and PE-labeled rabbit anti-human PD-L1 antibody (Clone D8T4X, Cat. no: 71391, Cell Signaling) were used. Mouse spleen cells were examined with a three-color assay, using FITC anti-mouse CD3 (Cat. no: 35-0032, Tonbo Biosciences), APC-eFluor780 anti-mouse CD19 (Cat. no: 47-0193-82, eBioscience) and APC anti-mouse PD-L1 (Clone MIH5, Cat. no: 17-5982-82, eBioscience) antibodies. To quantify and characterize the immune infiltrates in the syngeneic lymphoma microenvironment, mice were sacrificed, tumors harvested, and manually disrupted to generate single cells suspension. The following analyses were performed: 1) T-cell subpopulations with FITC anti-mouse CD3, PE anti-mouse CD4 (Cat. no: 100407, Biolegend), APC anti-mouse CD8a (Cat. no: 100711, Biolegend); 2) PDL1 expression in A20 tumor cells - APC-eFluor780 anti-mouse CD19 and APC anti-mouse PDL1. All experiments included pre-incubation with Fc blocking antibodies and the use of a viability dye; instrument compensation was performed with UltraComp eBeads™ (Cat.no: 01-2222-42, Invitrogen). Cells were acquired using a BD FACSCelesta equipped with BD FACSDiva v8.0.1. (BD Bioscience, San Jose, CA) or a Cytek Aurora (Cytek BioSciences) equipped with SpectroFlo software. Data was analyzed with FlowJo software v10.6.2 (FlowJo LLC, Ashlang, OR).

Immunoblotting analysis. Whole-cell lysates were extracted from DLBCL cell lines or primary human or murine cells, using NP40 lysis buffer. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immobilon-P polyvinylidene difluoride membrane (Cat. no: IPVH00010, Millipore, Burlington, MA). Membranes were immunoblotted at 4°C in the presence of 5% nonfat dry milk with the following antibodies: anti-PDL1 (Clone E1L3N, Cat. no: 13684), anti-phospho-STAT3 Y705 (Cat. no: 9131 or 9145), anti-phospho-STAT1 Y701 (Cat. no: 9167 or 7649), anti-STAT3 (Cat. no: 9139), anti-STAT1, (Cat. no: 9172) all from Cell Signaling Technology, Danvers, MA. Anti-mouse PDL1 (Cat. no: MAB90781), and anti-pCREB S133 (Cat. no: MAB6906) from R&D Systems, Minneapolis, MN), anti-CREB antibody (cat. no: 9197, Cell Signaling) and anti-β-Actin (Clone AC-74, Cat. no: A2228), Sigma-Aldrich, St. Louis, MO. The proteins were visualized using the SuperSignal® West Pico PLUS Chemiluminescent Substrate (Cat. no: 34580, ThermoScientific, Rockford, IL) followed manual X-ray film development or digital image capture (FluorChemR system, ProteinSimple, San Jose, CA).

RNA isolation, cDNA synthesis and q-RT-PCR. RNA was isolated from primary human lymph nodes, murine splenocytes and DLBCL cell lines using Trizol (Life Technologies, Carlsbad, CA). One microgram of RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Cat.no: 4368814, Applied Biosystems). Gene expression was measured on the QuantStudio 5 real-time PCR system (Applied Biosystems) using iTaq Universal SYBR Green Supermix (Cat.no: 1725121, Bio-Rad). The q-RT-PCRs were performed in triplicate and included a no-reverse transcriptase reaction and a no-template control (water) to test for genomic DNA amplification or contamination. Relative gene expression was calculated using the $2^{-\Delta\Delta}$Ct method. The oligonucleotide sequences are listed in Supplemental Table1.

Cyclic-AMP quantification. Intracellular cAMP levels were measured in DLBCL cell lines and HEK-293T cells following exposure to forskolin or DMSO for 1 hour using an enzyme-linked immunosorbent assay (ELISA) (Cat. no: KGE002B; R&D Systems); values were calculated against cAMP standards.

Luciferase Reporter Assay. A *PD-L1* promoter reporter construct (pGL3-PDL1-3kb) was obtained from addgene (Cambridge, MA). This construct, or the control vector pGL3basic, was co-transfected into HEK-293T cells with the pCMVβ-gal plasmid. After 18h of transfection, cells were treated with DMSO, forskolin (20μM) or IFN-γ (20ng/mL) for 6h, and IL-6 (50ng/ml) or IFN-γ (100ng/mL) for 2h. Cells were harvested, and luciferase and beta-galactosidase quantified.

Chemokine array profiling and cytokine quantification. DLBCL cell line SU-DHL2 (1×10^6/mL) was treated with DMSO or forskolin (40 μM) for 8h and supernatant obtained and used for the qualitative and semi-quantitative detection of 105 cytokines and chemokines included in the Proteome Profiler Human XL Cytokine Array Kit (Cat. no: ARY022B, R&D Systems), according to the manufacture's instruction. For specific detection of IL-6, IL-8 and IL-10 in SU-DHL2, OCI-Ly3, RIVA and U2932, quantikine ELISA kits were utilized (Cat. nos: D100B, D6050 and D8000C, R&D systems). The values were plotted into a standard curve generated with recombinant human IL-10, IL-6 or IL-8.

IL-10, IL-8 and IL-6 signal blocking. To study the functional role of IL-10, IL-8 and IL-6 on cAMP mediated PDL1 expression, IL-10, IL-8 or IL-6 signal was blocked following forskolin treatment in SU-DHL2 cell line. In brief, cells were exposed to DMSO, or concomitantly to forskolin (20-40uM for 4-8h) with isotype antibody, forskolin with anti-IL-10/anti-IL-10R (Cat. no: 16-7108-81, eBiosciences, Cat. no: 556012, BD Pharmingen), forskolin with anti-CXCR2 antibody (Cat. no: 89254, Abcam), and forskolin with anti-IL-6/anti-IL6R (Cat.no: AHR0061, clone B-R6, eBiosciences and Cat. no: MAB206, R&D Systems).

Cell Proliferation Assay. Mouse lymphoma A20 (2×10^4) cell were seeded in 96-well plates and treated with forskolin (40 μM) or roflumilast (10 μM) or their combination. CellTiter Proliferation assay was done using tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega).

Statistical analysis. *P* values were calculated with one-way ANOVA with Tukey's multiple comparisons test, or Student's *t*-test (two-tailed, equal variance). The Kaplan-Meir method was used to estimate the survival probability, and the log-rank (Mantel-Cox) test was used to compare the survival curves. Equal variance was calculated with an F-test (t-test) or with a Bartlett's statistics for equal variances (ANOVA). No samples or mice were excluded from statistical analysis. Statistical analyses were performed with the GraphPad Prism 8 software. *P* value ≤0.05 was considered significant

Supplemental Table 1 – Oligonucleotides sequences:

Name	Forward	Reverse
PDL1qRT-PCR-Human	CCTGCAGGGCATTCCAGAAA	TAGGTCCTTGGGAACCGTGA
IL-10 qRT-PCR-Human	GAGTCCTTGCTGGAGGACTTT	CGCCTTGATGTCTGGGTCTT
IL-6 qRT-PCR-Human	AGGCACTGGCAGAAAACAACCTG	TCTGCACAGCTCTGGCTTGTTC
IL-8 qRT-PCR-Human	GCCTTCCTGATTTCTGCAGCTC	TCCACTCTCAATCACTCTCAGTTC
TBP qRT-PCR-Human	TATAATCCCAAGCGGTTTGCTGCG	AATTGTTGGTGGGTGAGCACAAGG
PDL1 qRT-PCR-Mouse	TCAGATCACAGACGTCAAGCTG	TGGATCCACGGAAATTCTCTG
IL-10 qRT-PCR-Mouse	TGAAGACCCTCAGGATGCG	TCATGGCCTTGTAGACACCT
IL-6 qRT-PCR-Mouse	GACCTGTCTATACCACTTCACAAG	CCAGTTTGGTAGCATCCATCA
IL-8 qRT-PCR-Mouse	GCTACGATGTCTGTGTATTCAGGA	TCACCCATGGAGCATCAGGAT
TBP qRT-PCR-Mouse	CTGGAATTGTACCGCAGCTT	CAGTTGTCCGTGGCTCTCTT

Supplemental Figures legends:

Supplemental Figure 1. Cyclic-AMP signaling and PD-L1 expression in human DLBCL models. A) Quantification of intra-cellular cAMP levels in five ABC-DLBCL cell lines/PDX following 1h incubation with DMSO (ctrl) or forskolin (Frsk, 40μM). Data shown are mean ±SD of two biological replicates, cell lines, or three technical replicates, PDX. **B)** FACS analysis of cell surface expression of PD-L1 in ABC-like DLBCL cell line and PDX models following incubation with DMSO or forskolin (Frsk 20-40μM, 16h) – triplicate values used for MFI values in Fig. 1A. **C)** FACS analysis of cell surface expression of PD-L1 in GCB-like DLBCL cell line and PDX models following incubation with DMSO or forskolin (Frsk 40μM, 16h) **D)** Western blot (WB) analysis of PD-L1 expression in GCB-like DLBCL cell line and PDX models following incubation with DMSO or forskolin (Frsk 40μM, 16h). **E)** Quantification of intra-cellular cAMP levels in five GCB-DLBCL cell lines/PDXs following 1h incubation with DMSO (ctrl) or forskolin (Frsk, 40μM). Data shown are mean ±SD of three biological replicates. **F)** WB of phospho-CREB (S133) in GCB-DLBCL cell lines exposed to DMSO or forskolin (40μM, 4h). **G)** WB analysis of differential baseline PD-L1 expression in ABC-DLBCL cell lines. In panel **E**), *p<0.05, **p<0.01, two-sided Student's t-test.

Supplemental Figure 2. **Characterization of cAMP effects on the *PD-L1* promoter**. **Top.** Diagrammatic display of the PD-L1 promoter reporter construct – indicated are the putative CRE sites. **Bottom, left.** Quantification of luciferase activity in HEK-293T cells transfected with pGL3 basic or pGL3-*PD-L1* plasmids and exposed to DMSO, forskolin (20μM), or IFN-γ (20ng/mL) for 6h. Data shown are mean ±SD of three biological normalized by β-gal activity; ***p<0.001 two-sided Student's t-test. **Bottom, middle**. Quantification of intra-cellular cAMP levels in HEK-293T following 1h incubation with DMSO (ctrl) or forskolin (Frsk, 20μM). **Bottom, right.** WB of pCREB (S133) in HEK-293T after 1h, 2h or 6h incubation with DMSO or forskolin (Frsk, 20μM).

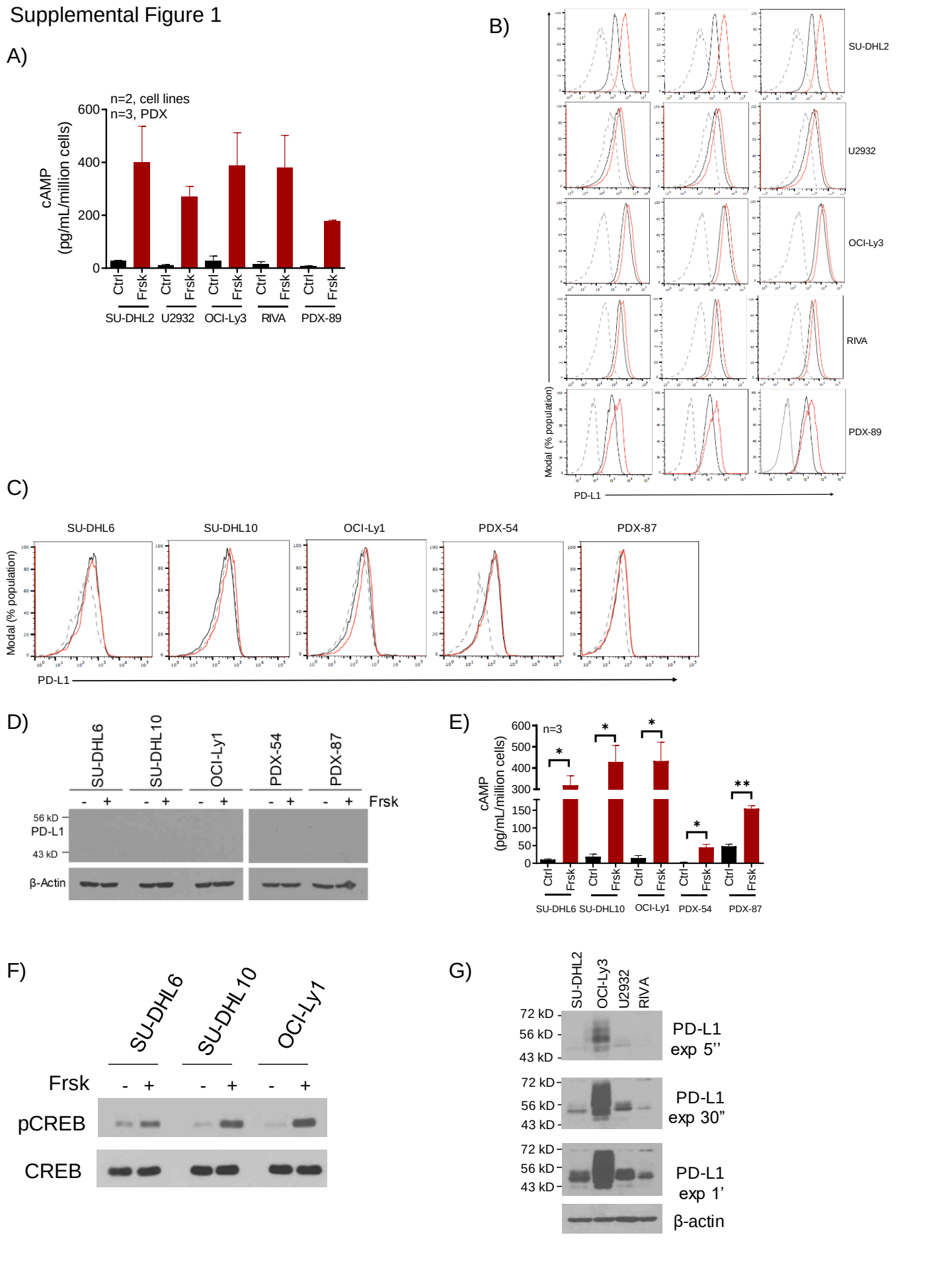
Supplemental Figure 3. Cyclic-AMP induces the expression and secretion of multiple cytokines that increase PD-L1 levels. A) q-RT-PCR quantification of *IL-10, IL-6* and *IL-8* in ABC-DLBCL cell models exposed to DMSO or forskolin (40μM) for 8h. Data shown are mean ±SD of triplicates.

*p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 – two-sided Student's t-test. **B)** q-RT-PCR quantification of *IL-10 , IL-6* and *IL-8* in the SU-DHL2 (IL-10 , IL-6) and RIVA (IL-8) DLBCL cell lines exposed to DMSO, forskolin (40μM) or forskolin + H-89 (20μM) for 4h. Data shown are mean ±SD of triplicates. ** p<0.01, **** p<0.0001 two-sided Student's t-test. **C) Top** - WB analysis of phospho (Y705) and total STAT3, and phospho (Y701) and total STAT1 in HEK-293T cells exposed to IL-10 (50ng/ml), IL-6 (50ng/ml), IL-8 (50ng/ml), or IFN-γ (100ng/mL) for 30 minutes and 1h. **Bottom -** luciferase activity in HEK-293T cells transfected with pGL3 basic or pGL3-*PD-L1* plasmids and exposed to vehicle, IL-6 (50ng/ml), or IFN-γ (100ng/mL) for 2h. Data shown are mean ±SD of three replicates normalized by β-gal activity; ***p<0.001, **** p<0.0001, two-sided Student's t-test **D)** q-RT-PCR quantification of *IL-10* (left) or *IL-6* (right) in GCB- or ABC-DLBCL models (cell lines and PDXs) exposed to DMSO or forskolin (40μM) for 4h. Each data point is the mean of quantifications performed in triplicate for each of the 10 models examined; mean ±SEM are shown. **E)** WB analysis of pSTAT3 (Y705) and STAT3 in GCB-DLBCL cell lines and PDX models following incubation with DMSO (-) or forskolin (40μM, 4h). **F)** WB analysis of pSTAT1 (Y701) and PD-L1 in GCB-DLBCL models exposed to IFNγ (100ng/ml) for 4h. ABC-type PDX-89 is a positive control for PD-L1 induction with IFNγ**. G)** Spearman correlation between *PD-L1* (*CD274*) and *IL-10*, *IL-6* and *IL-8* in non-small cell lung cancer, colon rectal adenocarcinoma, and melanoma. Spearman correlation between *PD-L1* (*CD274*) and *IL-10*, *IL-6, IL-6R* and *ILST (gp130)* in DLBCL. Data are form the TCGA collection and analyzed in the cBioPortal platform (https://www.cbioportal.org/).

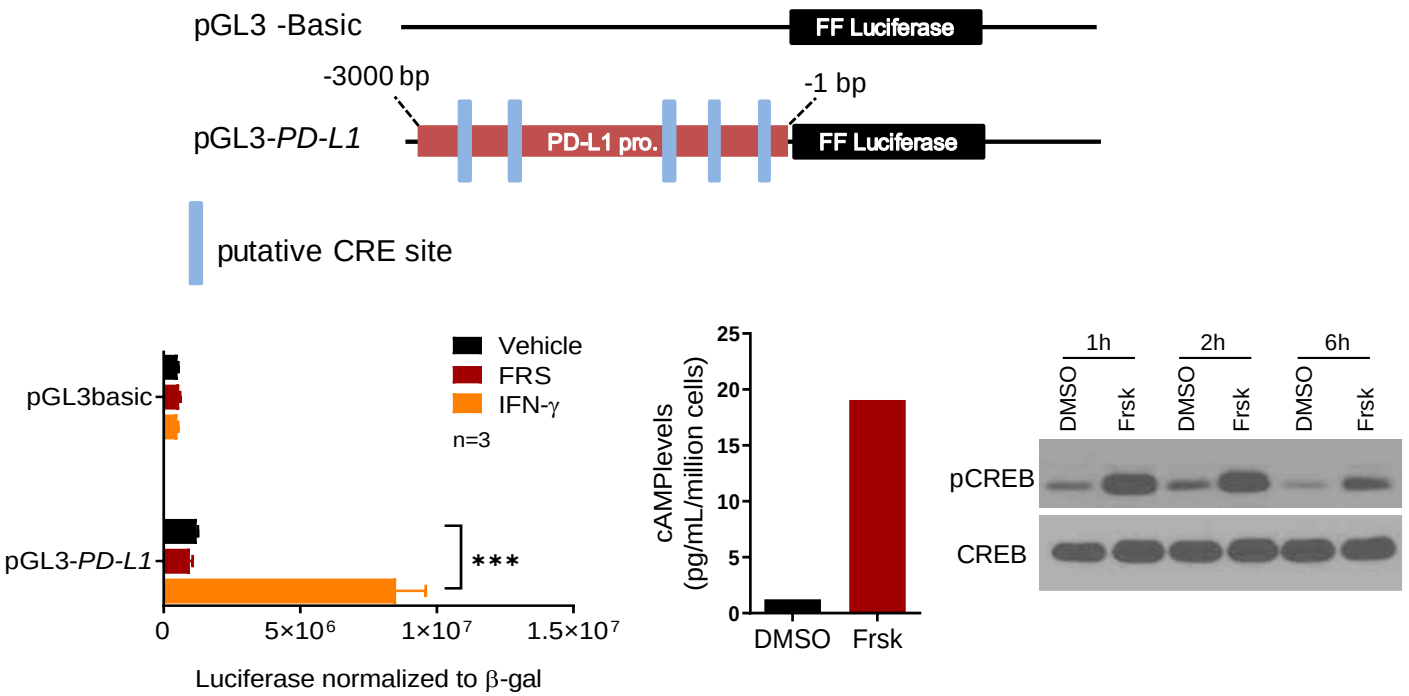
Supplemental Figure 4. Combination of PDE4 inhibition and checkpoint blockade in a syngeneic murine model of B cell lymphoma. A) Cell proliferation assay (MTS) of A20 cells following exposure to DMSO, forskolin (40μM), roflumilast (10μM) or their combination for 72h. Data are mean ±SD of two biological replicates, each quantified in technical triplicates. **B)** Survival curves (Kaplan-Meir survival probability) of tumor-bearing mice treated with vehicle control, roflumilast, anti-mouse PD-L1, or their combination (n=60 total, 15/treatment arm). P value is from log-rank (Mantel-Cox) test **C)** FACS analysis of CD4 expression in tumors harvested at the end of the therapeutic trial (n=47). Data shown are mean ±SEM of the percentage of CD4+ cell within the

CD3+ gate in each treatment arm, *p<0.05, ** p<0.01, - two-sided Student's t-test. **C)** FACS analysis of CD8 expression in tumors harvested at the end of the therapeutic trial (n=47). Data shown are mean ±SEM of the percentage of CD8+ cell within the CD3+ gate in each treatment arm, *p<0.05, two-sided Student's t-test. **D)** Mean fluorescence intensity (MFI) of PD-L1 in A20 lymphoma cells collected from Balb/c mice at the end of the therapeutic trial therapeutic trial (n=17). Data shown are mean ±SD, *p<0.05, ** p<0.01, one-way ANOVA with Tukey's multiple comparisons test.

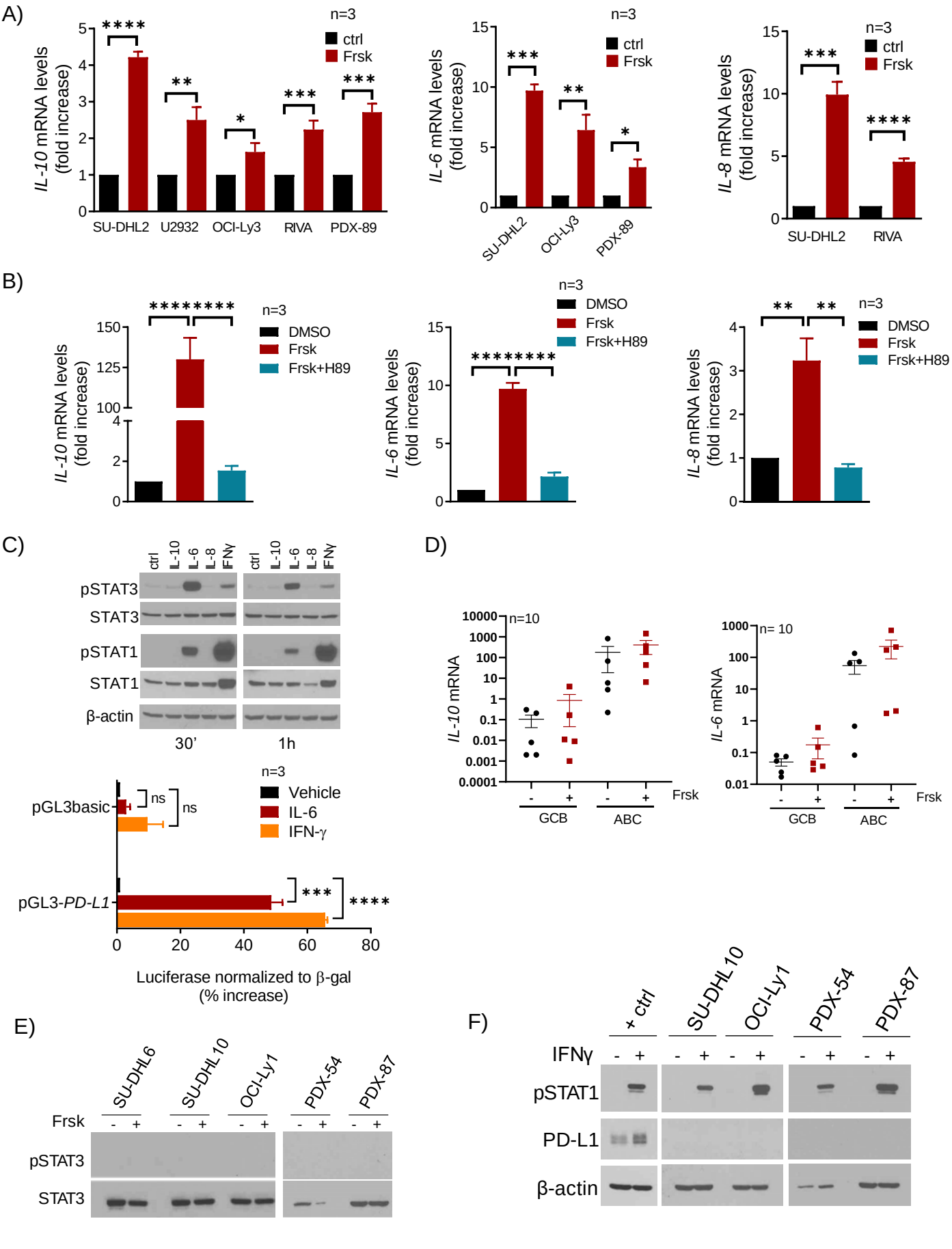
Supplemental Figure 1



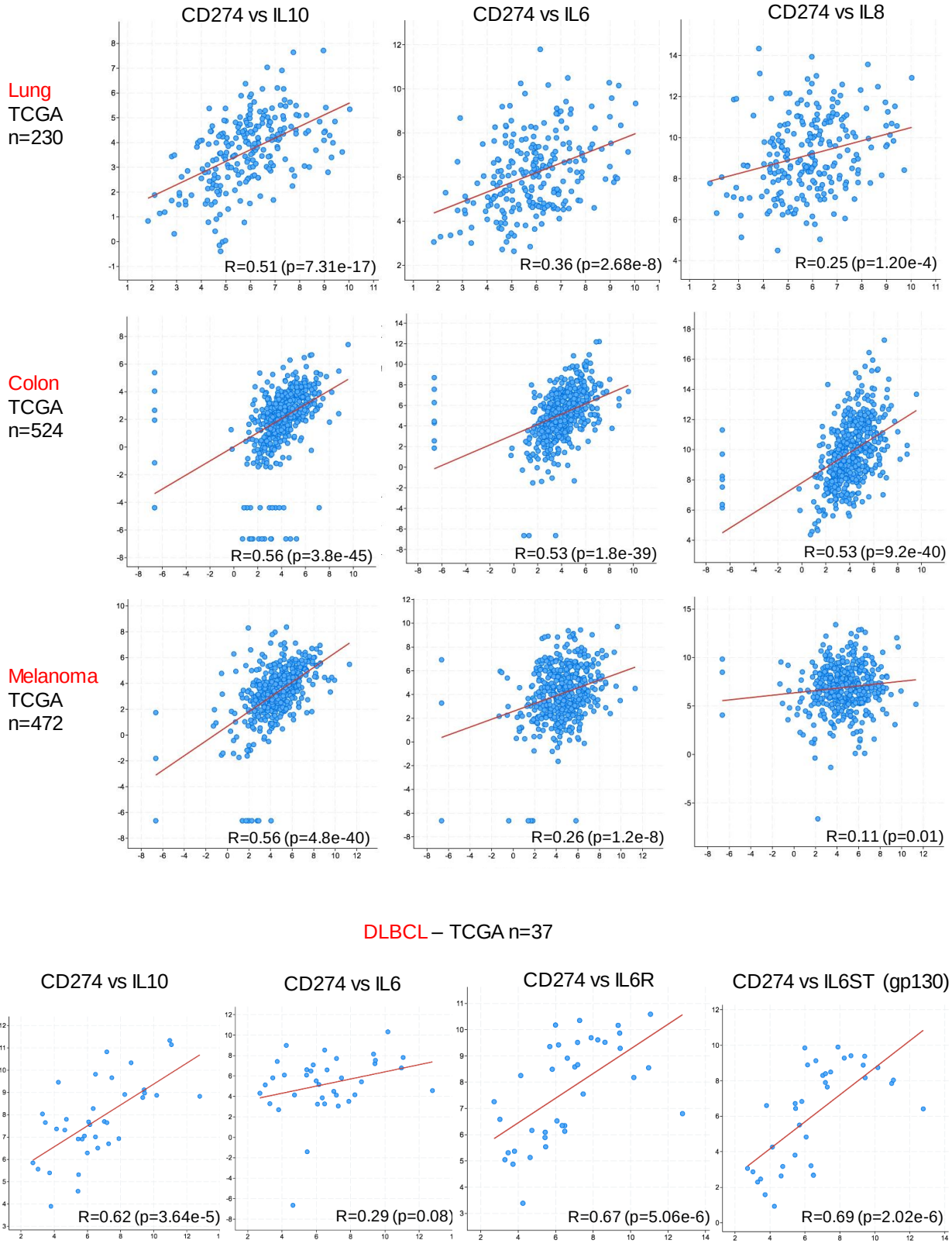
Supplemental Figure 2



Supplemental Figure 3



G)


CD274 vs IL10
CD274 vs IL6
CD274 vs IL8
Lung
TCGA
n=230
R=0.51 (p=7.31e-17)
R=0.36 (p=2.68e-8)
R=0.25 (p=1.20e-4)
Colon
TCGA
n=524
R=0.56 (p=3.8e-45)
R=0.53 (p=1.8e-39)
R=0.53 (p=9.2e-40)
Melanoma
TCGA
n=472
R=0.56 (p=4.8e-40)
R=0.26 (p=1.2e-8)
R=0.11 (p=0.01)
DLBCL – TCGA n=37
CD274 vs IL10
CD274 vs IL6
CD274 vs IL6R
CD274 vs IL6ST (gp130)
R=0.62 (p=3.64e-5)
R=0.29 (p=0.08)
R=0.67 (p=5.06e-6)
R=0.69 (p=2.02e-6)

Supplemental Figure 4