Research Article

Cancer Immunology Research

Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation

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Abstract

Tumor-associated macrophages (TAMs) express programmed cell death ligand 1 (PD-L1) and contribute to the immune-suppressive tumor microenvironment. Although the role of the PD-L1 and PD-1 interaction to regulate T-cell suppression is established, less is known about PD-L1 signaling in macrophages and how these signals may affect the function of TAMs. We used *in vitro* and *in vivo* models to investigate PD-L1 signaling in macrophages and the effects of PD-L1 antibody treatment on TAM responses. Treatment of mouse and human macrophages with PD-L1 antibodies increased spontaneous macrophage proliferation, survival, and activation (costimulatory molecule expression, cytokine production). Similar changes were observed in macrophages incubated with soluble CD80 and soluble PD-1, and in PD-L1$^{-/-}$ macrophages. Macrophage treatment with PD-L1 antibodies upregulated mTOR pathway activity, and RNAseq analysis revealed upregulation of multiple macrophage inflammatory pathways. *In vivo*, treatment with PD-L1 antibody resulted in increased tumor infiltration with activated macrophages. In tumor-bearing RAG$^{-/-}$ mice, upregulated costimulatory molecule expression by TAMs and reduced tumor growth were observed. Combined PD-1/ PD-L1 antibody treatment of animals with established B16 melanomas cured half of the treated mice, whereas treatment with single antibodies had little therapeutic effect. These findings indicate that PD-L1 delivers a constitutive negative signal to macrophages, resulting in an immune-suppressive cell phenotype. Treatment with PD-L1 antibodies reverses this phenotype and triggers macrophage-mediated antitumor activity, suggesting a distinct effect of PD-L1, but not PD-1, antibody treatment. *Cancer Immunol Res; 6(10); 1260–73.* ©*2018 AACR.*

Introduction

Immune checkpoint molecules maintain self-tolerance and prevent uncontrolled inflammation. However, expression of these molecules is often dysregulated in the tumor microenvironment (TME). This includes overexpression of inhibitory checkpoint molecules such as programmed cell death ligand 1 (PD-L1), which leads to suppression of T-cell activation and effector functions and interferes with T-cell control of tumors. PD-1 and its ligand, PD-L1, are inhibitory checkpoint molecules that suppress tumor immunity. Thus, interrupting the PD-1/PD-L1 signaling pathway with therapeutic antibodies can activate T-cell responses to tumors. For example, PD-L1 antibody treatment increases T-cell infiltration and IFNγ production and decreases tumor growth in mouse models (1). Blockade of either molecule with therapeutic antibodies induces antitumor responses in patients with cancer with several tumor types (2–5). Adverse effects observed in clinical trials were generally mild, including fatigue, diarrhea, and decreased appetite. Grade 3 adverse effects were occasionally reported, including hypothyroidism and liver abnormalities that were managed by steroids.

Studies have examined the role that PD-L1 signaling may play in regulating tumor growth. For example, PD-L1 signaling has been shown to promote the epithelial-to-mesenchymal transition in several tumor types (6, 7). Conversely, downregulation of PD-L1 expression was associated with decreased tumor metastasis (6, 7). Other groups have reported that PD-L1 acts as an anti-apoptotic receptor in response to Fas ligation, and that PD-L1 has been associated with cancer stem cell proliferation (8, 9).

PD-L1 signaling also regulates cellular functions in tumor cells. Cell metabolism is regulated by PD-L1 through Akt/mTOR phosphorylation, and PD-L1 blockade or knockdown results in decreased glycolysis, suggesting that PD-L1 signals constitutively in tumor cells and that this activity may be blocked by therapeutic PD-L1 antibodies (10). Increased ERK- and mTOR-mediated proliferation and survival of tumor cells has been reported following ligation of PD-L1 with PD-1 or when in culture with PD-1-expressing T cells (11, 12). Ligation of PD-L1 on dendritic cells using soluble PD-1 downregulated maturation-associated markers and a murine macrophage cell line showed greater immune-suppressive phenotype following PD-L1 antibody treatment (13, 14). Little is known, however, regarding PD-L1 signaling in macrophages, especially tumor-associated macrophages (TAMs). The density of PD-L1–expressing macrophages in tumors is predictive of the efficacy of both PD-1 and PD-L1 antibody therapy (15–17). In some cases, response rates as high as 80% have been observed in patients where at least 10% of tumor macrophages express PD-L1 (15).

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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The goal of the present study was to investigate the role that PD-L1 expression plays in regulating biological functions of macrophages, particularly TAMs. TAMs express PD-L1, and we reported that TAM PD-L1 expression was regulated by locally produced TNFα (18). In a screen of several different tumors in humans, PD-L1–expressing macrophages were more abundant than PD-L1–expressing tumor cells (15). Other studies found that PD-L1 antibody treatment induced antitumor activity even in models where PD-L1 was not expressed by the tumor cells themselves, suggesting that PD-L1 expression by macrophages may be a key element driving response to PD-L1 antibody treatment (16, 17). Therefore, we conducted *in vitro* studies to assess the impact of PD-L1 antibody treatment on macrophage survival and activation. We also conducted gene expression profiling of PD-L1 antibody–treated macrophages to elucidate signaling pathways. The effects of PD-L1 antibody treatment on TAM populations were then assessed in mouse tumor models, and other studies examined the effects of combined PD-1 and PD-L1 antibody treatment, based on the expectation that the two antibodies may elicit nonredundant antitumor activity. These studies indicate a constitutive negative signaling role for PD-L1 in both mouse and human macrophages, which was reversed by treatment with therapeutic PD-L1 antibodies. These findings have implications for understanding the effects of checkpoint molecule blockade with PD-L1 antibodies, and for selecting optimal combinations of checkpoint targeted antibodies.

Materials and Methods

Mice

Wild-type C57Bl/6 mice and Rag1^{tm1Mom}/J mice were purchased from The Jackson Laboratories and cared for in accordance with institutional and NIH guidelines. Mice were inoculated subcutaneously in the flank with 1×10^5 B16.F10 melanoma cells or PyMT breast carcinoma cells. On day 7, B16-inoculated mice were randomly placed into 3 treatment groups and tumor take between the groups was verified to be statistically similar. They received intraperitoneal injections of PBS, 250 μg isotype (Bio X Cell, BE0090) or PD-L1 antibody (Bio X Cell, BE0101) in a volume of 100 μL every 3 days for 9 days. Treatment for PyMT-inoculated Rag$^{-/-}$ mice started on day 18, and mice received injections of PBS, 100 μg isotype or PD-L1 antibody every 3 days for 9 days. Mice were euthanized when the first tumors reached a size of 15 mm in diameter. For combination blockade of PD-1 and PD-L1, mice were inoculated similarly and randomized on day 7. They received intraperitoneal injections of 100 μg PD-1 antibody (Bio X Cell, 0146), 100 μg PD-L1 antibody, or 100 μg PD-1 plus 100 μg PD-L1 antibodies every 3 days for 9 days then once a week for a total of 68 days. Mice were euthanized individually as the tumors reached 15 mm in diameter for a survival study, and tumor area was calculated as length × width. Remaining mice were euthanized at 68 days and verified to be tumor free. All animal studies were conducted in accordance with an Institutional Animal Care and Use Committee protocol approved by Colorado State University.

Monocyte isolation and macrophage culture

Tibias and femurs were collected from healthy mice and bone marrow cells were harvested as described previously (18). Tissues from PD-L1$^{-/-}$ C57Bl/6 mice were a kind gift from Raphael Nemenoff and Howard Li (University of Colorado Denver, Aurora, CO). Bone marrow cells were cultured in PermaLife bags (OriGen Biomedical, PL30) with 10 ng/mL rM-CSF (R&D Systems, 416-ML-010) for 1 week. A total of 50,000 M-CSF-cultured macrophages were washed with medium to remove M-CSF and plated in 96-well polystyrene cell culture plates (Corning, 353072) for 48 hours with or without treatment before use in experiments. Adherent bone marrow–derived macrophages were then harvested by pipetting with 2 mmol/L EDTA (Thermo Fisher Scientific, 15575020) in PBS.

For enrichment of human macrophages, monocytes were selected from peripheral blood mononuclear cells by adherence overnight and cultured for one week in 10 ng/mL rhM-CSF (PeproTech, 300-25).

Reagents for treatment of bone marrow–derived macrophages

PD-L1 antibody (Bio X Cell, BE0101) and irrelevant isotype-matched control antibody (Bio X Cell, BE0090) were used at 100 μg/mL, which represents the reported minimum desired plasma antibody concentration for patients treated with PD-L1 antibody (15). These antibodies were verified by the manufacturer to contain less than 0.002 EU/μg of endotoxin. A rat anti-mouse CD11b antibody (eBioscience, 16-0112-82) was used as a macrophage-specific antibody, but with a function unrelated to that of PD-L1. For human macrophages, PD-L1 antibody (BioLegend, 329702) and irrelevant isotype-matched control antibody (BioLegend, 400302) were used at 200 μg/mL. Atezolizumab (Tecentriq, NDC 50242-917-01) and control human IgG were also used at 200 μg/mL.

Recombinant mouse-soluble PD-1 chimera protein with a human IgG1 Fc portion was obtained from R&D Systems (1021-PD) and used at 100 μg/mL to match the PD-L1 antibody concentration. Recombinant mouse-soluble CD80 chimeric protein with a human Fc portion was obtained from BioLegend (555406) and used at 100 μg/mL. Donkey anti-human IgG Fc from Jackson ImmunoResearch (709-005-098) was used at 10 μg/mL to crosslink sPD-1 and sCD80, according to the manufacturer's suggestions. CD16/CD32 Fc blocking antibody (eBioscience, 16-0161-85) was used at 100 μg/mL to match the PD-L1 antibody concentration. LPS was purchased at Sigma-Aldrich (L2630) and used at 1 ng/mL to stimulate macrophages as a control.

Rapamycin (Sigma-Aldrich, R0395) was used at 10 μg/mL as an indirect inhibitor of mTOR signaling and Torin2 (Sigma-Aldrich, SML1224) was used at 0.15 μmol/L as a direct mTOR inhibitor (19, 20). IFNγ and TNFα from PeproTech (315-05 and 315-01A) were used to stimulate macrophages at 10 ng/mL.

Tumor cell line

B16.F10 melanoma cells and PyMT breast carcinoma cells were obtained from ATCC and cultured in ATCC-recommended media for up to several weeks per frozen aliquot. Cells were screened by PCR to ascertain that they were of murine origin but were not authenticated in the past year. PCR was also used to verify that the cells were free of *mycoplasma* contamination.

Tissue preparation for flow cytometry

Tumor tissues were processed to a single cell suspension, as described previously (18). Cells were immunostained using the following antibodies: Invitrogen: CD45 Pacific Orange (MCD4530), eBioscience: PD-L1 PE (12-5982-81), CD11b Pacific Blue (48-0112-82), CD86 PE (12-0862-83), and MHCII APC

(17-5321-81), Abd Serotec: F4-80 APC (MCA497A647), BD Pharmingen: Ly6C biotin (557359) and Ly6G FITC (551460). Cells were also stained with appropriately matched isotype antibodies to ensure specificity of immunostaining.

To quantitate intracellular cytokine production, macrophages were treated with protein secretion inhibitor Brefeldin (BioLegend, 420601) for 4 hours prior to staining. The cells were fixed with 4% PFA (Affymetrix, 19943) and permeabilized with 0.25% Saponin in FACS buffer (1% BSA in PBS with 0.05% sodium azide) prior to immunostaining for IL12 PE (BD Pharmingen, 554479) and TNFα PE (eBioscience, 12-7321-81). Antibodies from Cell Signaling Technologies were used to stain for intracellular levels of mTOR (2972S) and p-mTOR (2971S), and secondary donkey anti-rabbit IgG FITC (Jackson, 711-546-152) was used to detect primary antibody binding.

Human macrophages were immunostained with CD14 APC (Bio-Rad MCA1568A647), CD40 PE (eBioscience, 12-0409-41), and CD86 FITC (BD Pharmingen, 560958). Matched isotype control antibodies were also used to ensure specificity of immunostaining.

Immunofluorescent tissue staining and imaging

Tumor tissues were prepared for immunofluorescent staining as described previously (18). Antibodies for F4-80 (AbD Serotec, MCA497A647) and MHC class II (eBioscience, 12-5321-82) were used. Controls included immunostaining with appropriate concentrations of irrelevant isotype-matched antibodies.

Proliferation assay

A Click-iT assay kit was used to measure proliferation of macrophages (Thermo Fisher Scientific, C10420). EdU was added the same day as PD-L1 antibody treatment to be incorporated into proliferating cells for 48 hours prior to analysis. Cells were then detached, stained, and proliferation was evaluated by flow cytometry or immunofluorescent imaging as described above.

Western blotting

A standard Western blotting protocol from Bio-Rad was followed. Briefly, macrophages were treated with medium, irrelevant isotype antibody, or PD-L1 antibody for 5 hours. Samples were prepared under nonreducing, denaturing conditions and 6 μg total protein was loaded into a gel (Bio-Rad, 4561084). Antibodies from Cell Signaling Technologies for Akt (4691S) and pAkt (4060S) were used to probe for their respectable proteins, followed by peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson, 111-035-033). An antibody for β-actin (Sigma, A5441) was used as a loading control, followed by donkey anti-mouse secondary antibody (Jackson, 715-035-150). Densitometric analysis to quantify band area was completed using ImageJ software and values were normalized to β-actin.

RNAseq analysis

Sample collection. Bone marrow was collected from 9 mice and cultured as described above. Macrophages were treated with medium only, irrelevant isotype or PD-L1 antibody antibodies for 24 hours, and total RNA was extracted using RNeasy micro Kit (Qiagen, 74104). RNA was submitted to Novogene for RNA sequencing (RNAseq).

Quality control (QC) of RNA. Samples were tested for quality control by Agilent 2100 Bioanalyzer system and by agarose gel electrophoresis. Sample RNA integrity number ranged from 7 to 8.9.

cDNA library construction and QC. mRNA was enriched using oligo(dT) beads and fragmented randomly, then cDNA was synthesized by mRNA template and random hexamers primer, after which a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H, and DNA polymerase I were added to initiate the second-strand synthesis. After a series of terminal repair, a ligation, and sequencing adaptor ligation, the double-stranded cDNA library was completed through size selection and PCR enrichment. The cDNA library was quantified by Agilent 2100 to test the insert size to ensure concentration was over 2 nmol/L. qPCR was also used to quantify to a greater accuracy.

RNA sequencing. Libraries were run on Illumina PE150 (HiSeq) with 250 to 300 bp insert cDNA library for 20M raw reads/sample. Sequence data have been deposited in the Gene Expression Omnibus with the accession code GSE116564.

Analysis. Raw data were filtered by removing reads containing adapters and reads containing $N > 10\%$ by Novogene, Phred score >30. The filtered reads obtained by RNAseq from Novogene were analyzed using Partek Flow software, version 6.0. Filtered reads were aligned with STAR pipeline aligned against RefSeq Transcripts 83-2017-11-01 mouse whole genome. Counts were calculated using Partek E/M. Transcript counts were normalized using Partek total count method per sample and then an offset of 0.0001 was added to avoid zero counts. Normalized transcript counts were used to generate PCA plots. To detect differentially expressed genes, ANOVA was performed on the normalized transcript counts. Further biological interpretations including gene ontology enrichment and pathway analysis were then performed. Functional analysis was generated using Ingenuity pathway analysis (IPA, Qiagen Inc.) Version 01-12. IPA analysis settings included standard filters for molecules and relationships in mouse (provided in IPA) and experimentally observed confidence, prior to filtering for tissue and primary cells.

Statistical analysis

Statistical comparisons between those data sets with two treatment groups were done using nonparametric t tests (Mann–Whitney test). Comparisons between 3 or more groups were done using ANOVA, followed by Tukey multiple means posttest. Analyses were done using Prism7 software (GraphPad) and statistical significance was determined for $P < 0.05$. Statistical comparisons for survival analysis were done with Kaplan–Meier and log-rank (Mantel–Cox) tests, with statistical significance determined as $P < 0.01$. For RNAseq analysis, ANOVA was performed on normalized transcript counts and differentially expressed genes were filtered using P value with false discovery rate ≤ 0.05 and fold change ≤ -2 or ≥ 2.

Results

Macrophage proliferation and hypertrophy following PD-L1 antibody treatment

To examine the effects of PD-L1 antibody on macrophage phenotype and function, *in vitro* CSF-1 generated bone marrow macrophages (mice) and monocyte-derived macrophages (human) were utilized because these macrophages express high

levels of PD-L1 (Supplementary Fig. S1A). We found that macrophages in our culture system did not express detectable PD-1 (Supplementary Fig. S1B). However, we observed that cultured bone marrow macrophages expressed membrane-bound CD80 (Supplementary Fig. S1C). We observed that macrophages cultured in the presence of PD-L1 antibody were more numerous and larger than untreated macrophages or macrophages treated with control antibodies (Fig. 1A).

To quantify the increase in macrophage numbers and size following PD-L1 antibody treatment, we assessed macrophage proliferation (Fig. 1B). Macrophage proliferation in the presence of PD-L1 antibody was significantly increased compared with untreated and irrelevant antibody–treated cells (Fig. 1C and D). Live-cell measurements of macrophages cultured with PD-L1 antibody demonstrated that macrophage size and numbers increased significantly over time (Fig. 1E and F). Increased macrophage survival was demonstrated using an MTT assay (Fig. 1G). We concluded that PD-L1 antibody treatment increased macrophage proliferation, survival, and size.

PD-L1 antibody treatment induces macrophage activation

We next determined whether PD-L1 antibody treatment produced macrophage activation. Compared with isotype antibody–treated macrophages, PD-L1 antibody treatment significantly upregulated expression of the costimulatory molecules CD86 and MHC II, consistent with macrophage activation (Fig. 2A). This effect was titratable with increasing concentrations of PD-L1 antibody (Fig. 2B). Macrophage changes were first apparent after 48 hours of treatment (Fig. 2C). PD-L1 antibody–treated macrophages also increased production of TNFα and IL12 (Fig. 2D), consistent with PD-L1 antibody treatment inducing production of inflammatory macrophages.

Similar changes were observed following PD-L1 antibody treatment of human macrophages (Supplementary Fig. S2A and S2B). For example, the human therapeutic PD-L1 antibody atezolizumab (Tecentriq) mediated changes in macrophage survival, whereas a noncommercial PD-L1 antibody exerted significant but less pronounced activity (Supplementary Fig. S2C and S2D). Treatment with the noncommercial PD-L1 antibody upregulated expression of costimulatory molecules CD40 and CD86 (Supplementary Fig. S2E), whereas atezolizumab did not (Supplementary Fig. S2F).

Soluble CD80 more effective than soluble PD-1 at altering macrophage phenotype

Next, ligands for PD-L1 were investigated for their ability to induce the same effects as PD-L1 antibody treatment. Macrophage treatment with sPD-1 and sCD80 altered macrophage morphology (Fig. 3A) and produced larger cells, consistent with the changes induced by incubation with PD-L1 antibody (Fig. 3B). sPD-1 treated macrophages increased expression of costimulatory molecule CD86, but not MHC II, suggesting partial macrophage activation. However, treatment with sCD80 significantly increased expression of CD86, MHC II, and TNFα (Fig. 3C). As mentioned previously, the macrophages in our culture system did not express detectable PD-1 but expressed large amounts of membrane-bound CD80 (Supplementary Fig. S1B and S1C). These results suggest that CD80 may be a more important ligand than PD-1 for PD-L1 interaction *in vivo*, and that treatment with PD-L1 antibodies may mimic the macrophage CD80–PD-L1 interaction.

Fc receptor antibody and a macrophage integrin antibody fail to activate macrophages

The specificity of observed changes mediated by PD-L1 antibodies were investigated by treating macrophages with antibodies to known cell surface receptors, including CD11b and FcRII/III. Changes in macrophage morphology, proliferation, or expression of costimulatory molecules were not observed following treatment with CD11b antibody or FcRII/III antibodies (Supplementary Figs. S3 and S4). Furthermore, pretreatment of macrophages with anti-FcRII/III prior to incubation with PD-L1 antibody did not abrogate the changes mediated by PD-L1 antibody treatment (Supplementary Figs. S3 and S4). In addition, macrophages were incubated with LPS to rule out possible effects of LPS contamination. These macrophages did not recapitulate the PD-L1 antibody treatment effects on morphology (Supplementary Fig. S5A), increased plate confluence in culture (Supplementary Fig. S5B), or increased survival (Supplementary Fig. S5C).

PD-L1 signals constitutively inhibit mTOR pathway signaling

The previous results indicated that incubation with PD-L1 antibodies affect PD-L1 signaling to macrophages. These effects could be explained by two possible pathways. One, PD-L1 antibodies (and PD-L1 ligands) may stimulate PD-L1 signaling to macrophages to induce proliferation and activation. Alternatively, PD-L1 antibodies and ligands may interrupt constitutive signaling by PD-L1, to then induce the phenotypic changes. To elucidate which of these two pathways was operative, studies were conducted using bone marrow–derived macrophages generated from PD-L1$^{-/-}$ mice. These macrophages exhibited increased proliferation (Fig. 4A) and upregulated MHC II expression compared with wild-type macrophages (Fig. 4B). These findings are most consistent with the idea that the PD-L1 molecule signals constitutively and negatively in macrophages, and that ligation of the receptor with PD-L1 antibodies inhibits this negative signaling, resulting in macrophage proliferation, survival, and spontaneous activation.

Previous studies have reported involvement of the mTOR pathway in PD-L1 signaling in tumor cells (10–12), and mTOR signaling has been found to regulate metabolic programming of antigen presenting cells in normal tissues (21). Therefore, the effects of treatment with mTOR inhibitors on macrophage activation induced by PD-L1 antibodies were evaluated. Treatment with rapamycin or torin2 significantly inhibited the changes induced by PD-L1 antibody treatment. Treatment with rapamycin and torin2 blocked PD-L1 antibody-induced proliferation, TNFα production, and expression of MHC II (Fig. 4C).

Next, we examined Akt and mTOR phosphorylation in cultured macrophages, because PD-L1 signaling maintains mTOR pathway signaling in murine tumor cells (10). We found increased p-Akt (Fig. 4D) and p-mTOR (Fig. 4E) expression in macrophages treated with PD-L1 antibodies. Together, these results suggest that PD-L1 constitutively signals to block mTOR pathway signaling. Genetic elimination of PD-L1 expression, or blockade of signaling by PD-L1 antibodies, appears to remove inhibition of this pathway, resulting in macrophage activation and proliferation.

PD-L1 antibody treatment changed macrophage transcriptome profiles

To understand the changes generated in macrophages by PD-L1 antibody treatment, we conducted RNAseq and transcriptomic

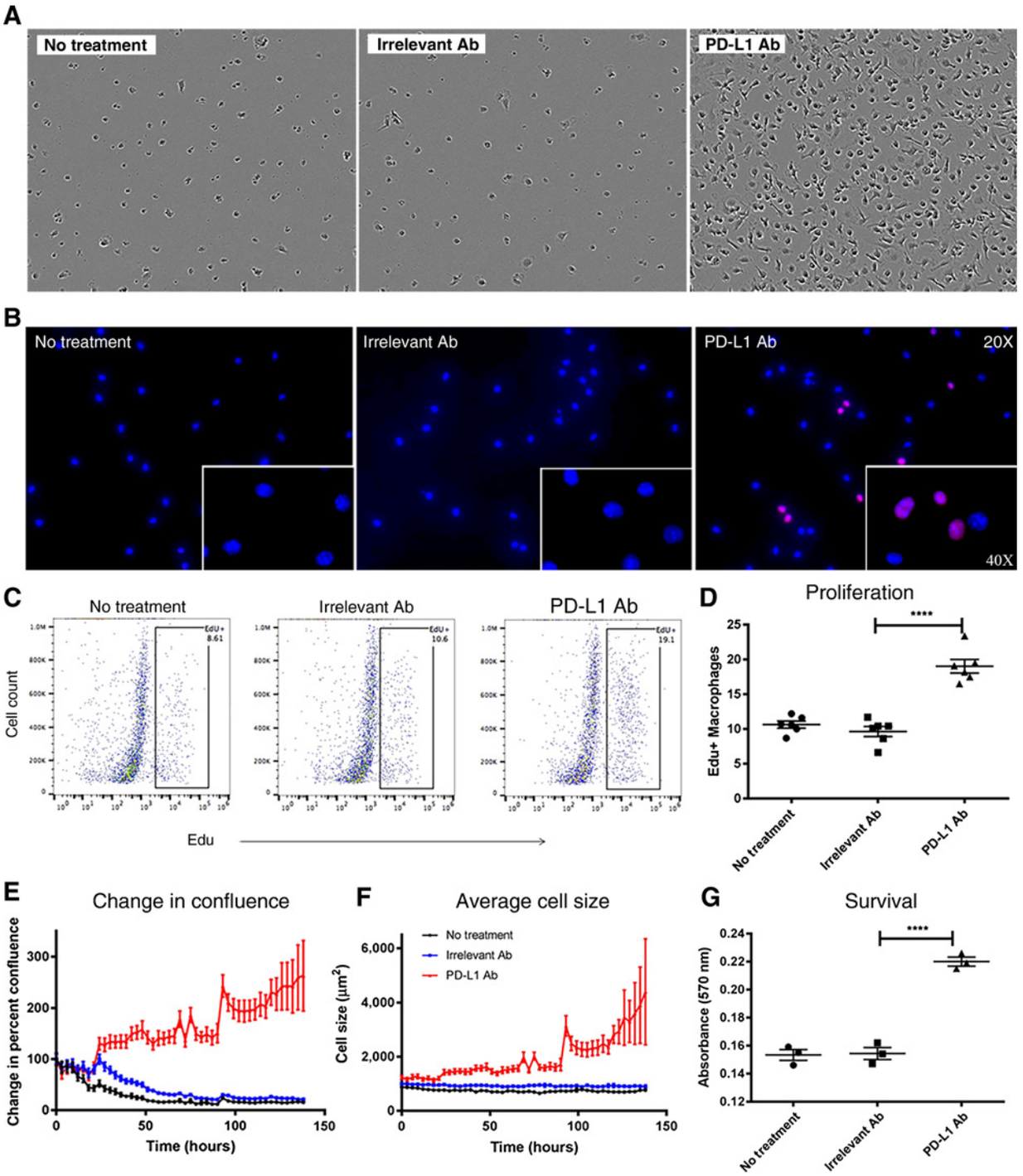

Figure 1.

Macrophage proliferation and size increase with PD-L1 antibody treatment. Macrophages were treated with medium only, irrelevant isotype, or PD-L1 antibody. After 6 days, images of the macrophages were taken **(A)**. Proliferation was measured using EdU incorporation **(B)** and quantified using flow-cytometric analysis **(C)** to be statistically compared using one-way ANOVA and Prism7 software **(D)**. Changes in confluence **(E)** and average cell sizes **(F)** were measured every 3 hours for the 6 days with an IncuCyte ZOOM live-cell imaging system. Lastly, an MTT was performed to measure survival of the cells and mean absorbances of MTT substrate were compared statistically with one-way ANOVA and Prism7 software **(G)**. Statistically significant differences were denoted as ****, $P < 0.0001$. Similar results were obtained in three independent experiments.

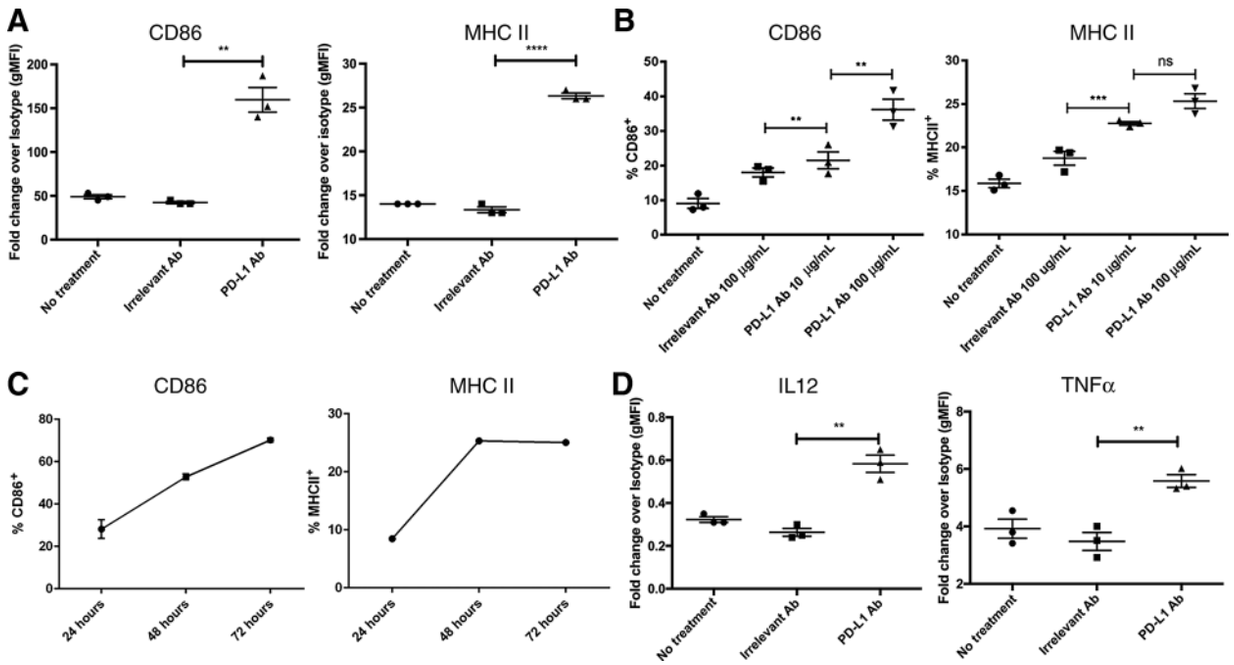

Figure 2.

PD-L1 antibody treatment induces macrophage activation. Macrophages were treated with medium only, irrelevant isotype, or PD-L1 antibody for 48 hours. They were stained for costimulatory molecule expression (CD86 and MHCII) by flow cytometry, and the geometric mean fluorescence intensity (gMFI) is shown as fold increase over an irrelevant isotype stain **(A)**. PD-L1 antibody was titrated **(B)** and macrophages were harvested at different time points following treatment with 100 μg/mL PD-L1 antibody in **C**. Finally, macrophages were permeabilized for staining of intracellular cytokines (IL12 and TNFα) by flow cytometry in **D**. Fold changes were compared using one-way ANOVA and Prism7 software. Statistically significant differences were denoted as **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$. These data are representative of three experiments with similar results.

analysis. The transcriptomic expression profiles of macrophages treated with PD-L1 antibody were distinct from those of control macrophages or those treated with isotype antibodies (Fig. 5A). There were 1,823 differentially expressed genes identified in macrophages following PD-L1 antibody treatment, with 643 genes upregulated and 1,180 genes downregulated compared with isotype antibody–treated macrophages (Fig. 5B). Gene ontology analysis identified upregulated inflammatory immune processes (Fig. 5C) and downregulated cellular processes (Fig. 5D).

IPA software was used to evaluate the pathways regulated by PD-L1 antibody treatment in macrophages. This analysis revealed that the top 10 signaling pathways altered by PD-L1 antibody treatment included 3 upregulated inflammatory pathways, 1 downregulated anti-inflammatory pathway, 1 upregulated survival and proliferation pathway, and 2 downregulated apoptosis pathways (Fig. 5E). The top upregulated pathway with the strongest statistical significance was the "Type I Diabetes Mellitus" pathway, which includes components of the macrophage inflammatory response such as TNFα, iNOS, and IL1β (Supplementary Fig. S6A). The second most upregulated pathway was "LXR/RXR activation," which also involves increased TNFα expression as well as upregulated inflammatory mediators iNOS, IL1, IL1β, and IL6 (Supplementary Fig. S6B). "PPAR signaling" was downregulated, including with decreased PDGF signaling (which promotes an immune-suppressed environment; ref. 22), and decreased PPARγ expression, which skews macrophages toward an anti-inflammatory phenotype (refs. 23, 24; Supplementary Fig. S6C). "TNFR2 signaling" was upregulated, with increased TNFR2 and A20 expression and an association with increased cell proliferation and survival (refs. 25, 26; Supplementary Fig. S6D). Also downregulated were two apoptosis pathways associated with decreased calpain and SERCA levels (refs. 27, 28; Supplementary Fig. S6E and S6F).

The top differentially expressed genes in PD-L1 antibody–treated macrophages were identified. The 25 genes most upregulated genes in PD-L1 antibody–treated macrophages are depicted in Fig. 5F. These genes were upregulated up to 115-fold over isotype antibody–treated macrophages. The most highly upregulated gene was found to be *Serpinb2*, a gene upregulated in macrophages following LPS stimulation and associated with increased cell survival (29). Serpinb2-deleted mice show impaired macrophage infiltration and an alternative/anti-inflammatory macrophage phenotype (30), suggesting this protease promotes inflammatory macrophage function. The second most upregulated gene was *Saa3* (serum amyloid A3), expression of which increases in response to acute inflammation. *Saa3* drives proinflammatory macrophage differentiation (31, 32). Proinflammatory macrophage markers *Cd38*, *Il1a*, *Nos2*, and *Il6* were also upregulated. Other upregulated genes included *Slfn4* and *Slc7a2*, which are also upregulated during macrophage activation (33, 34), and upregulated chemotaxis genes *Ccl7* (MCP3), *Cxcl3* (MIP-2β), *Ccl2* (MCP-1), and *Fpr2* (35). Finally, upregulated *Dll4* and *Tarm1* promote proinflammatory activation and cytokine secretion by macrophages (36, 37).

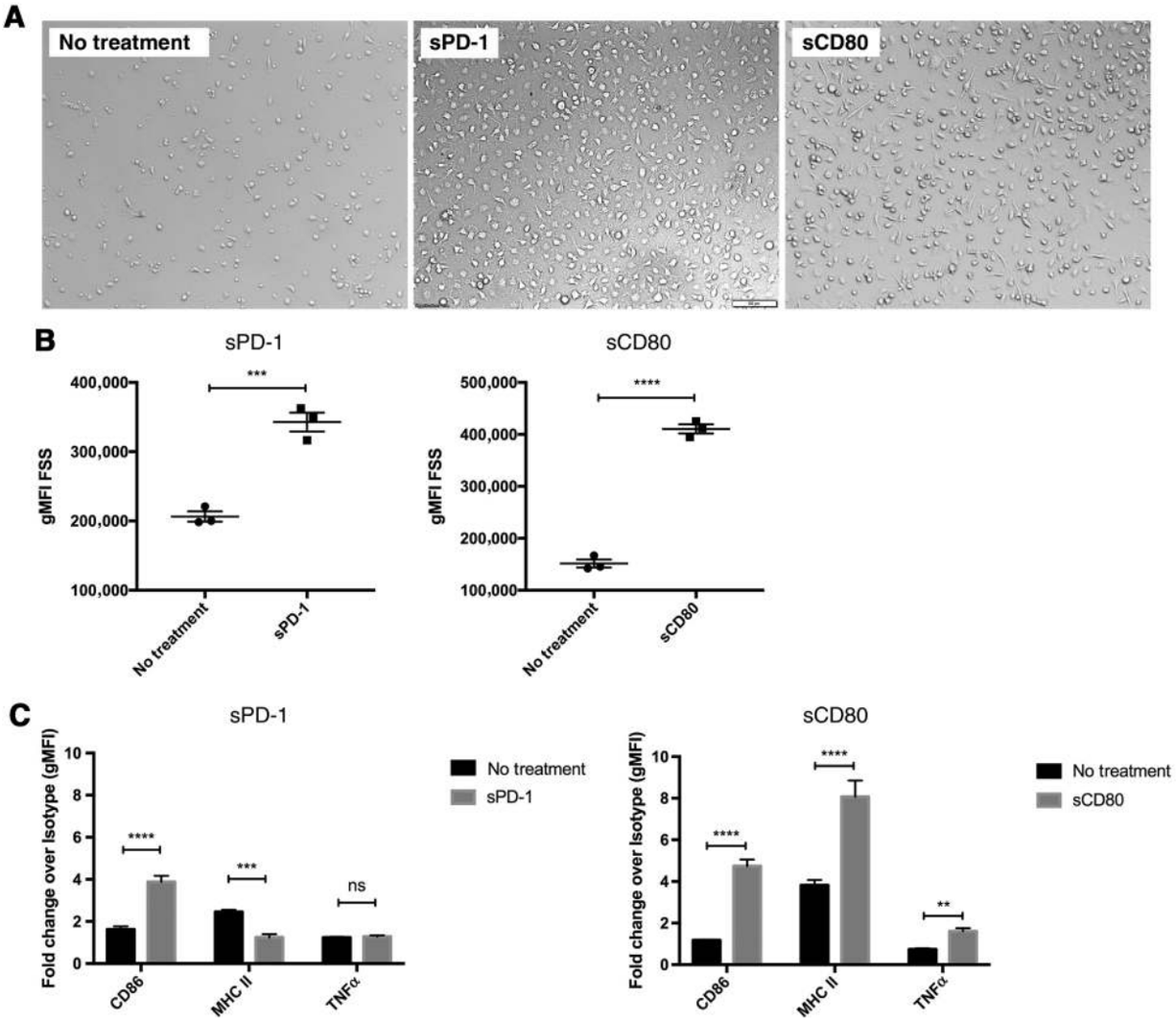

Figure 3.

Treatment of macrophages with sCD80 is more effective than treatment with crosslinked sPD-1 at altering macrophage phenotype and activation. Macrophages were treated with crosslinked sPD-1 or sCD80 alone for 48 hours, and images were taken of the cells to assess changes in morphology **(A)**. Size of the cells was quantified by flow cytometry in **B** and compared using nonparametric *t* test and Prism7 software. In **C,** costimulatory molecule expression (CD86 and MHC II) and intracellular TNFα production were measured by flow cytometry. Geometric mean fluorescence intensity (gMFI) is shown as fold increase over an irrelevant isotype stain, and fold changes were compared using two-way ANOVA and Prism7 software. Statistically significant differences were denoted as **, $P < 0.005$; ***, < 0.0005; ****, < 0.0001. Similar results were obtained in two repeated experiments.

The top 25 downregulated genes in PD-L1 antibody–treated macrophages are also depicted in Fig. 5F. Of these, a gene characteristic of anti-inflammatory macrophages (*Slco2b1*) was strongly downregulated (38). We also found downregulated expression of *Angptl4*, which codes for a protein that is decreased in proinflammatory macrophages (39). Finally, downregulated *Pparg, Tle1,* and *Clec10a* further suggest a suppressed anti-inflammatory phenotype of these macrophages (23, 40, 41).

Accumulation of activated macrophages in tumors after PD-L1 antibody treatment

Studies were conducted next in mice with tumors to determine the relevance of the preceding *in vitro* observations. These studies were done using the B16 melanoma tumor model because TAM in these tumors express PD-L1 (18). Treatment with PD-L1 antibody triggered a significant increase in numbers of TAM-infiltrating tumors, whereas numbers of macrophages in the spleen and lymph nodes were not altered (Fig. 6A). Increased numbers of tumor-infiltrating T cells (both $CD4^+$ and $CD8^+$ T cells) were observed in tumors from PD-L1 antibody–treated animals (Fig. 6B). TAM isolated from PD-L1 antibody–treated mice had significantly increased MHC II expression (Fig. 6C). There was also an increase in the numbers of TAM (F4-80^+ macrophages) in tumor tissues of PD-L1 antibody–treated mice (Fig. 6D).

The next question to be addressed was whether the increase in numbers of activated TAM in tumors of PD-L1 antibody–treated

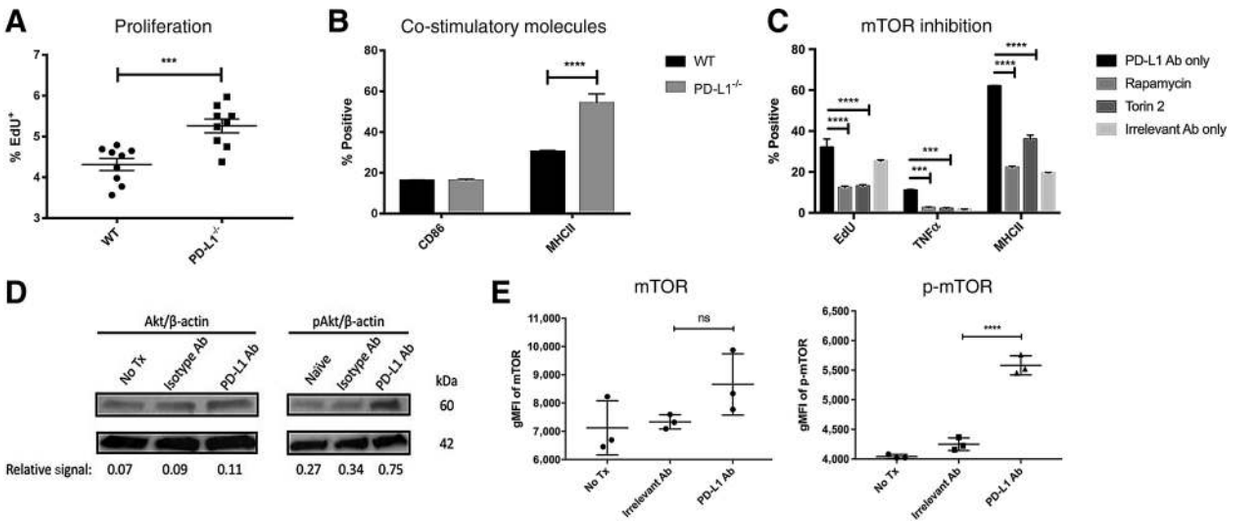

Figure 4.

PD-L1 signals constitutively in macrophages to inhibit mTOR pathway signaling. Macrophages from wild-type and PD-L1$^{-/-}$ mice were cultured for 1 week as described in Materials and Methods. Proliferation was measured by EdU incorporation **(A)** and costimulatory molecule expression (CD86 and MHC II) was measured by flow cytometry **(B)**. In **C,** wild-type macrophages were treated with PD-L1 antibody in combination with mTOR inhibitors rapamycin and torin2 for 48 hours prior to phenotyping by flow cytometry. In **D,** lysates from wild-type macrophages treated with medium, irrelevant isotype, or PD-L1 antibody were used for assessment of Akt phosphorylation by Western blot. Lastly, wild-type macrophages treated with medium, irrelevant isotype, or PD-L1 antibody were also used for intracellular staining of mTOR and p-mTOR levels by flow cytometry **(E)**. Statistical comparison for **(A)** was completed using nonparametric *t* test, for **B** and **C** using two-way ANOVA, and for **E** using one-way ANOVA. All statistical comparisons were completed using Prism7 software. Statistically significant differences were denoted as ***, $P < 0.0005$; ****, $P < 0.0001$, and similar results were obtained in two additional, independent experiments.

mice was mediated indirectly via T-cell–targeted effects or directly by PD-L1 antibody treatment of macrophages. Therefore, tumors were established in RAG$^{-/-}$ mice, using a more slow-growing tumor model (PyMT) to allow for a greater treatment interval. In mice lacking functional T cells, we did not observe changes in numbers of TAM (Fig. 6E) but we did find a significant increase in CD86^{+} and MHCII^{+} macrophages compared with isotype antibody–treated mice, consistent with T-cell–independent macrophage activation (Fig. 6F). PD-L1 antibody treatment also resulted in significant slowing of tumor growth, consistent with a T-cell–independent effect of PD-L1 antibody treatment (Fig. 6G).

Combined therapy is more effective than treatment with PD-L1 or PD-1 antibodies alone

Given the preceding evidence for a T-cell–independent antitumor effect of PD-L1 antibody treatment, we hypothesized that combined treatment with PD-L1 and PD-1 antibodies would not be redundant and would induce greater antitumor activity than treatment with either antibody alone. To address this question, B16 melanoma tumor-bearing mice with established cutaneous tumors (confirmed in all treated animals) received treatment with PD-L1 or PD-1 antibodies, or the combination of both antibodies. We observed that the combination of PD-L1 and PD-1 antibodies induced early tumor regression and, eventually, complete tumor rejection in 50% of animals, whereas single antibody treatment induced rejection in only 6% of animals (Fig. 7A and B). Furthermore, survival was improved in combination antibody-treated mice, with 50% of animals surviving to 68 days after treatment, compared with 13% for PD-L1–only treated animals and 0% for PD-1–only treated animals. These findings provide evidence that the antitumor activity of PD-L1 antibody treatment is distinct from that of PD-1 antibody treatment.

Discussion

PD-L1 is a coinhibitory checkpoint molecule known for its role in dampening T-cell responses. However, previous clinical trials found that PD-L1 antibody treatment in patients whose tumor cells do not express PD-L1 can still be effective in inducing tumor regression, suggesting a tumor-independent effect of the treatment (16, 17). Treatment of tumor-bearing mice with PD-L1 antibody decreases tumor growth in mice deficient in T cells, which points to a lymphocyte-independent mechanism of action (42). This phenomenon was attributed to direct effects of PD-L1 antibodies on tumor cell growth through alterations in mTOR pathway signaling. Thus, there is precedent for antitumor effects induced by PD-L1 antibody treatment that may not be related to T-cell PD-1 expression. Several reports have suggested additional roles for PD-L1 in regulating tumor cell and dendritic cell activity (6–14), but little is known concerning the role of PD-L1 in regulating macrophage function.

Key findings from the studies reported here are that PD-L1 antibody–treated macrophages exhibited greater proliferation, survival, and activation compared with control or irrelevant antibody-treated macrophages. For example, PD-L1 antibody-treated macrophages exhibited upregulation of costimulatory molecules and spontaneous proinflammatory cytokine production. Similar effects were also noted in human macrophages treated with PD-L1 antibodies, indicating that the effects observed are not restricted only to mouse myeloid cells. CSF-1 generated

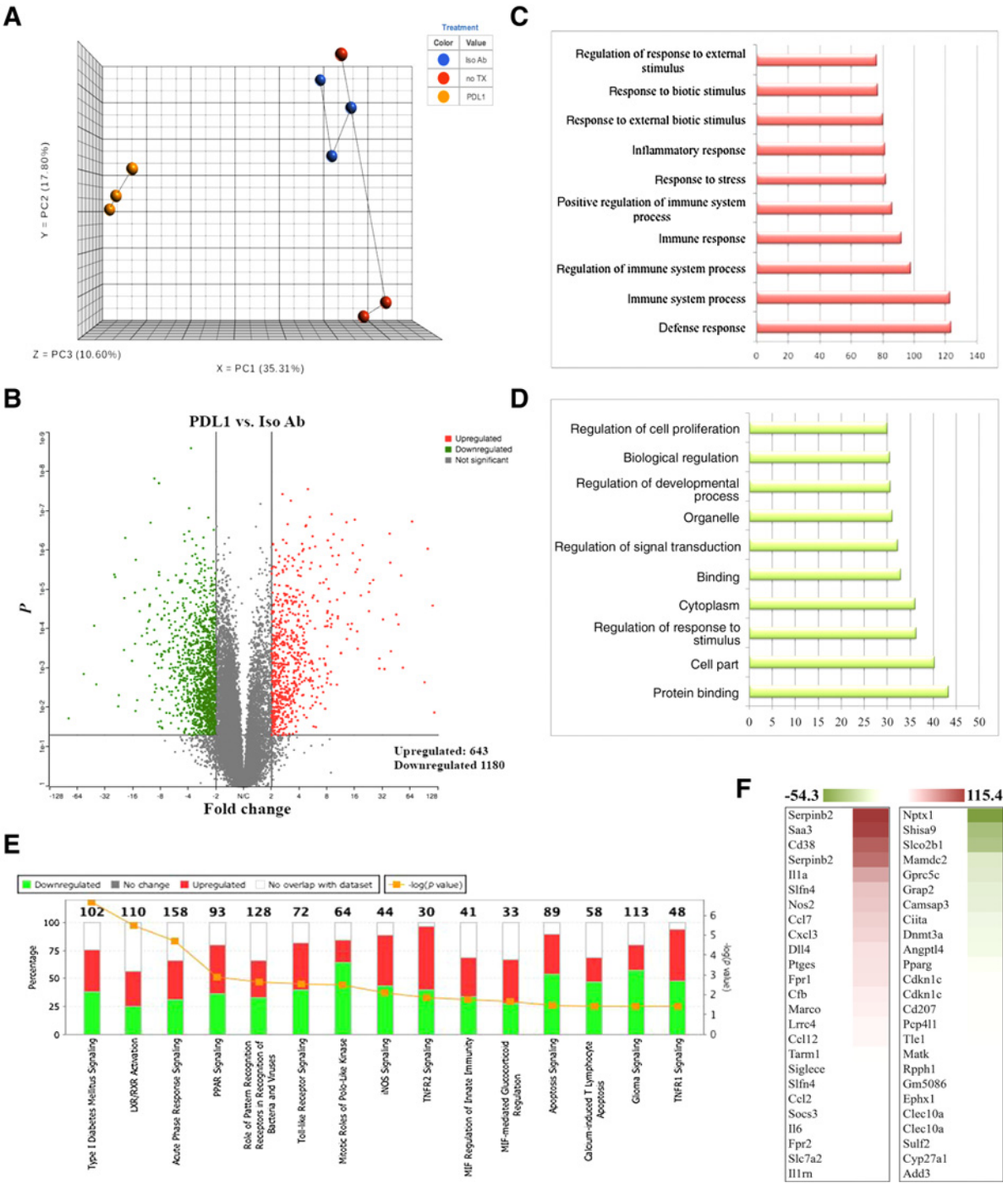

Figure 5.

Transcriptome profiling of PD-L1 antibody–treated macrophages showed inflammatory phenotype, increased survival and proliferation, and decreased apoptosis. Macrophages were treated with medium only, irrelevant isotype antibody, or PD-L1 antibody for 24 hours prior to RNA extraction for RNA sequencing analysis. The PCA plot **(A)** depicts the relationship and grouping of the samples based on global gene expression with medium only in red, isotype antibody in blue, and PD-L1 antibody in yellow. The volcano plot **(B)** shows the number of genes upregulated and downregulated on PD-L1 antibody–treated macrophages compared with isotype antibody–treated macrophages for *P* value with FDR $\leq$ 0.05 and fold change ≤ -2 (left, green) or ≥ 2 (right, red). Gene Ontology enrichment analysis was used to classify all significantly upregulated **(C)** and downregulated **(D)** genes into biological processes, with the enrichment score on the x-axis. IPA was next used to identify altered signaling pathways **(E)** with the yellow line depicting statistical significance, and upregulated values in red and downregulated values in green. **F,** The top 25 upregulated (left, red) and downregulated (right, green) genes. These data were generated using macrophages from 9 mice, with 3 mice in each treatment group.

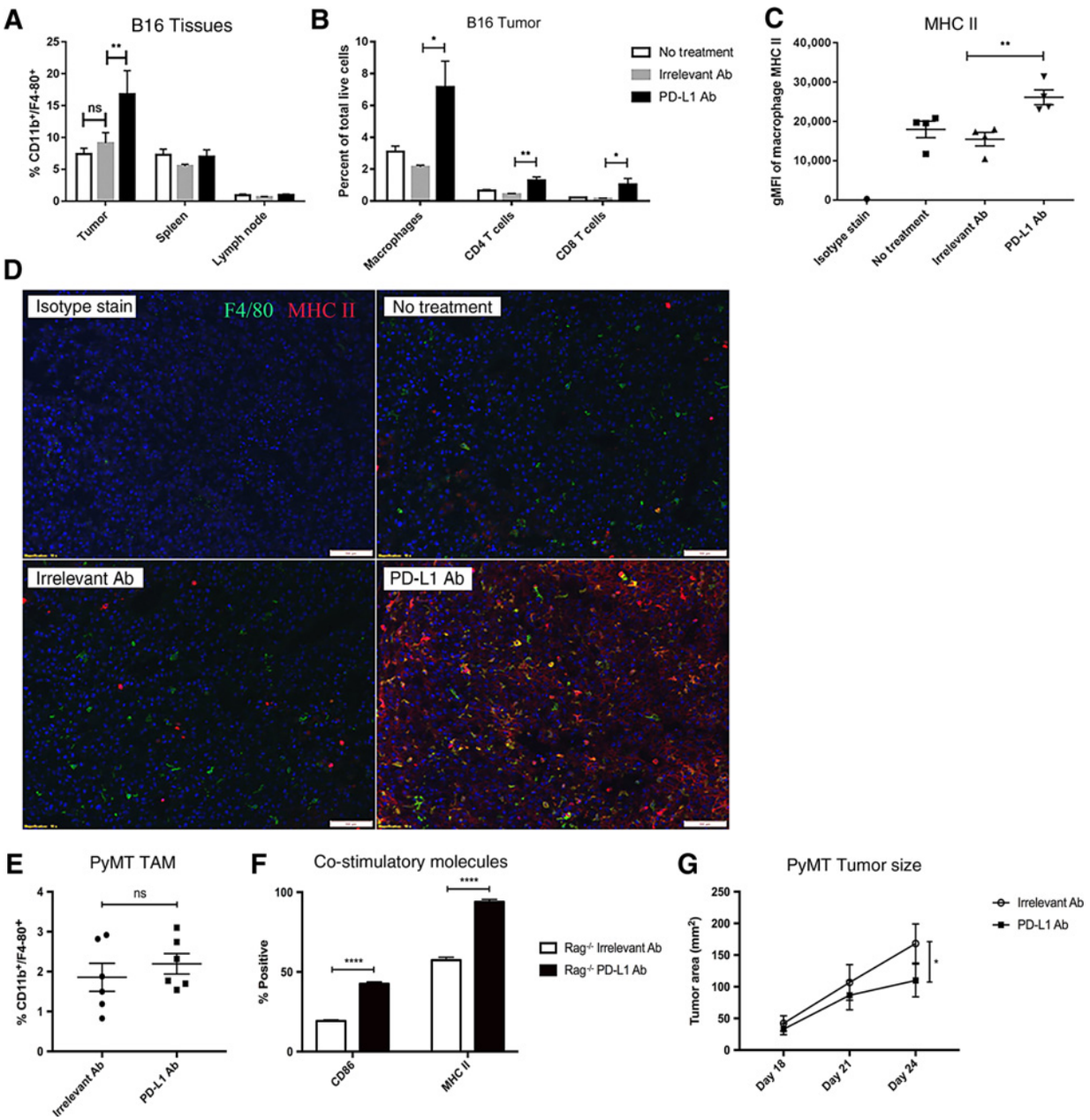

Figure 6.

Accumulation of activated macrophages in tumors following PD-L1 antibody treatment and inhibited tumor growth in Rag$^{-/-}$ mice treated with PD-L1 antibody. B16 melanoma cells were injected into C57Bl/6 mice and the mice were treated with PBS, irrelevant isotype, or PD-L1 antibody. Tumor, spleen, and lymph node tissue was harvested for flow-cytometric analysis of macrophage populations **(A)** and percentages of tumor macrophages and T cells are shown in **B**. We also measured surface expression of tumor macrophage MHC II **(C)** by flow cytometry, and tumor tissues were stained for F4-80 (green) and MHC II (red) expression before counterstaining with DAPI (blue) for imaging at 10× magnification **(D)**. Statistical comparison of cell numbers and expression of surface markers were conducted by two-way ANOVA using Prism7 software. These data are representative of two repeated experiments with four mice in each group. Next, PyMT breast carcinoma cells were injected into Rag$^{-/-}$ mice and the mice were treated with irrelevant isotype or PD-L1 antibody. Tumor tissue was harvested for flow-cytometric analysis of macrophage percentages **(E)** and surface expression of costimulatory molecules **(F)**. Tumor growth was also compared **(G)**. Statistical comparison of cell numbers was completed using nonparametric *t* test and both expression of surface markers and tumor sizes were compared by two-way ANOVA using Prism7 software. Statistically significant differences were denoted: *, $P < 0.05$; **, $P < 0.005$; ****, $P < 0.0001$, and these are pooled data from 2 experiments for a total of 6 mice in each group.

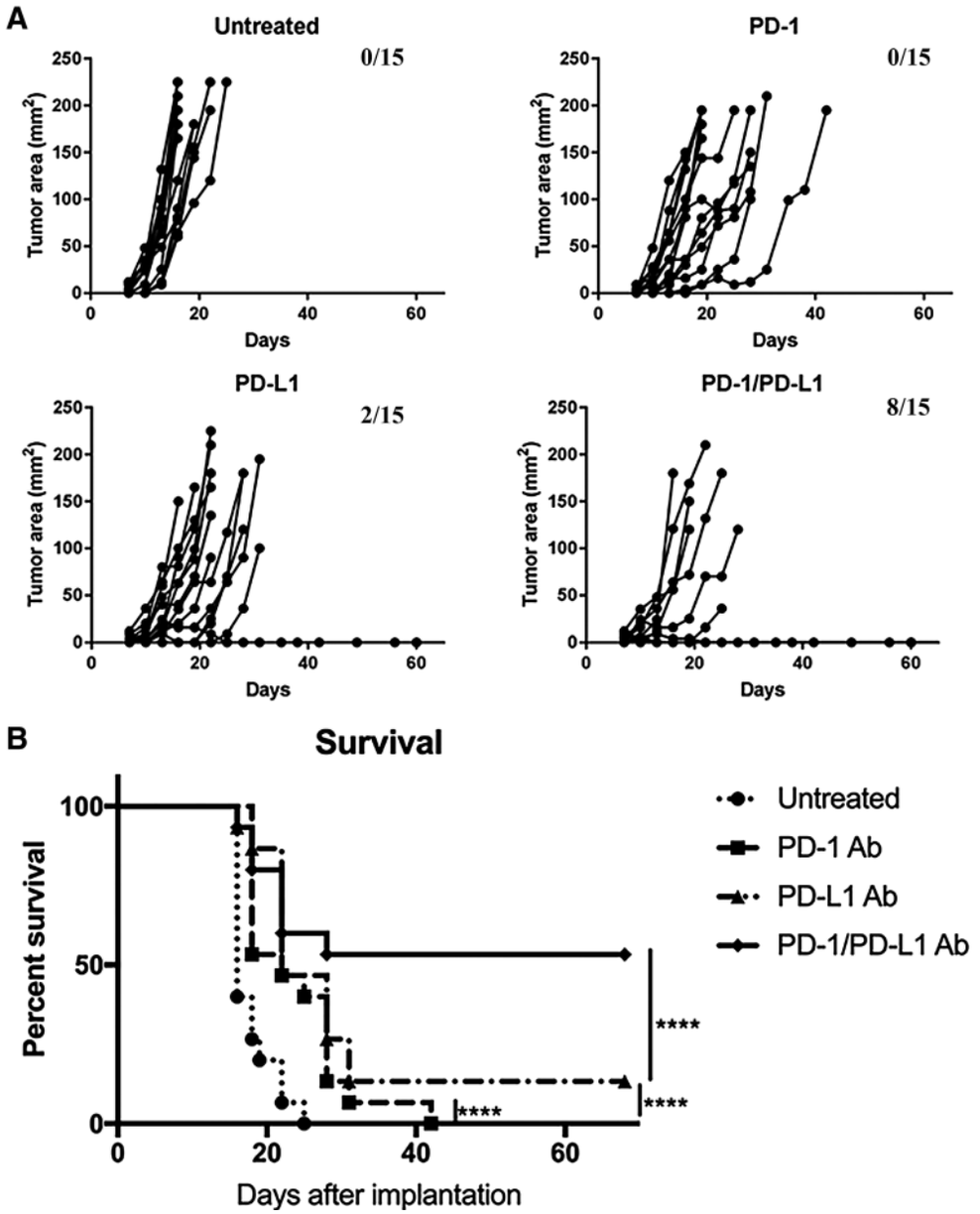

Figure 7.

Combined therapy with PD-1/PD-L1 antibodies induces early tumor regression and tumor-free survival. B16 were implanted into C57Bl/6 mice and mice were treated with PD-1 antibody, PD-L1 antibody, or a combination of PD-1 and PD-L1 antibodies. Tumor growth was measured every 3 days, and the number of tumor-free mice at the end of the study is noted for each group **(A)**. Survival curves are shown in **B**. Plots represent pooled data from two individual experiments for a total of 15 mice per group. Survival curves were compared by Kaplan–Meier and log-rank (Mantel–Cox) test using Prism7 and statistically significant differences were denoted as ****, $P < 0.0001$.

macrophages are known for their relative inability to produce proinflammatory cytokines, even following LPS stimulation (43). Overall, the picture that emerges is that PD-L1 antibody–treated macrophages are activated and proliferating, with a proinflammatory phenotype.

Our studies also reveal that PD-L1 exerts constitutive signaling effects on macrophages, leading to suppression of activation and inhibiting survival. For example, macrophages from PD-L1$^{-/-}$ mice exhibited spontaneous proliferation and activation, though PD-L1 antibody treatment resulted in a more pronounced activation phenotype. In addition, the effects of PD-L1 antibody treatment could be recapitulated with soluble CD80 and to a lesser degree with soluble PD-1, suggesting that the native ligands for PD-L1 serve to positively regulate macrophage activation and survival by interrupting the constitutive negative signals delivered by PD-L1.

These findings expand the known costimulatory role of CD80 in T-cell activation to also implicate CD80 in stimulating macrophage activation via interaction with PD-L1. Although PD-1 is known to restrain T-cell and NK cell function, our findings suggest that PD-1 can also partially activate macrophages following binding to PD-L1. Thus, the ability of PD-L1 ligands CD80 and PD-1 to partially block negative signals by PD-L1 and thereby activate macrophages provides feedback control essential to maintaining overall immune homeostasis. An immune system wherein macrophage function was continuously suppressed by unrestrained inhibitory signals from PD-L1, without mechanisms to allow counterregulation and fine tuning of the pathway, would likely result in deleterious, unrelieved macrophage suppression.

It is unclear why soluble PD-1, or genetic ablation of PD-L1 expression, did not fully recapitulate the phenotype of PD-L1 antibody–treated macrophages. It is possible that the PD-L1 antibodies or soluble CD80 interrupt PD-L1 signaling more efficiently than soluble PD-1. In the case of PD-L1$^{-/-}$ mice, compensatory pathways to regulate macrophage activation may have arisen during mouse development, thereby dampening the activated phenotype of macrophages in these mice.

We also observed that not all PD-L1 antibodies triggered macrophage proliferation and activation equivalently, as is the case for antibody disruption of the PD-1 and PD-L1 interaction. For example, a different murine PD-L1 antibody clone (MIH5) failed to recapitulate the macrophage-activating effects of the 10F.9G2 clone. Thus, not all PD-L1 therapeutic antibodies are expected to exert equivalent macrophage targeted activity *in vivo*, suggesting that screening for this activity *in vitro* could be a facet of the PD-L1 antibody development process.

Our studies also suggest that regulation of the mTOR pathway may be a component of the mechanism by which PD-L1 regulates macrophage functions. For example, inhibiting the mTOR pathway partially reversed the macrophage-activating effects of PD-L1 antibody treatment. Our studies also revealed upregulated mTOR pathway activity in macrophages following treatment with PD-L1 antibody. The Akt/mTOR signaling pathway regulates macrophage proliferation, activation, and metabolism (44, 45). Therefore, one of the effects of PD-L1 antibody treatment may be to block PD-L1 constitutive signaling and reverse the nonproliferating, immunologically inactive default pathway of macrophages, especially TAM. It is also possible that treatment with PD-L1 antibody blocks the interaction of PD-1 with PD-L1 on macrophages, leading to spontaneous macrophage activation. For example, it has been reported that the PD-1 interaction with PD-L1 functions to inhibit phagocytosis and antitumor activity of human and mouse TAM (46).

Transcriptome profiling of PD-L1 antibody–treated macrophages produced a picture of macrophages activated by multiple pathways to become activated, inflammatory, proliferating, and longer-lived macrophages. For example, PD-L1–treated macrophages expressed upregulation of chemoattractant and survival genes (*A20*, *Serpinb2*, *Ccl7*, *Cxcl3*, *Ccl2*, and *Fpr2*) as well as classic inflammatory genes (*Cd38*, *Il1a*, *Nos2*, and *Il6*). Highly downregulated genes included anti-inflammatory and apoptosis genes (*Slco2b1*, *Angptl4*, *Pdgf*, *Pparg*, *Tle1*, *Clec10a*, and *Capn1*). Furthermore, IPA yielded multiple upregulated inflammatory pathways, including "Type I Diabetes Mellitus," "LXR/RXR activation," "PPAR signaling," and "TNFR2 signaling." We also found activation of key components of the mTOR signaling pathway, evidenced by increased phosphorylation of Akt and mTOR.

In tumor-bearing mice, PD-L1 antibody treatment triggered an increase in both the numbers and activation of TAM. The PD-L1 antibody effects on macrophages were restricted to tumor tissues and did not occur in other macrophage-populated organs such as the spleen and lung. These findings suggest that the effects of PD-L1 antibody treatment will be primarily observed in tissues such as tumors that are exposed to high levels of PD-1 or CD80, or other macrophage-suppressive molecules. Furthermore, PD-L1 treatment exerted antitumor activity in mice lacking T cells (RAG$^{-/-}$ mice), consistent with T-cell–independent, macrophage-dependent antitumor activity, though a role for other immune cell types such as NK cells cannot be completely excluded. Reprogramming macrophages to a proinflammatory phenotype can inhibit tumor progression and metastasis (47, 48), consistent with our findings that PD-L1 antibody–treated TAM had antitumor effects *in vivo*.

PD-1– and PD-L1–blocking antibodies are established as effective immunotherapeutics for treating cancer. The effects of each antibody were previously assumed to be interchangeable and mediated solely by interrupting T-cell suppression via PD-1 signaling (2–5). However, our findings suggest that PD-L1 and PD-1 antibodies do not function in a completely overlapping manner for tumor immunotherapy, and that PD-L1 antibodies exert distinctive, T-cell–independent effects on tumor immunity. Thus, the combination of PD-1 and PD-L1 antibodies, which heretofore would have been considered redundant, appears to exhibit synergistic antitumor activity. For example, combined treatment with PD-1 and PD-L1 antibodies in mice with established B16 tumors resulted in a significant increase in complete tumor rejection (50% of animals), compared with 0% of PD-1 antibody–treated animals and 10% of PD-L1 antibody–treated animals. The potency of combined PD-1 and PD-L1 antibody treatment is likely due to the targeting of distinct, nonoverlapping cell populations in the tumor. Thus, combined therapy with PD-1 and PD-L1 antibodies may be warranted, including in patients who have failed treatment with PD-1 antibodies alone.

In summary, we provide evidence here that PD-L1 is a negative signaling molecule in macrophages, and that blocking PD-L1 signals can trigger macrophage proliferation, survival, activation, and antitumor activity in tumor tissues. This property of macrophage expressed PD-L1 may therefore be utilized therapeutically as another target for immunotherapy, in addition to blocking the PD-L1 and PD-1 interaction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G.P. Hartley, S.W. Dow
Development of methodology: G.P. Hartley, L. Chow, W.H. Wheat, S.W. Dow
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.P. Hartley, L. Chow
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.P. Hartley, L. Chow, D.T. Ammons, W.H. Wheat, S.W. Dow
Writing, review, and/or revision of the manuscript: G.P. Hartley, L. Chow, S.W. Dow

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.P. Hartley, D.T. Ammons, W.H. Wheat
Study supervision: G.P. Hartley, S.W. Dow

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