Supplemental Methods

Tumor models and cells: B16F10 and BPD6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 0.1mM non-essential amino acids (NEAA) and 1mM sodium pyruvate. YuMM5.2 cells were maintained in DMEM/F12 media supplemented with 10% FBS. The cells were replated 3 times/week at a confluency of 1:10 and were kept in a 37°C incubator at 5% CO_2. For subcutaneous tumor models B16F10 ($5X10^4$ or $1x10^5$), YuMM5.2 ($5x10^5$) and BPD6 ($5x10^5$) cells were injected into the right flank of the mice. For iBP mice, tumors were induced with a single intradermal dose of 4-hydroxytamoxifen in DMSO (150µg/mouse). Tumors were measured thrice weekly using an electronic caliper. Tumor volumes were calculated by the formula V=Lx(WxW)/2. For iBP mouse tumors, volumes were calculated by the formula V=LxBxH. For tumor growth rate studies mice were euthanized when the tumors reached a maximum size of $2000mm^3$ as specified by the Duke IACUC.

Ovariectomy: Eight days prior to tumor inoculation, 7 weeks old C57BL/6J or 5 weeks old (iBP) female mice were subjected to ovariectomy or sham surgery. Mice were anesthetized in an inhalation chamber (2% Isoflurane) and maintained in half the dose of isoflurane (1%) via nose cone throughout the surgical process. Prior to surgery, mice were administered a 5mg/kg dose of carprofen subcutaneously. The area below the ribs was shaved with an electronic razor and the skin was sterilized by rubbing with betadine and alcohol (3X alternating). A horizontal incision was made through the skin above the ovary fat pad, followed by a vertical incision through the abdominal muscle wall. The ovary was externalized and removed using a cauterizing scissors (or returned if sham). Fat pad was replaced, and muscle walls were opposed and sutured (1-2 stitches). Following suturing 1 drop of bupivacaine (0.25%) was added on top of the incision site. The skin was opposed, and a wound clip was placed on the incision site. This was repeated for the other ovary. The mouse was then removed from anesthesia and kept in a clean cage and monitored until conscious.

The mice were monitored for recovery for 10 days. On day 7 after surgery the mice were supplemented with either placebo or E2 (0.01mg/60days continuous release, Innovative Research of America, FL, Sarasota) pellets with the help of a trocar.

Single Cell RNA sequencing: In total, 10,000 cells were loaded on the 10x Genomics Chromium Controller Single-Cell Instrument (10x Genomics) mixed with reverse transcription reagents along with gel beads and oil to generate single-cell gel beads in emulsions (GEMs). GEM-RT was performed in an Eppendorf Mastercycler Pro (cat#950030020, Eppendorf): 53 °C for 45 min; 85 °C for 5 min; then held at 4 °C. After reverse transcription, GEMs were disrupted and the single-stranded cDNA was purified using Dynabeads MyOne Silane beads (cat#37002D, Thermo Fisher Scientific). cDNA was amplified using the Eppendorf Mastercycler Pro (cat#950030020, Eppendorf): 98 °C for 3 min; cycled 11X: 98 °C for 15 s, 67 °C for 20 s, and 72 °C for 1 min; 72 °C for 1 min; held at 4 °C. The amplified cDNA product was purified with the SPRIselect Reagent Kit (0.6 × SPRI) (cat#B23318, Beckman Coulter). Indexed sequencing libraries were constructed using the reagents in the Chromium Single-Cell 3′ version 3 Library Kit, following these steps: (1) fragmentation, end repair and A-tailing; (2) SPRIselect cleanup; (3) adapter ligation; (4) post ligation cleanup with SPRIselect; (5) sample index PCR; (6) PostindexPCR cleanup. The barcoded sequencing libraries were analyzed using quantitative PCR (cat#KK4824, KAPA Biosystems Library Quantification Kit for Illumina platforms). Sequencing libraries were transferred to the Duke University Center for Genomic and Computational Biology (GCB) and were loaded on a NovaSeq6000 (Illumina) for sequencing.

scRNA seq data analysis: Sequencing data was de-multiplexed, trimmed, filtered, aligned, and quantified using the Cell Ranger pipeline (10X Genomics). Reads were aligned to CSC mm10 transcriptome and UMI count matrices for each sample was obtained. The Seurat v3.1 package was used to count matrices. For each sample, cells that express <200 or >2000 genes and cells that express >5% of mitochondrial genes were removed. Highly variable genes were identified and used for principal component analysis. Cell subpopulations were identified using the

'FindNeighbors' function with first 30 PCs and 'FindClusters' function from Seurat R package with default resolution parameters. Cells were then clustered and visualized using uniform manifold approximation and projection (UMAP) (1). DE Wilcoxon test analysis was used to identify genes that define a cluster using known cell type signatures (**Supplementary File I**) and genes that differ within clusters between treatments.

Pseudotime Analysis: To infer the developmental trajectories in the monocyte/macrophage lineages, Monocle3 was used to perform the pseudotime analysis where UMAP coordinates from Seurat were used as input (2). We used the graphtest function implemented in Monocle3 to find genes that vary with pseudotime where genes with $q < 0.01$ were identified as pseudotime-dependent genes. Cells were divided into four pseudotime blocks (e.g., 0-5, 5-10, 10-15 and 15-21) based on their pseudotime estimate.

Immunoblotting: Cells were washed three times with 2 ml of ice-cold PBS and lysed with 0.15 ml of phospho-RIPA lysis buffer (Tris-HCl pH 7.5, 50mM; NaCl, 150 mM; NP-40, 1%; Sodium deoxycholate, 0.5%; SDS, 0.05%; EDTA, 5mM; Sodium fluoride, 50mM; Sodium pyrophosphate, 15mM; β-glycerophosphate, 10mM; Sodium orthovanadate, 1mM) with protease inhibitor cocktail (Millipore-Sigma, P-8340). 50µg of B16F10 and YuMM5.2 cell lysates and 25µg of MCF7 cell lysates were denatured and resolved by SDS-PAGE. Proteins were transferred to Odyssey Nitrocellulose Membrane (Cat no # 926-31092, LI-COR Biosciences). Primary antibodies used were anti-ERα (6F11, 1:1000, Leica cat # 6F11); anti-actin (Cell Signaling, cat no #8457, dilution 1:20000). Secondary antibodies used were HRP-conjugated anti-mouse IgG (1:5000) Catalog # 1706516 and anti-rabbit IgG (1:5000) Catalog #1706515 , BioRad) and protein bands were visualized by Western Lightning Plus Ecl system (Catalog # ORT2655 and ORT2755 Perkin Elmer).

Quantitative PCR of tumor infiltrating myeloid cells: Tumor infiltrating myeloid cells were isolated from iBP tumors using a CD11b isolation kit (catalog # 18970, StemCell Technologies).

RNA was isolated using RNA Aqueous Micro kit (catalog # 1931, Ambion) followed by cDNA synthesis using an iScript cDNA synthesis kit (Cat# 170-7691). Quantitative amplification was performed using Sybr Green (Cat# 1725124, Bio-Rad) and a CFX-384 Real Time PCR detection system. Primers used are listed in **Supplementary Table I**.

Absolute quantification of *Esr1* mRNA: Full-length *Esr1* mRNA was generated by *in vitro* transcription from a T7 promoter present upstream of *Esr1* construct (pcDNA-*Esr1*) using the MaxIScript T7/SP6 *in vitro* transcription kit (Catalog # AM1322, Thermo Fisher). *Esr1* mRNA generated by *in vitro* transcription was purified using BioRad Aurum RNA isolation kit and reverse transcribed to cDNA using iScript cDNA synthesis kit. cDNA generated from this reaction was used to generate standards (7.5ng-.075fg range) and the absolute amount of RNA present in the BMDM isolated from *Esr1*$^{f/f}$ and *Esr1*$^{f/f}$;LysMCre mice was determined by plotting Ct values generated from BMDM cDNA to the standard cDNA.

siRNA transfection: B16F10 (50,000 cells/ml) and YuMM5.2 (50,000 cells/ml) cells were transfected with either a scrambled siRNA (Catalog # AM4637, Thermo Fisher Scientific) or *Esr1* (50 nM) siRNA using the Dharmafect 1 transfection agent according to the manufacturer's instruction. Cells were collected for downstream analysis after 48 hrs (RNA) or 72 hours post transfection (protein). The sequences of the siRNAs used are included in supplementary table II.

Proliferation assay: For proliferation assays, B16F10 cells were plated in DMEM media (without phenol red) supplemented with 10% charcoal-stripped FBS. Cells were plated at a concentration of 1000 cells/well of a 96-well plate for 2 days in 200µl of media. After 2 days, 50µl of media was removed and replenished with 50µl of fresh media containing 4X concentration of vehicle (DMSO), E2, or E2+fulvestrant at stated concentrations. Cells were collected every 24 hours by discarding the media from the plates. Plates were frozen at -80°C. Frozen plates were thawed at room temperature and 100µl of water was added to each plate to mediate cell lysis. Cell numbers were determined by the addition of 100µl of (conc.) DNA dye Hoechst 333258 dye (Sigma Cat #

94403) in TNE buffer (10mM Tris, 2M Nacl and 1mM EDTA) and the fluorescence was read at an excitation of 360 nm and an emission at 465 nm using a microplate reader.

Single cell isolation from tumors: Tumors were isolated, minced on a petri dish in media (DMEM+5%FBS) and then enzymatically digested by the addition of 100µg/ml DNase I (D5025-150KU, Sigma-Aldrich) and 1mg/ml collagenase (Collagenase A, Cat# 10103586001, Sigma-Aldrich) for 30 mins-45 mins. For iBP models, isolated tumors were sliced into large chunks and subjected to mechanical digestion in a gentleMACS Dissociator for 30 seconds twice. Following this, tumors were digested with an enzyme cocktail containing DNase I, collagenase, and hyaluronidase (100µg/ml) (H6254, Sigma-Aldrich) for 40 minutes following a second round of mechanical digestion for 30 seconds (twice). The cells were then filtered through a 40µm strainer to produce single cell suspensions and the enzymes were diluted by addition of additional media then spun down to remove media. Red blood cells were lysed with the addition of ACK lysis buffer (Cat# A1049201, ThermoFisher Scientific) for 4 minutes at room temperature. Following red blood cell lysis cells were washed with PBS before proceeding to flow cytometry staining or magnetic bead-based isolation.

Flow cytometry staining: Single cells suspensions (10^6 cells in 50µl) were incubated with Live/dead fixable dead cell stain in PBS for 10 mins at 4°C. Cells were spun down at 1000xg and were incubated with anti-CD16/32 (Catalog# 14-0161-85, ThermoFisher Scientific) in flow buffer (10gms BSA in 1L PBS + NaN3?) for 15 mins. Following this, cells were stained with an antibody cocktail in Brilliant Stain Buffer (Cat# 566349, ThermoFisher Scientific). The antibodies used are listed in **Supplementary Table III**. For intracellular staining cells were fixed and permeabilized using the eBioscience Foxp3 Transcription Factor Staining Buffer Set (Cat# 00-5523-00, ThermoFisher Scientific) followed by intracellular staining with the desired antibody for 30 minutes at 4°C. Multicolor flow cytometry was performed in BD Fortessa 16 color analyzer. The FACS results were analyzed by FlowJo_V10 software (FlowJo, LLC).

***In vitro* bone marrow-derived macrophage differentiation**: For this purpose, bone marrow cells were aseptically collected from 8-10 weeks old female C57BL/6J mice by crushing the femurs and tibias in PBS, 1% PBS and 2mM EDTA. Cells were added to ACK buffer to lyse the red blood cells for 2 minutes with intermediate vortexing. The solution was filtered through a 40µm strainer to remove bone fragments. To differentiate bone marrow cells to macrophages the cells were plated in DMEM media (100%) or DMEM media (70%) and 30% tumor-conditioned media, supplemented with 10% heat-inactivated charcoal-stripped serum in the presence of 30 ng/ml M-CSF (Cat# 312-02, PeproTech). After 3 days cells were supplemented with 50% of respective fresh media. On day 6 the media was removed and replaced with fresh media. When the cells are fully differentiated to macrophages on day 7, they are treated overnight with either DMSO, E2 (1nM) or E2+fulvestrant (100nM). For polarization, cells were further treated with LPS (100ng/ml, Cat L2630, Sigma Aldrich) and IFNγ (20ng/ml, Cat# 315-05, PeproTech) (24 hours) for M1 polarization or IL4 (10ng/ml, cat #214-14, PeproTech) for M2 polarization.

T cell proliferation assay and staining: $CD3^+$ T cells were isolated from the spleens of C57BL/6J or P-mel mice with magnetic bead-based T cell isolation kit (Cat # 19851, StemCell Technologies). T cells from naïve mice were stained with 5µM CFSE (Cat# C34554, ThermoFisher Scientific) for 5 minutes in PBS + 5% CFS with rapid vortexing following which the cells were washed twice with PBS. Stained T cells were then counted and plated in 96-well plates coated with anti-CD3 antibody (0.5µg/ml, Cat# 19851, ThermoFisher Scientific) anti-CD28 antibody (1µg/ml, Cat# 16-0281-86, Thermo Fisher Scientific) at desired density (250,000 T cells/50,000 CD11b or BMDM) in the presence of IL2 (50ng/ml) (Cat# 212-12, PeproTech). 72 hours after plating the cells were incubated with protein transport inhibitors brefeldin (Cat# 00-4506-51, Thermo Fisher Scientific) and monensin (Cat#00-4505-51, Thermo Fisher Scientific) for 6 hours at a final concentration of 2µM monensin and 3µg/ml brefeldin after which they were collected and were processed for staining for flow cytometry.

Flow cytometry staining of grafted tumor infiltrating T cells: For assessment of TIL function/cytotoxicity, T cells from established YuMM5.2 tumors were isolated after 14 days of tumor growth. For assessment of IFNγ and granzyme-B production by TILs, the isolated TILs were incubated with ionomycin (1mg/ml) and phorbol myristate acetate (20ng/ml) for 4 hours in the presence of protein transport inhibitors (brefeldin and monensin) at 37°C and 5% CO2. Surface and intracellular staining were performed as described in the section describing flow cytometry staining.

T cell depletion with α-CD8 antibody: For the purpose of CD8 depletion, C57BL/6J mice were injected with 200µg/mouse of a rat anti-CD8 antibody (clone YTS169.4, cat # BE0017 BioXCell) or rat IgG2b anti-KLH isotype control (clone LTF2, cat # BE0090BioXCell) diluted in sterile PBS, 24 hours before tumor injection and every 4 days after tumor injection. The efficiency of CD8 depletion was analyzed at the end of the experiment by collecting cardiac blood and performing flow cytometry for T cell subpopulations.

Fulvestrant treatment: Mice were injected with fulvestrant (Cat# HY-13636 MedChemExpress) 2 days after tumor injection at a dose of 25mg/kg via intramuscular route. After the initial injection fulvestrant treatment was administered to the mice every 5 days. Corn oil (Cat # C0136, Spectrum Chemical MFG Corp) was used as vehicle for fulvestrant which was administered at the same frequency to all other animals.

Anti-PD1 tumor studies: Age matched C57BL/6J mice harboring B16F10 tumors were treated with α-PD1 (clone RMP-14, cat # 0146 BioXCell) or rat IgG2a (clone 2A3, cat # BE0089 BioXCell) at 250µg/mouse by i.p. injections starting at day 10 after tumor inoculation and every 3 days until the end point was reached.

Macrophage depletion by clodronate liposomes: C57BL/6J mice were injected with B16F10 tumors into the right flank. 24hrs prior to tumor injection mice were injected with 1mg of clodronate liposomes (Liposoma B.V.) in 200µl of PBS per mouse via intravenous route. Liposomes were further administered 7 days and 14 days after tumor injection. The efficiency of intratumoral

macrophage depletion was verified by flow cytometry when the control group reached a tumor size of ~1000mm^3.

Analysis of human correlates: Raw RNA-sequencing data from Gide *et al.,* VanAllen *et al*. and Hugo *et al*. were downloaded from the European Nucleotide Archive (ENA) accession number PRJEB23709, Gene Expression Omnibus (GEO) accession number GSE78220, and dbGAP accession number phs000452.v2.p1 (3-5). Results were aligned and quantified relative to reference genome hg38 using a STAR-Salmon pipeline as previously described (6) and upper quartile normalized.

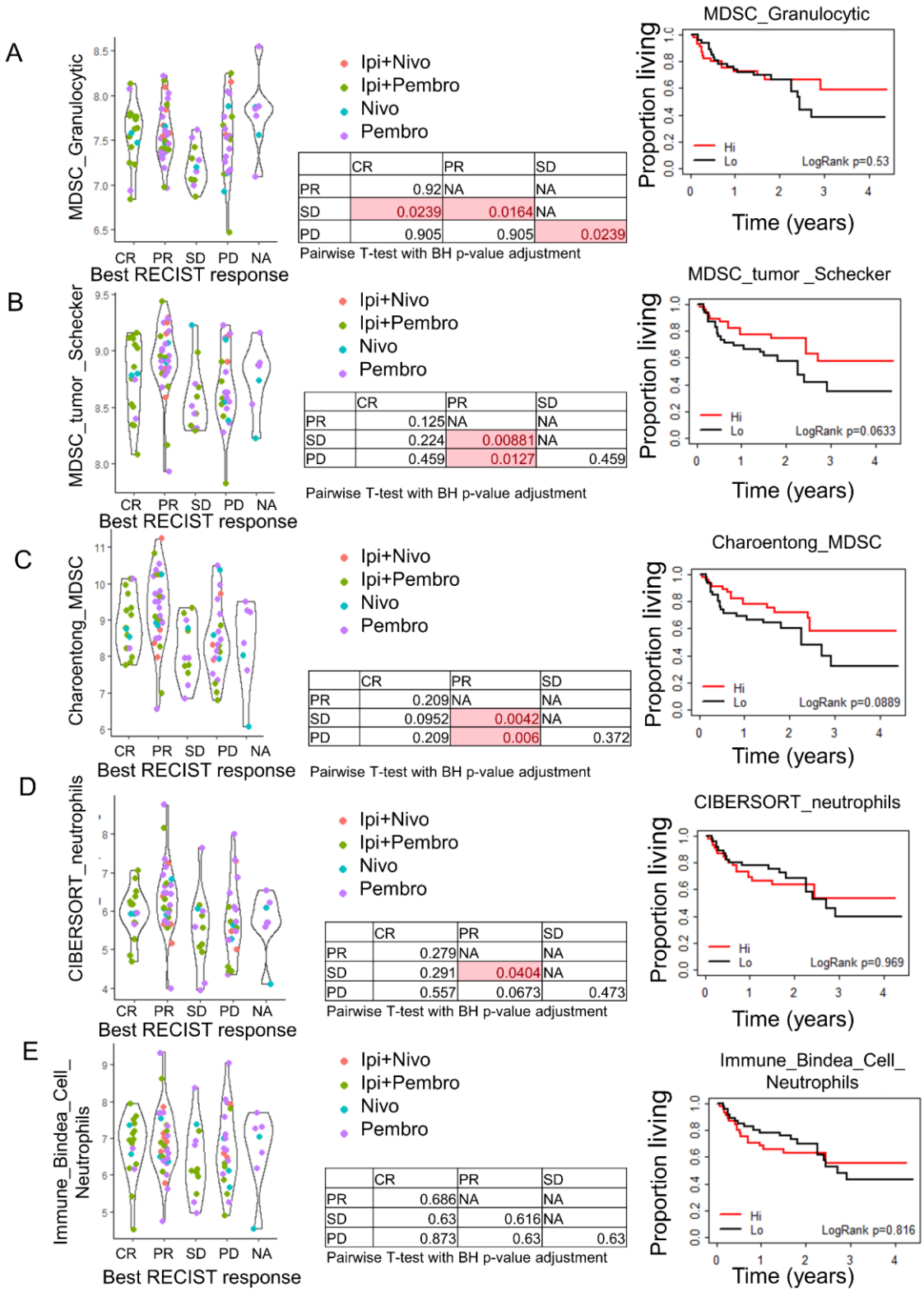
Hematopoietic immune cell relative fractions were determined from RNA expression data using CIBERSORT (7). Cell populations were determined using the LM22 signature from CIBERSORT using 100 permutations and disabling quantile normalization.

Survival analyses were performed using the ‘survival’ package’ analysis with R-. Patient populations were partitioned using median expression values and compared using the log-rank test.

References:

1. Liu Z. Visualizing Single-Cell RNA-seq Data with Semisupervised Principal Component Analysis. *Int J Mol Sci.* 2020;21(16).
2. Cao J, Spielmann M, Qiu X, Huang X, Ibrahim DM, Hill AJ, et al. The single-cell transcriptional landscape of mammalian organogenesis. *Nature.* 2019;566(7745):496-502.
3. Gide TN, Quek C, Menzies AM, Tasker AT, Shang P, Holst J, et al. Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy. *Cancer Cell.* 2019;35(2):238-55 e6.
4. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science.* 2015;350(6257):207-11.
5. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell.* 2016;165(1):35-44.
6. Hollern DP, Xu N, Thennavan A, Glodowski C, Garcia-Recio S, Mott KR, et al. B Cells and T Follicular Helper Cells Mediate Response to Checkpoint Inhibitors in High Mutation Burden Mouse Models of Breast Cancer. *Cell.* 2019;179(5):1191-206 e21.
7. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods.* 2015;12(5):453-7.

Supplemental Figure 1



	CR	PR	SD
PR	0.92	NA	NA
SD	0.0239	0.0164	NA
PD	0.905	0.905	0.0239

	CR	PR	SD
PR	0.125	NA	NA
SD	0.224	0.00881	NA
PD	0.459	0.0127	0.459

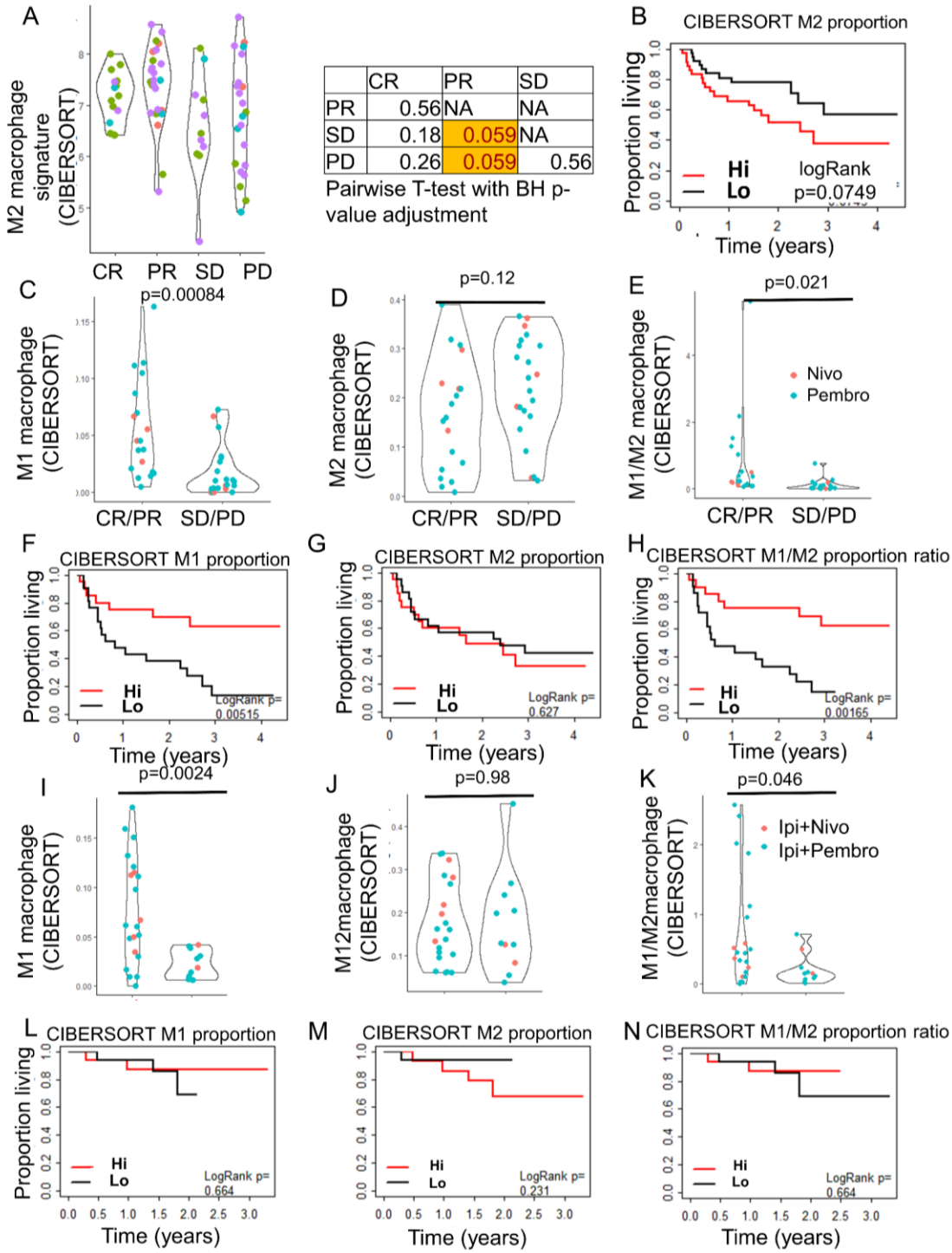
	CR	PR	SD
PR	0.209	NA	NA
SD	0.0952	0.0042	NA
PD	0.209	0.006	0.372

	CR	PR	SD
PR	0.279	NA	NA
SD	0.291	0.0404	NA
PD	0.557	0.0673	0.473

	CR	PR	SD
PR	0.686	NA	NA
SD	0.63	0.616	NA
PD	0.873	0.63	0.63

Supplemental Figure 1. Intratumoral MDSC do not predict ICB responses in melanoma patients Related to Figure 1 (A-E) Relative proportion of MDSC/neutrophils determined by applying published MDSC signatures from (A) PMID: 21954284, (B) PMID: 23152559, (C) PMID. 28052254, (D) PMID.25822800 and (E) PMID.24138885 and applied to analyze data from melanoma patients parsed by their response to pembrolizumab/nivolumab treatment alone. CR=complete response; PR=partial response; SD= stable disease; PD= progressive disease obtained from Gide *et al* dataset.

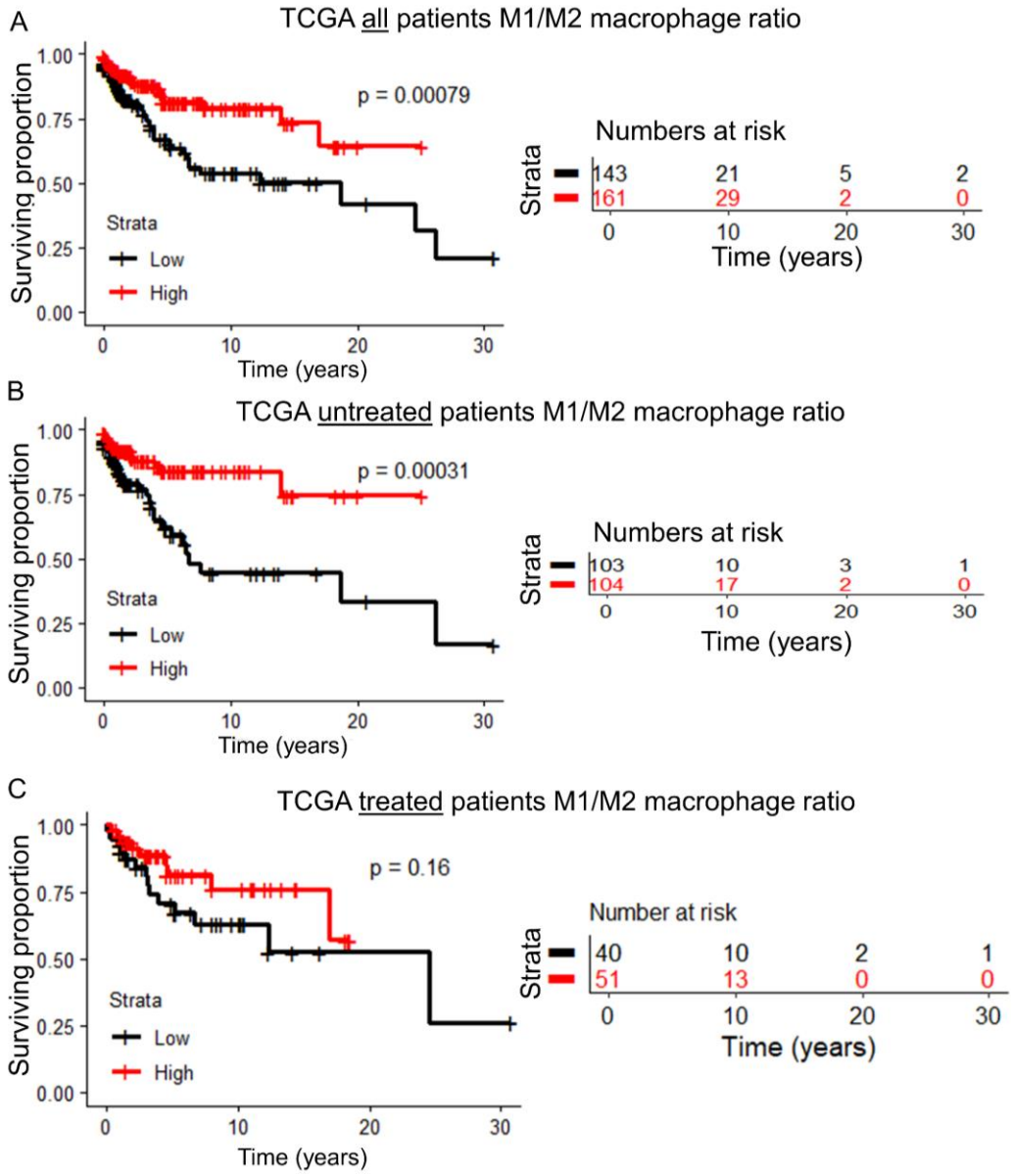
Supplemental Figure 2



	CR	PR	SD
PR	0.56	NA	NA
SD	0.18	0.059	NA
PD	0.26	0.059	0.56

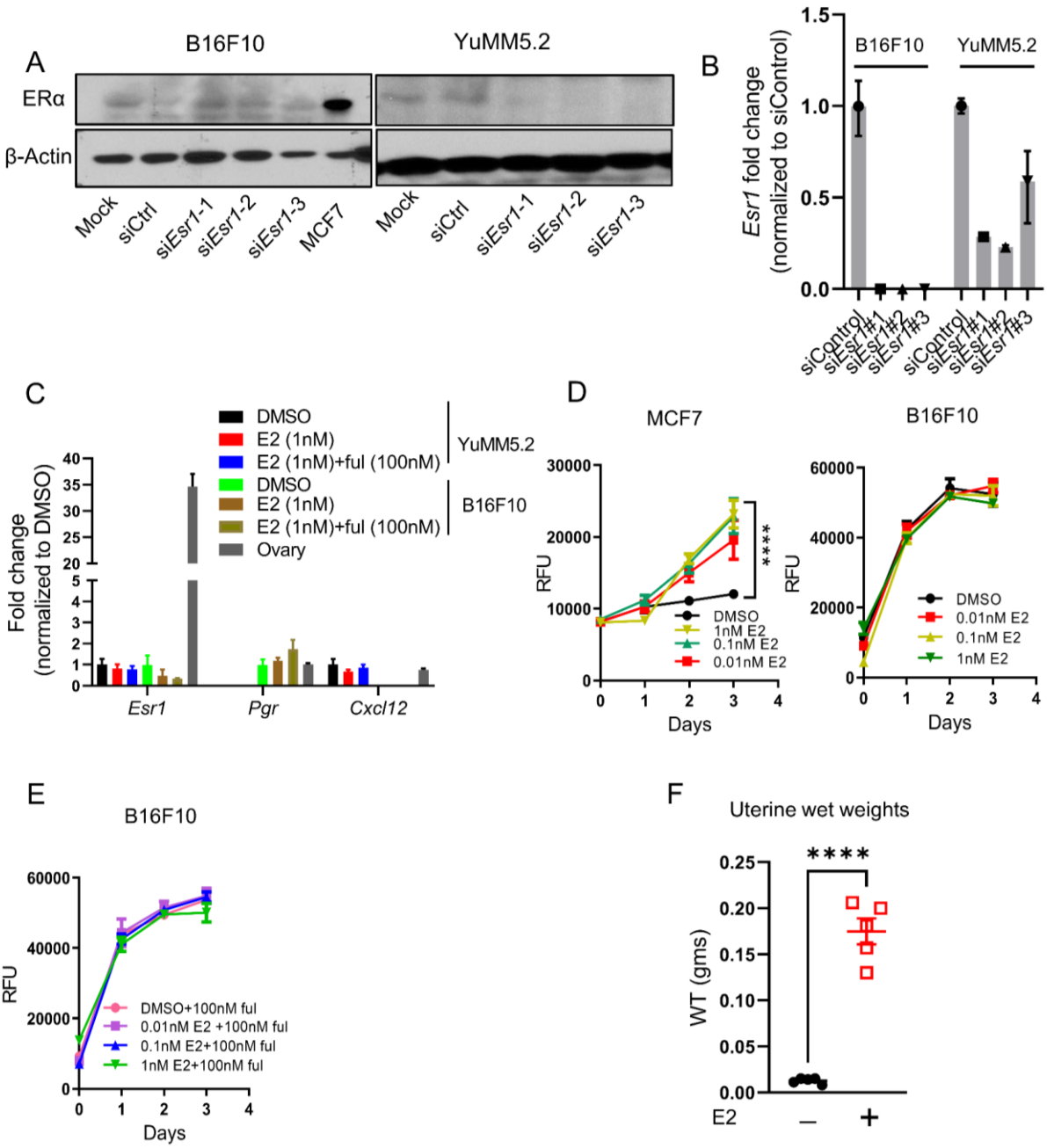
Supplemental Figure 2. Decreased M1/M2 ratio compromises benefit to immunotherapy in melanoma patients. Related to Figure 1 (A-B) Relative proportion of M2 macrophages or the ratio of M1/M2 macrophages as determined by CIBERSORT in melanoma patients parsed by their response to pembrolizumab/nivolumab treatment alone. CR=complete response; PR=partial response; SD= stable disease; PD= progressive disease obtained from Gide *et al* dataset (A). Median overall survival in all patient cohorts (Gide *et al.*) treated with immunotherapy with either high (upper 50%) or low proportions (low 50%) of M2 macrophages as determined by CIBERSORT (B). **(C-E)** Relative proportion of M1 and M2 macrophages or the ratio of M1/M2 macrophages as determined by CIBERSORT in melanoma patients parsed by their response to pembrolizumab/nivolumab treatment alone. **(F-H)** Median overall survival in all patient cohorts treated with anti-PD1 monotherapy (pembrolizumab or nivolumab) with either high (upper 50%) or low relative proportion (lower 50%) of M1 or M2 macrophages or M1/M2 macrophage ratio as determined by CIBERSORT. **(I-K)** Relative proportion of M1 and M2 macrophages or the ratio of M1/M2 macrophages in melanoma patients parsed by their response to Ipilimumab+Nivolumab and Ipilimumab+Nivolumab combination therapies. CR=complete response; PR=partial response; SD=stable disease; PD= progressive disease obtained from Gide *et al* dataset. (**L-N**) Median overall survival in all patient cohorts treated with combination immunotherapy (Ipilimumab+Nivolumab and Ipilimumab+Nivolumab) with either high or low proportions M1 or M2 macrophages or M1/M2 macrophage ratio.

Supplemental Figure 3



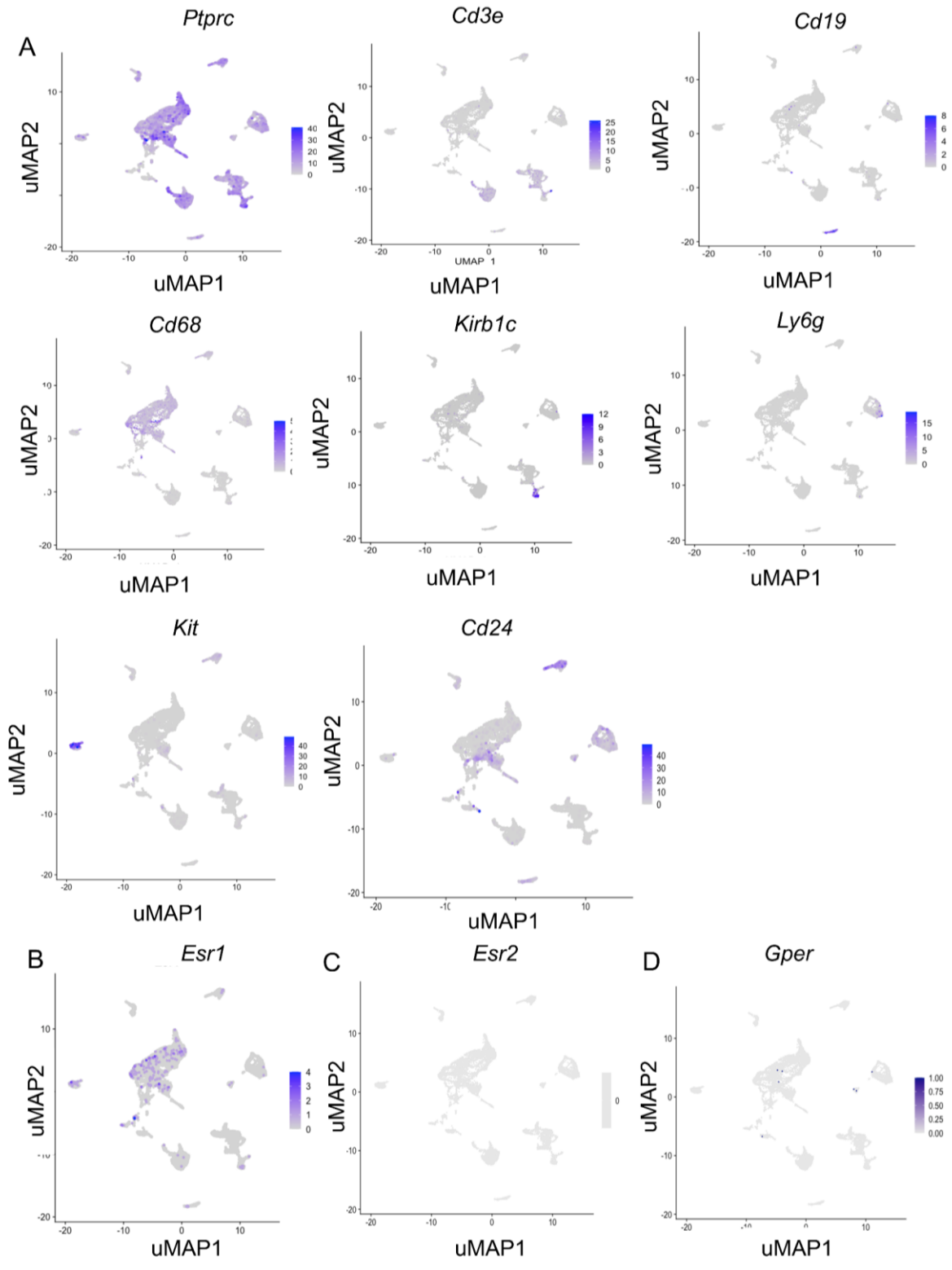
Supplemental Figure 3. Intratumoral M1/M2 macrophages dictate survival outcomes in melanoma patients. **Related to Figure 1**(**A-C**) Median overall survival of all (A) treated (B) or untreated (C) melanoma patients with either high (upper 50%) or low M1/M2 (lower 50%) M1/M2 macrophage ratio as obtained from TCGA SKCM dataset.

Supplemental Figure 4



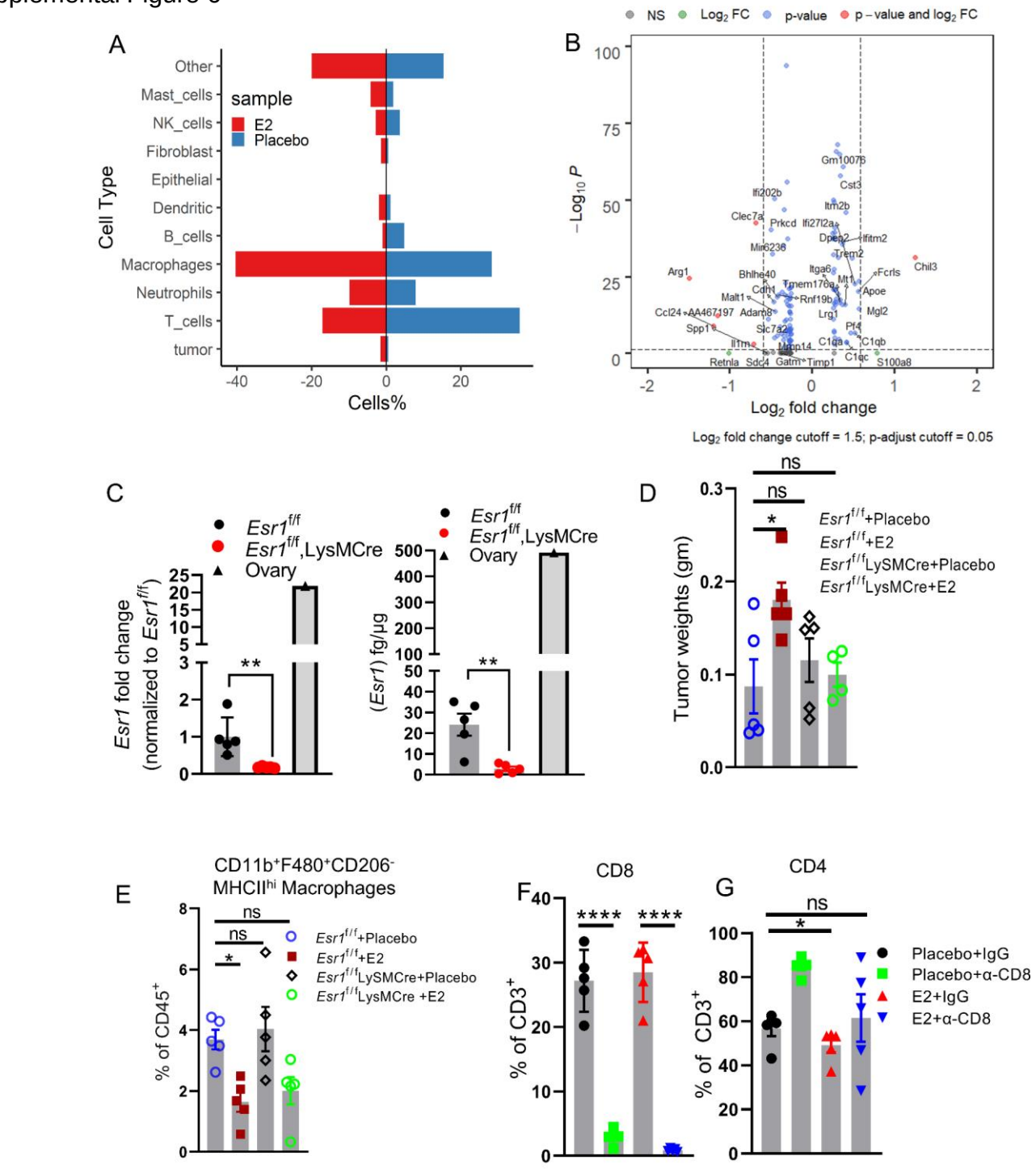
Supplemental Figure 4. Melanoma tumor cells do not respond to E2 *in vitro*. Related to Figure 2 (A) Immunoblot of ERα in melanoma cell lines B16F10 and YuMM5.2. ERα+ MCF7 cells served as a positive control. Cells were treated either mock transfected or transfected with scrambled control or three different siRNA targeting *Esr1*. **(B)** Quantitative PCR analysis of *Esr1* gene expression in B16F10 and YuMM5.2 cell lines from experiments described in (A) *Esr1* was first normalized to *Rplp0* and then Esr1 knockdown samples were normalized to *a* non-targeting control. RNA was collected from both cell lines after 48 hours of siRNA transfection. **(C)** Quantitative PCR of *Esr1* and ER target genes *Pgr* and *Cxcl12* in YuMM5.2 and B16F10 cells treated with either DMSO, E2 (1nM) or E2 (1nM)+fulvestrant (100nM) for 16 hours. Mouse ovary served as a positive control. Individual targets were normalized to *Rplp0* and then treated samples (E2 and ful) and ovary samples were normalized to DMSO. **(D-E)** Proliferation of B16F10 cells over 3 days when treated with either vehicle DMSO or E2 (0.01nM-1nM) (E, right) or E2 (0.01-1nM)+ fulvestrant (100nM) (F). ERα+ MCF7 cells served as a positive control (E, left) **(F)** Uterine wet weights from mice that were ovariectomized and supplemented with placebo or estrogen pellets. Data are expressed as individual data points and represented as mean ±S.E.M. Significance is calculated by Student's t test (G) two-way ANOVA (E and F) - followed by Bonferroni's multiple correction. ****p<0.0001

Supplemental Figure 5



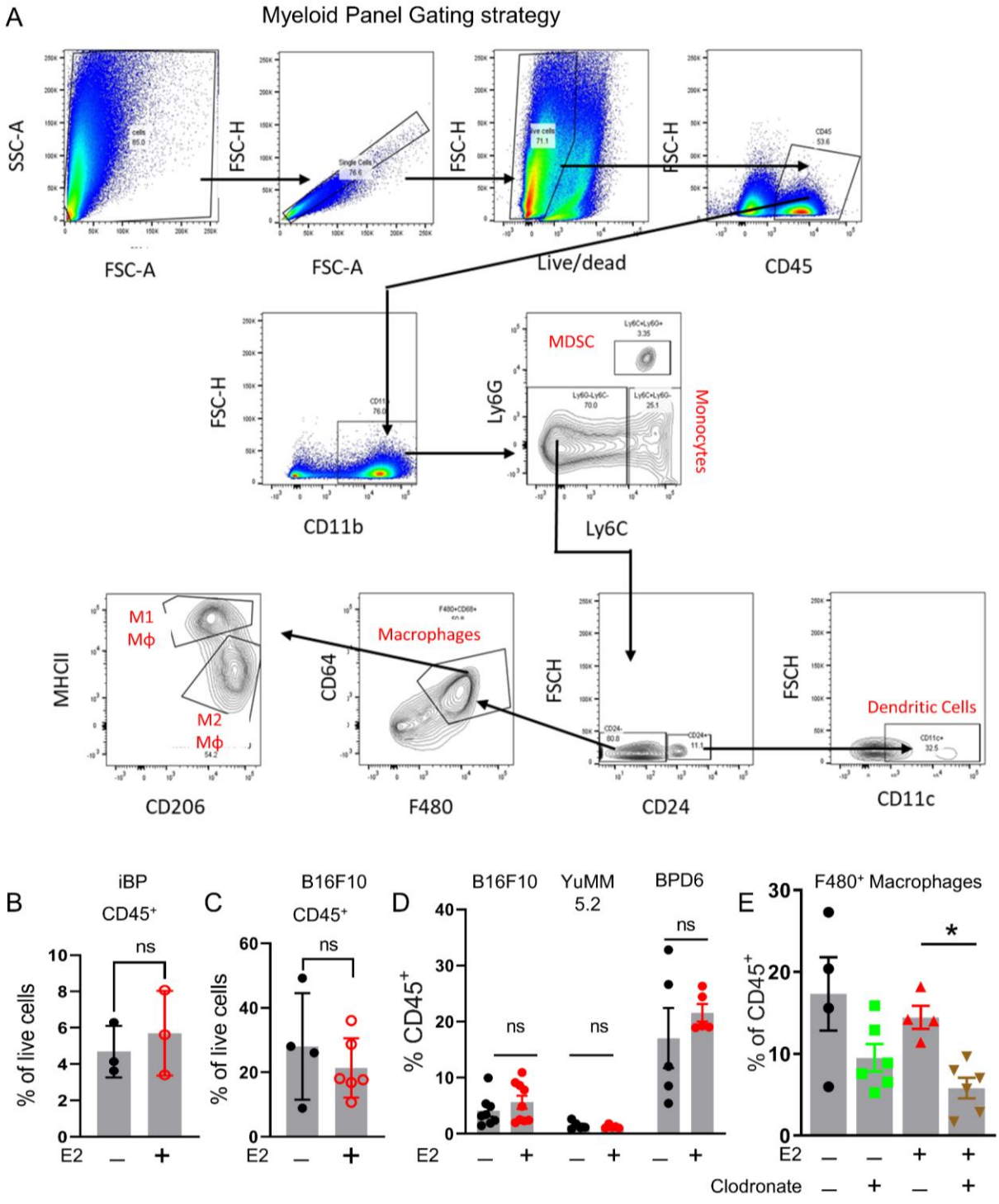
Supplemental Figure 5. Identification of immune cell types from scRNA seq. Related to Figure 3 (A) Uniform manifold approximation and projection (UMAP) plots of expression of marker genes that define each cluster *Ptprc* (all immune cells), *Cd3e* (all T cells), *Cd68* (monocytes and macrophages), *Kirb1c* (NK cells), *Kit* (Mast Cells), *Cd24* (DC), *Cd19* (B cells) and *Ly6g* (granulocytes). **(B-D)** UMAP plots of *Esr1* (B), *Esr2* (C), and *Gper* (D) in tumor infiltrating immune cells ($CD45^{+}$) isolated from placebo and E2 treated tumors

Supplemental Figure 6



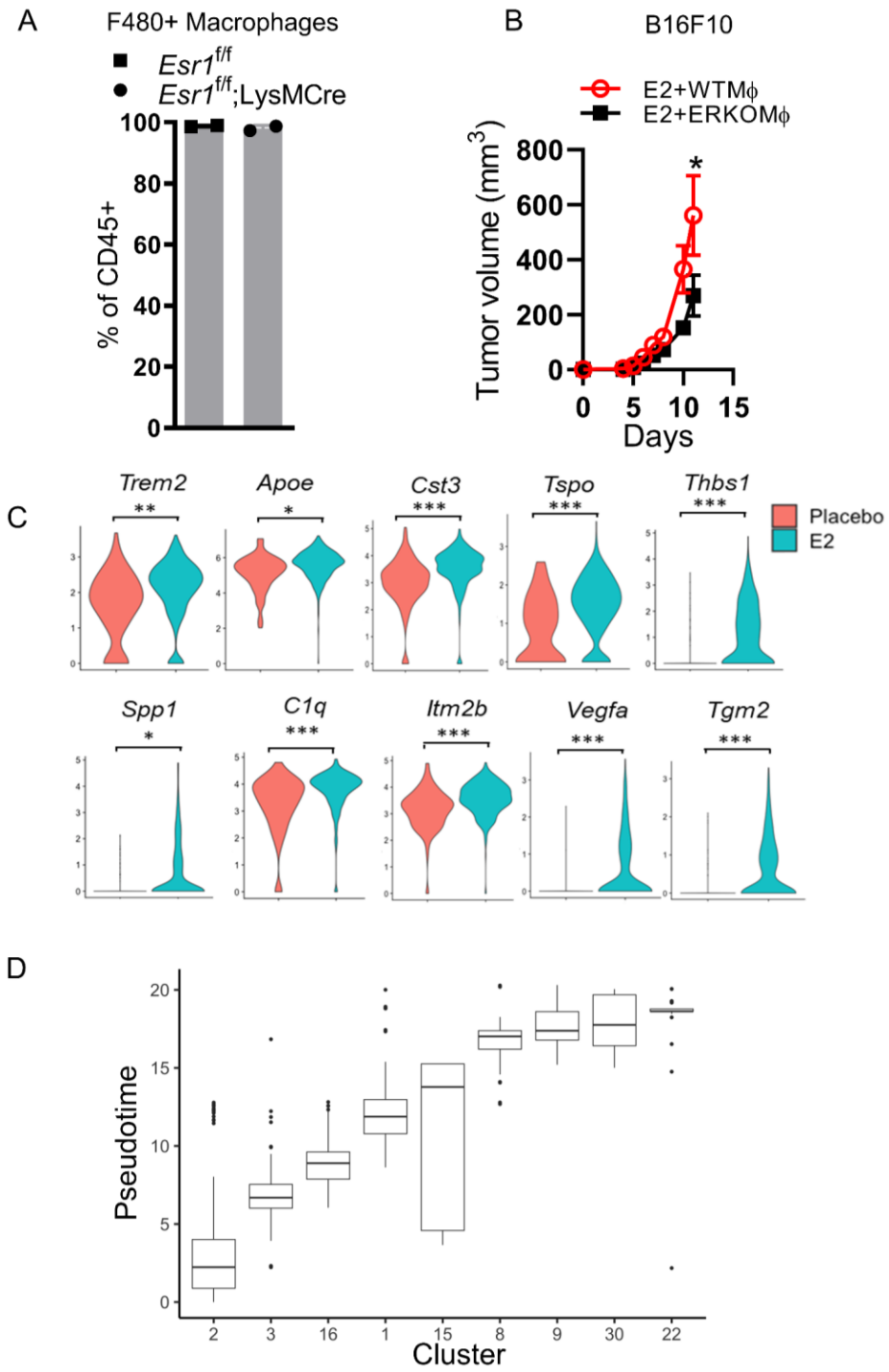
Supplemental Figure 6. E2 affects myeloid cell function. Related to Figure 3 (A) Percentage of different immune cell types determined by scRNAseq in placebo and E2- treated melanoma tumors. **(B)** Violin plot demonstrating top 20 up and down-regulated genes in CD68^{+} cluster as determined by scRNA sequencing **(C)** Quantitative real time PCR of *Esr1* expression in bone marrow derived macrophages isolated from *Esr1*$^{f/f}$ (black), n=5 and *Esr1*$^{f/f}$;LysMCre (red), n=5 mice (left). *Esr1* was normalized to the internal house-keeping control *Rplp0 and then Esr1*$^{f/f}$;LysMCre and ovary samples were normalized to *Esr1*$^{f/f}$ samples. Absolute quantification of *Esr1* from the same experiment. Mouse ovary was used as a positive control (right). **(D)** Weights of tumors resulting from implanting YuMM5.2 cells in *Esr1*$^{f/f}$ (n=5) and *Esr1*$^{f/f}$;LysMCre (n=5) mouse in the presence or in absence of E2. **(E)** Percentage of F480^{+}CD206^{-}MHCII^{hi} macrophages in tumor infiltrating immune cells isolated from YuMM5.2 tumors implanted in *Esr1*$^{f/f}$+placebo, *Esr1*$^{f/f}$;LysMCre+placebo, *Esr1*$^{f/f}$+E2 and *Esr1*$^{f/f}$;LysMCre+E2 mice. Data are expressed as individual data points and represented as mean±S.E.M. Significance is calculated by one-way ANOVA followed by Bonferroni's multiple correction. **(F-G)** Quantification of CD8^{+} and CD4^{+} T cells derived from peripheral blood of mice used in experiment 2E. (n=5 mouse per group). Data are represented as mean±S.E.M. Significance is calculated by Student's t test (B) one-way ANOVA (C, D, E and F) followed by Bonferroni's multiple correction. *p<0.05, **p<0.01 and ***p<0.001.

Supplemental Figure 7



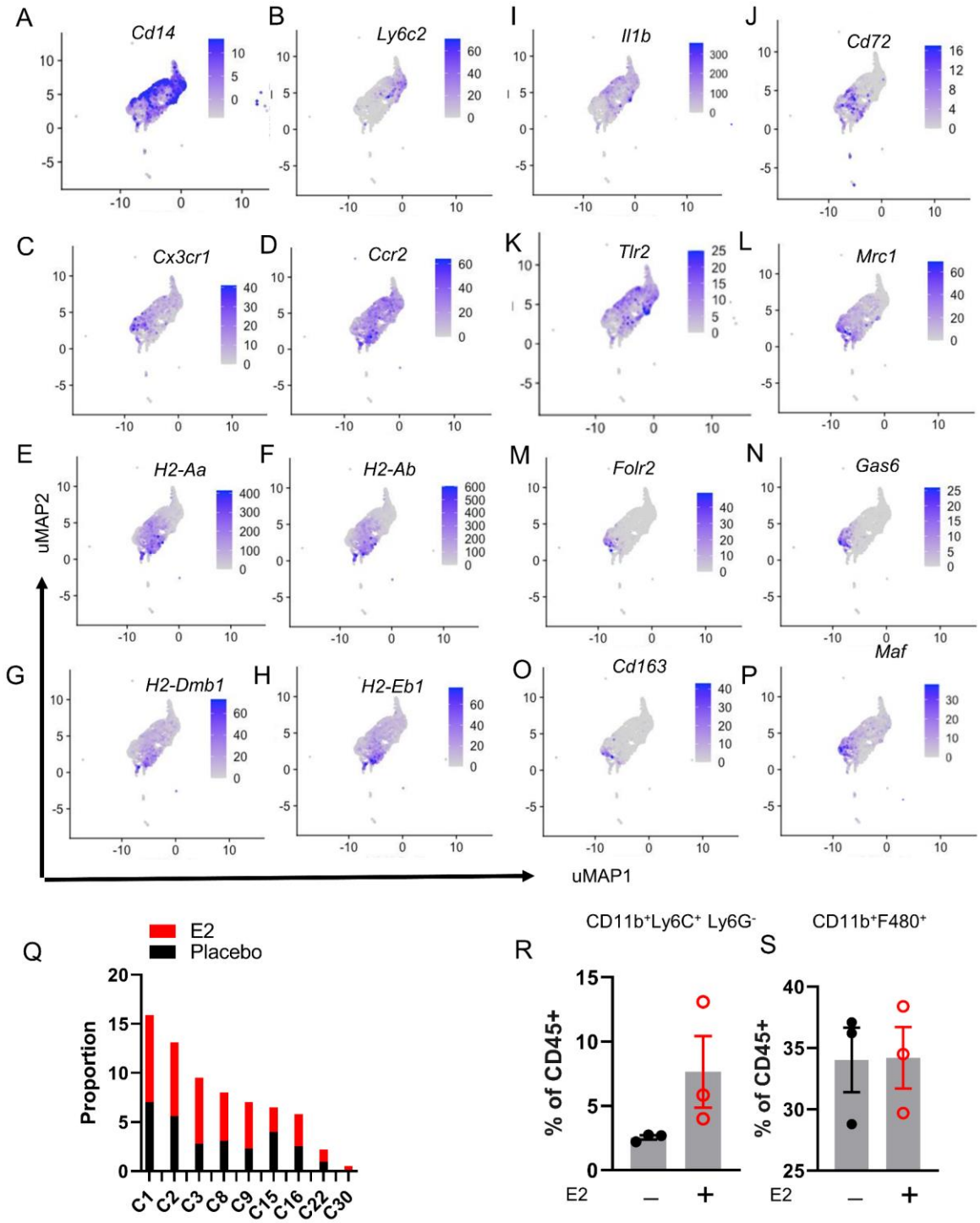
Supplemental Figure 7. E2 regulates TAM function in melanoma tumor microenvironment. Related to Figure 4 (A) Gating strategy for tumor infiltrating myeloid cells. **(B-C)** Percentage of $CD45^+$ tumor infiltrating immune cells isolated from iBP (n=3) and B16F10 (n=4-6) syngeneic tumors treated with placebo or E2. **(D)** Quantification of tumor infiltrating MDSCs ($Ly6C^+$ $Ly6G^+$) in B16F10, YuMM5.2 and BPD6 tumors. **(E)** Quantification of intratumoral macrophages upon clodronate mediated depletion of myeloid cells. Data are expressed as individual data points and represented as mean±S.E.M. Significance is calculated by one-way ANOVA followed by Bonferroni's multiple correction. *p<0.05

Supplemental Figure 8



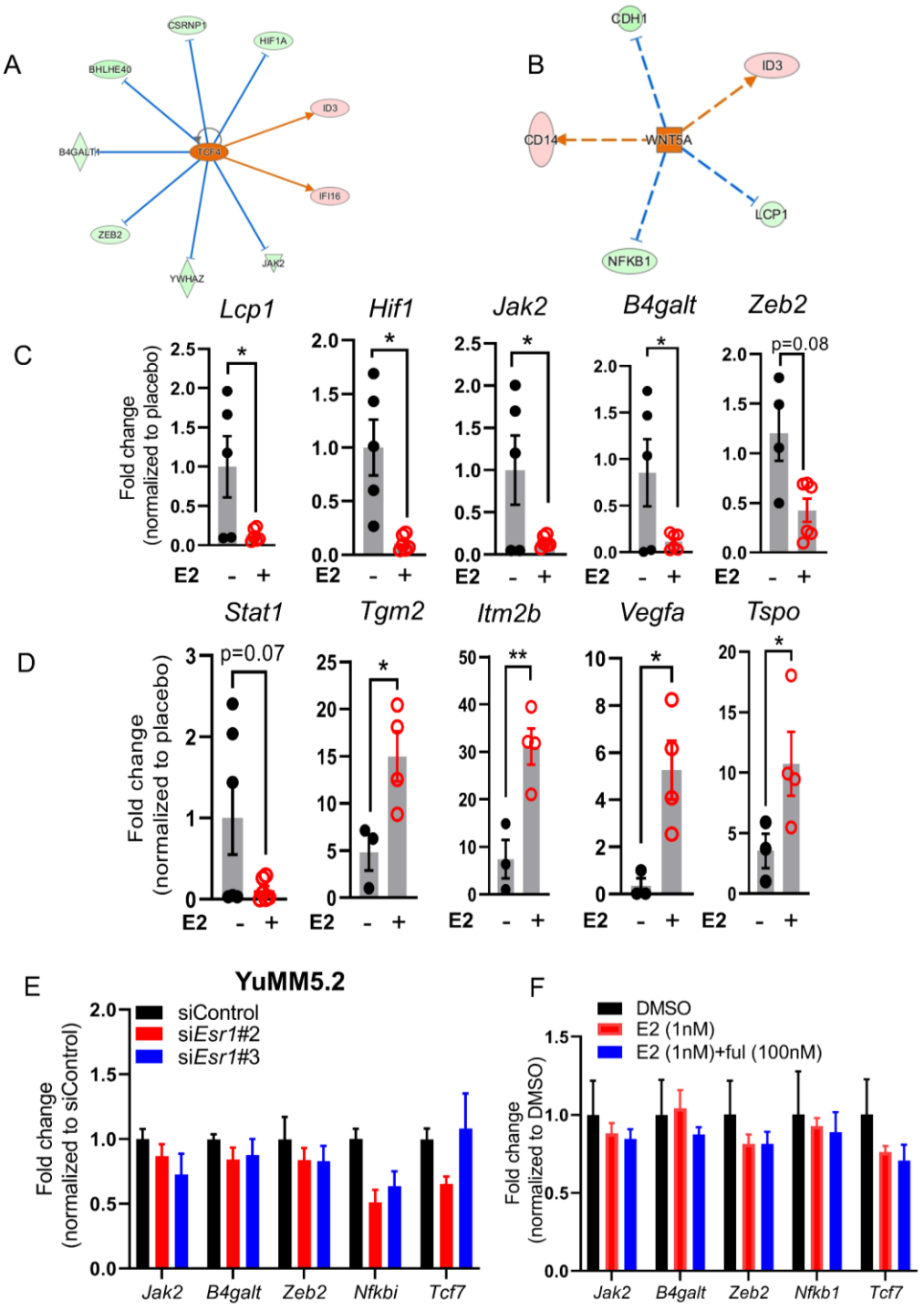
Supplemental Figure 8. Depletion of ERα in myeloid cells suppresses melanoma tumor growth. Related to Figure 4 **(A)** Percentage of F480^{+} macrophages from *in vitro* differentiated BMDM of genotypes *Esr1*$^{f/f}$ and *Esr1*$^{f/f}$;LysMCre used for macrophage tumor cell co-mixing experiments. **(B)** Syngeneic tumor growth of B16F10. Cells when co-mixed with BMDM from (*Esr1*$^{f/f}$;LysMCre) and its littermate controls (*Esr1*$^{f/f}$) mice. Mice of both genotypes were ovariectomized and supplemented with E2 pellets and injected with B16F10 (1X10^{5}) cells + BMDM at 1:1 ratio. (*Esr1*$^{f/f}$ BMDM+B16F10)- E2 (red, n= 10) and (*Esr1*$^{f/f}$;LysMCre, BMDM+B16F10)-E2 (black, n= 10). **(C)** Violin plots of monocyte/macrophage genes from placebo and E2 treated tumors as determined by scRNA seq. **(D)** Distribution of different clusters along the pseudotime axis.Data are expressed as mean± S.E.M. Significance is calculated by Student's t test (A) two-way ANOVA (B) - followed by Bonferroni's multiple correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Supplemental Figure 9



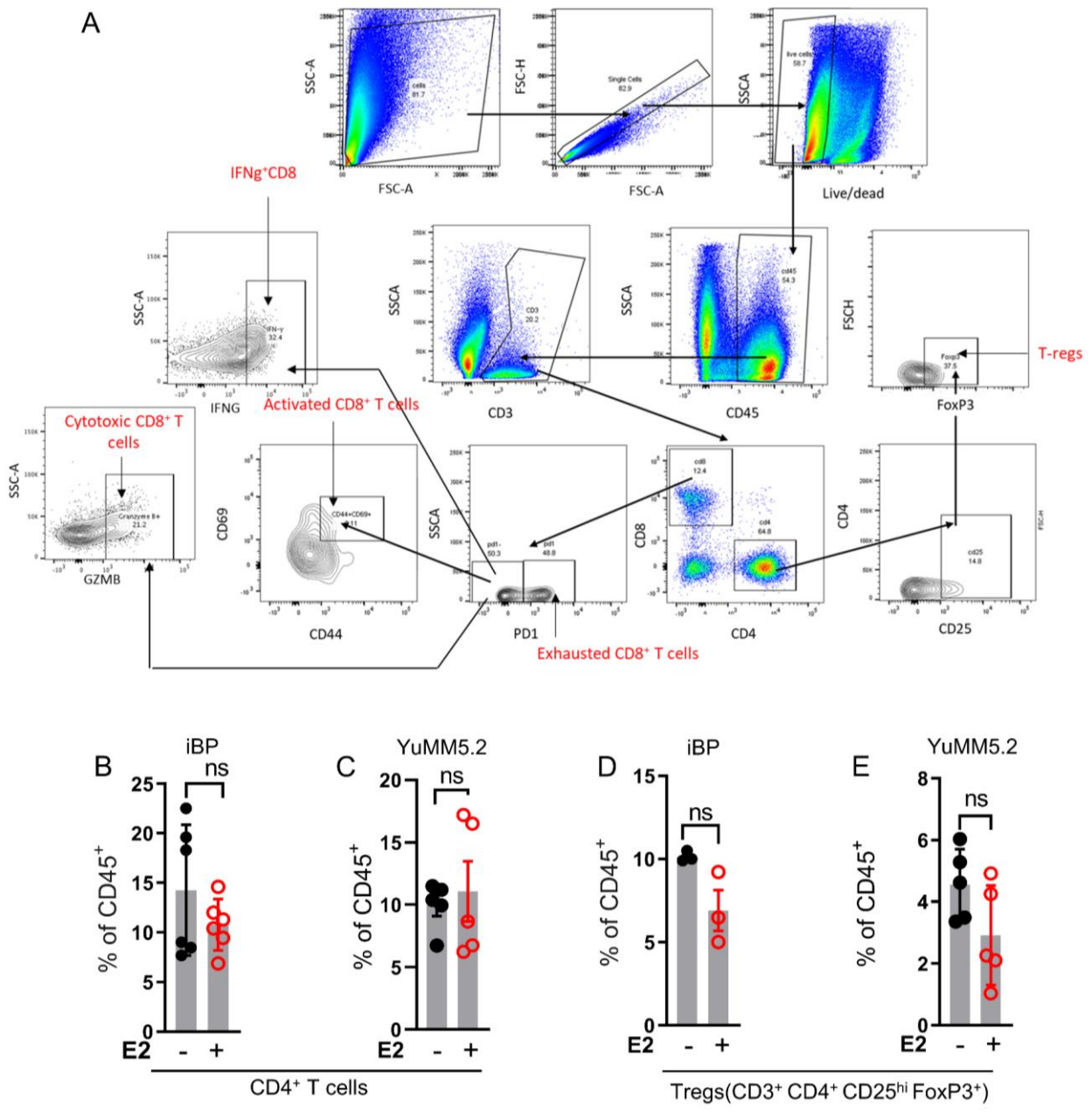
Supplemental Figure 9. Identification of monocyte/macrophage subclusters from CD68⁺ cells. Related to figure 4 (A-P) Expression of marker genes in different monocyte/macrophage subclusters. **(Q)** Comparison of the proportion of cells from each cluster shown Figure 3N in tumors from placebo (black) and E2 (red) treated mice. **(R-S)** Percentage of intratumoral ($Ly6C^+/Ly6G^-$) monocytes and $F480^+$ macrophages as determined by flow cytometry from iBP tumors treated with placebo or E2.

Supplemental Figure10



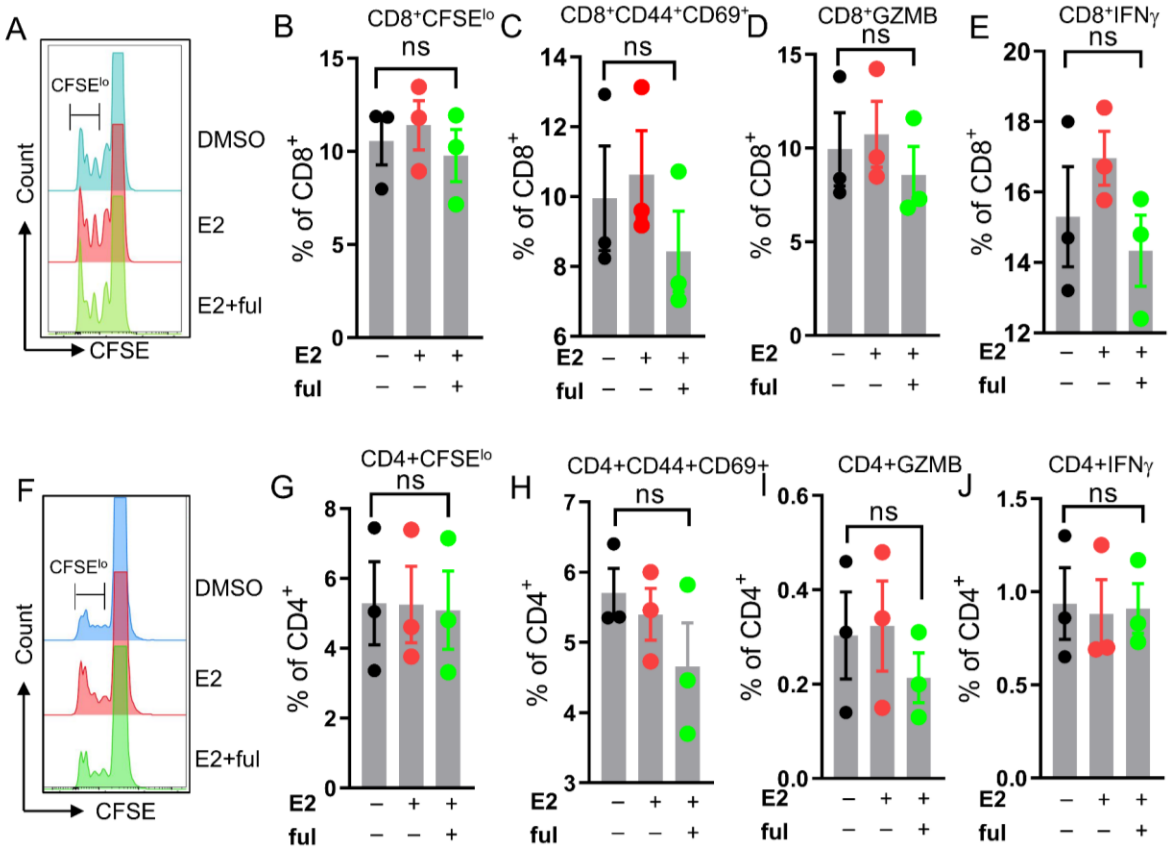
Supplemental Figure 10. E2 regulates Wnt5A/TCF4 pathways in myeloid cells. Related to Figure 4 (A-B) Bubble plots showing the upregulation of TCF4 and WNT5A signaling in tumor associated macrophages as determined by upstream regulator analysis of DEGs in the macrophage cluster by ingenuity pathway analysis (IPA) software red bubbles = upregulated; green bubbles = downregulated, solid line = direct target; dashed line = indirect target. **(C)** Quantitative real time PCR of WNT5A and TCF4 target genes in tumor infiltrating myeloid cells isolated from iBP tumors treated with placebo and E2 (n=6 per group). **(D)** Quantitative real time PCR of genes associated with angiogenesis from CD11b^{+} tumor infiltrating myeloid cells isolated from iBP tumors treated with placebo (black) and E2 (red) (n=3 per group). All target genes were normalized to Rplp0 and then E2 samples were normalized to placebo. **(E and F)** Quantitative real time PCR of genes associated with Wnt5A-β catenin targets in YuMM5.2 melanoma tumor cells depleted for *Esr1* with siRNA or treated with DMSO, E2 (1nM) or E2 (1nM) + ful (100nM). All target genes were normalized to *Rplp0* and then si*Esr1* (E) *or* DMSO (F) samples were normalized to si Control. Data are represented as mean±S.E.M. Significance is calculated by Student's t test (C and D). *p<0.05, **p<0.01 and ***p<0.001.

Supplemental Figure 11



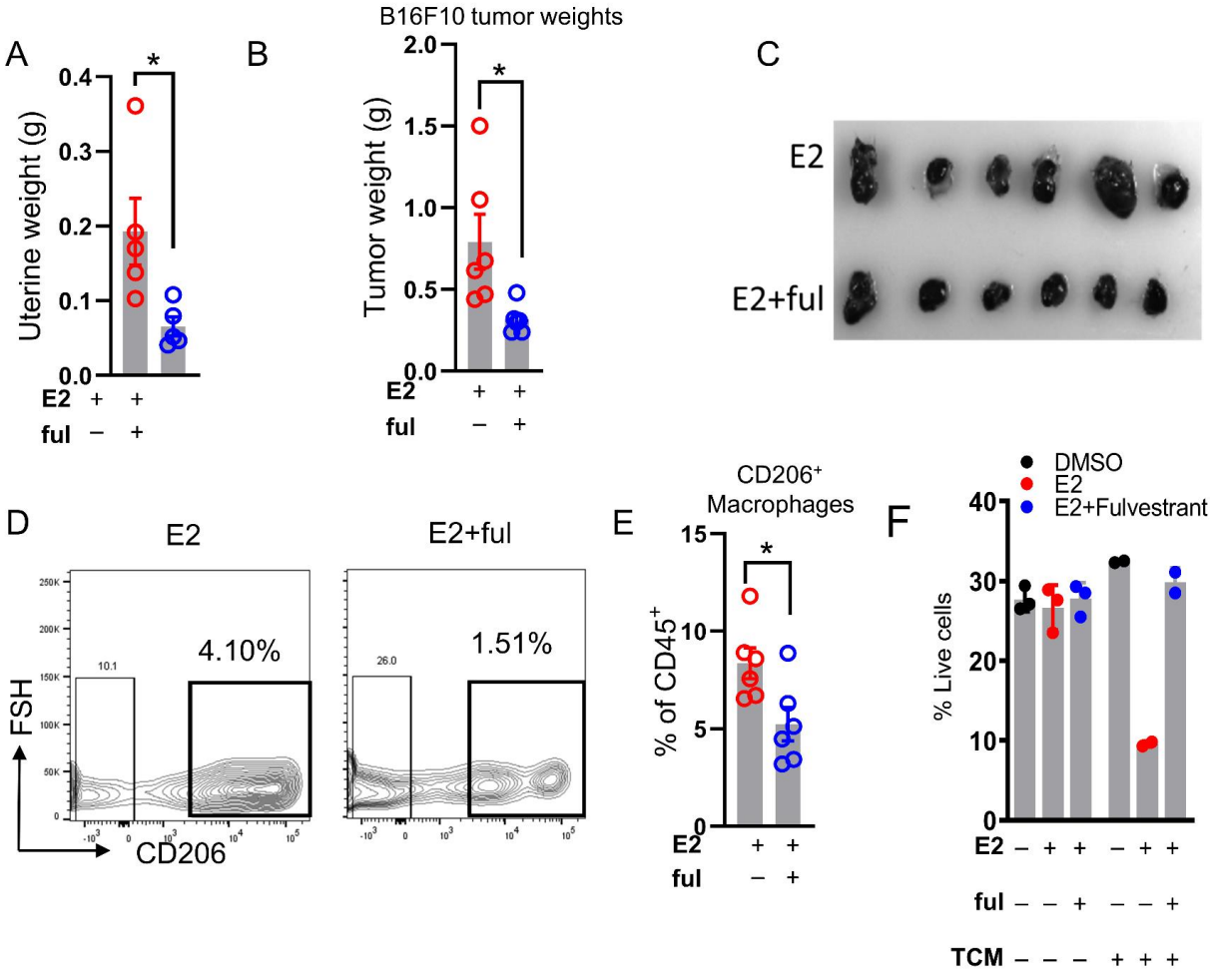
Supplemental Figure 11. E2 treatment does not affect CD4⁺ T cell infiltration. Related to Figure 5 (A) Gating strategy for identifying tumor infiltrating T cells. **(B)** Quantification of all CD3⁺ T cells from iBP tumors treated with placebo and E2 as determined by scRNAseq. **(C-D)** Quantification of percentage of tumor infiltrating CD4⁺T cells in B16F10 and YuMM5.2 tumors. **(E-F)** Quantification of percentage of tumor infiltrating $CD4^{+}CD25^{hi}FoxP3^{+}$ T cells (Tregs) in B16F10 and YuMM5.2 tumors. Data are expressed as individual data points and are represented as mean± S.E.M. Significance is calculated by Student's t test. *p<0.05, **p<0.01 and ***p<0.001

Supplemental Figure 12



Supplemental Figure 12. E2 has no direct effect on T cell functionality. Related to Figure 5 (A, B, F and G) Representative CFSE dilution plots demonstrating T cell proliferation and quantification of CFSE stain from $CD8^+$ (A-B) and $CD4^+$ (F-G) (n=3) cells. T cells were isolated from naïve WT C57BL/6J mice and sub optimally activated with CD3/D28 and IL2 for 3 days in the presence or absence of E2 (1nM) and E2 (1nM)+ful (100nM). **(C and H)** Percentage of $CD44^+CD69^+$ activated $CD8^+$ T cells (C) and $CD4^+$ T cells (H) from experiments described above. **(D and I)** Percentage of $GZMB^+$ activated $CD8^+$ T cells (D) and $CD4^+$ T cells (I) from experiments described above. **(E and J)** Percentage of $IFN\gamma^+$ activated $CD8^+$ T cells (E) and $CD4^+$ T cells (J) from experiments described above. Data are expressed as individual data points and represented as mean±S.E.M. Significance was calculated by one way ANOVA.

Supplemental Figure 13



Supplemental Figure 13. Fulvestrant treatment reverses E2 induced melanoma tumor growth. Related to Figure 6 (A) Uterine wet weights of E2 treated mice bearing B16F10 tumors and treated with either vehicle or fulvestrant. **(B-C)** Weights and photographs of tumors resulting from implanting B16F10 cells in E2 supplemented C57BL/6J mouse that were treated with either vehicle or fulvestrant (n=6). **(D-E)** Representative flow cytometry analysis of tumor infiltrating M2 macrophages isolated from B16F10 tumors treated with either vehicle or fulvestrant (D). Quantification of total CD206$^+$ macrophages from the same experiment (n=6) (E). **(F)** CFSE+ CD8 T cells in from T cells:BMDM co-cultures where BMDM were differentiated on either normal media or tumor conditioned media and then treated with DMSO, E2 (1nM) or E2 (1nM)+ful (100nM) and polarized to the M2 lineage. Data are expressed as individual data points and represented as mean±S.E.M. Significance is calculated by Student's t test (A, B, E). *p< 0.05.

Supplementary Table I.

Details of qPCR primers (mouse)

Gene id	Forward Primer	Reverse Primer
Hif1	CCTGCACTGAATCAAGAGGTTGC	CCATCAGAAGGACTTGCTGGCT
Jak2	GCTACCAGATGGAAACTGTGCG	GCCTCTGTAATGTTGGTGAGATC
B4galt	GCAACTCGACTATGGCATCTACG	CGGAATGAGGTCCACATCACTG
Stat1	GCCTCTCATTGTCACCGAAGAAC	TGGCTGACGTTGGAGATCACCA
Zeb2	GCAGTGAGCATCGAAGAGTACC	GGCAAAAGCATCTGGAGTTCCAG
Tgm2	GAAGGAACACGGCTGTCAGCAA	GATGAGCAGGTTGCTGTTCTGG
Tspo	GAGCCTACTTTGTACGTGGCGA	GCTCTTTCCAGACTATGTAGGAG
Itm2b	CATCAGTGTGCCTGTACCAGAG	GAGGAATCACGTAGCACTTGTCC
Vegfa	CTGCTGTAACGATGAAGCCCTG	GCTGTAGGAAGCTCATCTCTCC
Lcp1	TCTGTGCCAGACACGATTGACG	GAGGCAGAGTTCAGAGCCAAGT
Tcf7	CCTCTCATCACCTACAGCAACG	CTGGAGACAGTGGGTAATACGG
Esr1	AGGTGCCCTACTACCTGGAG	GTCTCTCTCGGCCATTCTGG
Cxcl12	CATCCATCCATCCATCCA	TTCAGGGTCATGGAGACAGT
Pgr	AGGTCTACCCGCCATACCTT	CGCCATAGTGACAGCCAGAT
Rplpo	AGATTCGGGATATGCTGTTGGC	TCGGGTCCTAGACCAGTGTTC

Supplementary Table II

Sequences of siRNA

Gene id	Sequences
si *Esr1*#1	AUAUUCAGAAUAGAUCAUGgg
si*Esr1*#2	UGUGCUUCAACAUUCUCCCtc
si*Esr1*#3	UGCUUAAUCACAAGAGGGCtt

Supplementary Table III.

Antibodies used for flow cytometry staining.

Reagents	Clone	Source	Catalog number	Working concentration
Live/Dead Staining dye		Invitrogen	L34964	1:200
BV650 anti mouse CD45	30-F11	Biolegend	103139	1:800
PerCpCy5.5 anti mouse CD3	17A2	BD	560572	1:50
AF647 anti human/mouse Granzyme B	GB11	Biolegend	515406	1:50
AF700 anti mouse/human CD44	IM7	Biolegend	103026	1:100
APC-Cy7 anti hamster CD69	H1.2F3	Biolegend	104526	1:100
BV650 anti rat CD8	53-6.7	Biolegend	100742	1:100
BV785 anti rat CD4	RM4-5	Biolegend	100552	1:100
BV711 anti rat IFNg	XMG1.2	Biolegend	505835	1:50
PE anti rat FOXP3	FJK-16s	eBioscience	12-5573-82	1:100
BV510 anti mouse PD1	29F1A12	Biolegend	135241	1:100
APC anti rat CD25	PC61.5	eBioscience	17-0251-82	1:100
PE anti rat CD11b	M1/70	Biolegend	101208	1:50
AF488 anti mouse CD206	C06C2	Biolegend	141710	1:100
BUV496 anti rat CD24	M1/69	BD	564664	1:100
PerCPCy5.5 anti mouse monoclonal CD64	X54-5/7.1	Biolegend	139308	1:100
APC anti rat F4/80	BM8	Biolegend	123116	1:100
APC Cy7 anti hamster CD11c	HL3	BD	561241	1:50
PE-CY7 anti rat MHCII	M5/114-15.2	eBioscience	25-5321-82	1: 600
BV711 anti rat Ly6 C	HK1.4	Biolegend	128037	1:100
BV786 anti mouse Ly6 G	1A8	Biolegend	127645	1:100