Supplemental Figures

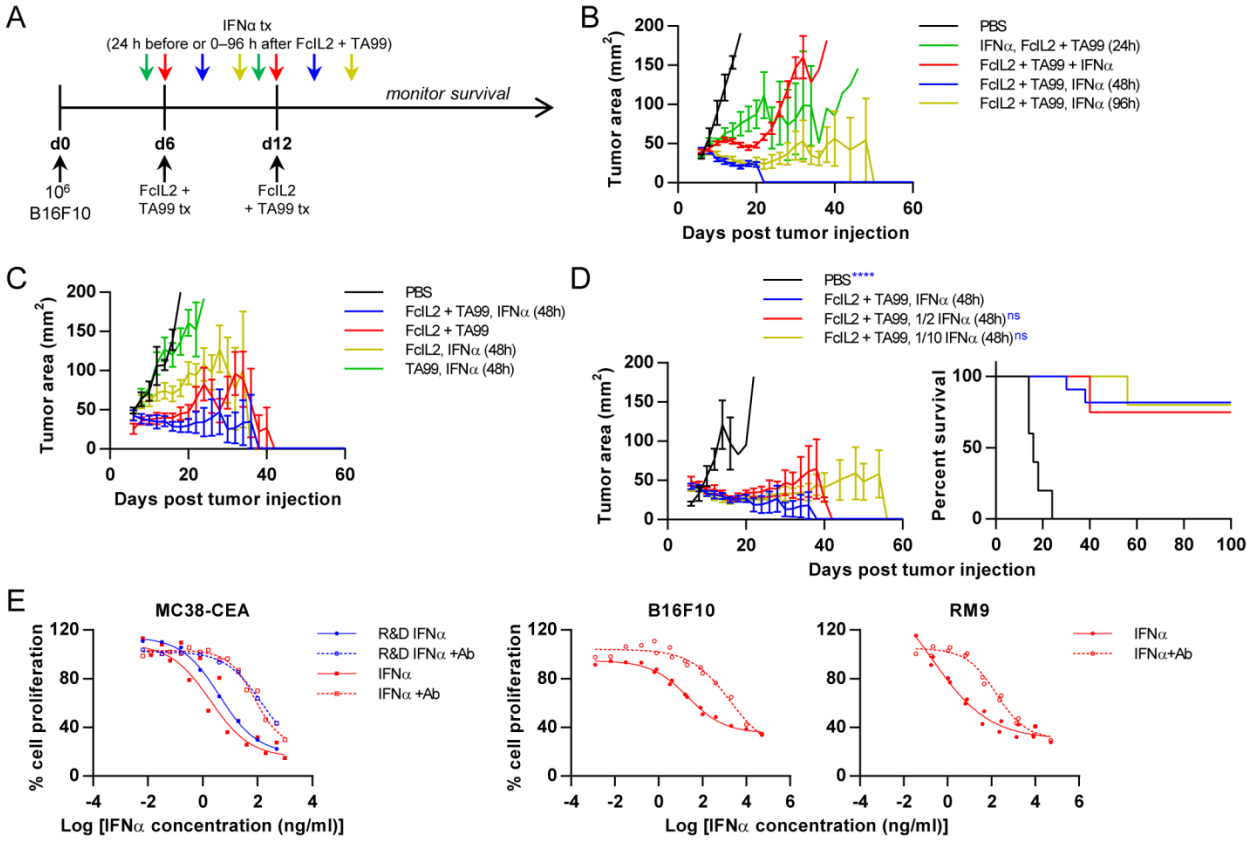

Figure S1. IFNα Inhibits *In Vitro* Tumor Cell Proliferation and Synergizes *In Vivo* with FcIL2 + TA99 in a Schedule- but Not Dose- Dependent Manner, Related to Figure 1

(A) Experimental setup for tumor survival studies. Mice were injected s.c. with 10^6 B16F10 melanoma cells, then treated on days 6 and 12 with i.v. PBS or 25 μg FcIL2 + 100 μg TA99. Mice given FcIL2 + TA99 also received 50 μg IFNα administered 24 h earlier (green arrows), simultaneously (red arrows), 48 h later (blue arrows), or 96 h later (yellow arrows).

(B and C) Tumor growth curves for mice treated as described in Figures 1A and 1B. n = 5–13 per group.

(D) Tumor growth and Kaplan-Meier survival curves for mice injected s.c. with 10^6 B16F10 melanoma cells, then treated on days 6 and 12 with i.v. PBS or 25 μg FcIL2 + 100 μg TA99 and 50, 25, or 5 μg of IFNα 48 h later. The FcIL2 + TA99, IFNα (48 h) curves comprise data pooled from Figures 1A and 1B (n = 11). For the other treatment conditions, n = 4–5 per group. ns, not significant; ****p < 0.0001 versus the corresponding color group in the legend.

(E) *Left:* MC38-CEA colon carcinoma cells were treated with varying concentrations of IFNα for 72 h, after which cell viability/proliferation were measured by WST-1 assay. The observed effects were sensitive to inhibition by an IFNα-neutralizing antibody (+Ab). Symbols indicate individual data points (normalized to control cells cultured without IFNα); lines indicate fits to a four-parameter logistic curve. IC_{50} values were determined to be 4.0 ng/ml for IFNα obtained commercially (solid blue) and 1.9 ng/ml for IFNα produced in house (solid red) from n = 4 independent experiments performed in triplicate. *Right:* In a similar fashion, the antiproliferative effects of IFNα on B16F10 melanoma and RM9 prostate cancer cells *in vitro* were assayed (n = 2 independent experiments performed in triplicate).

Data represent mean ± SEM.

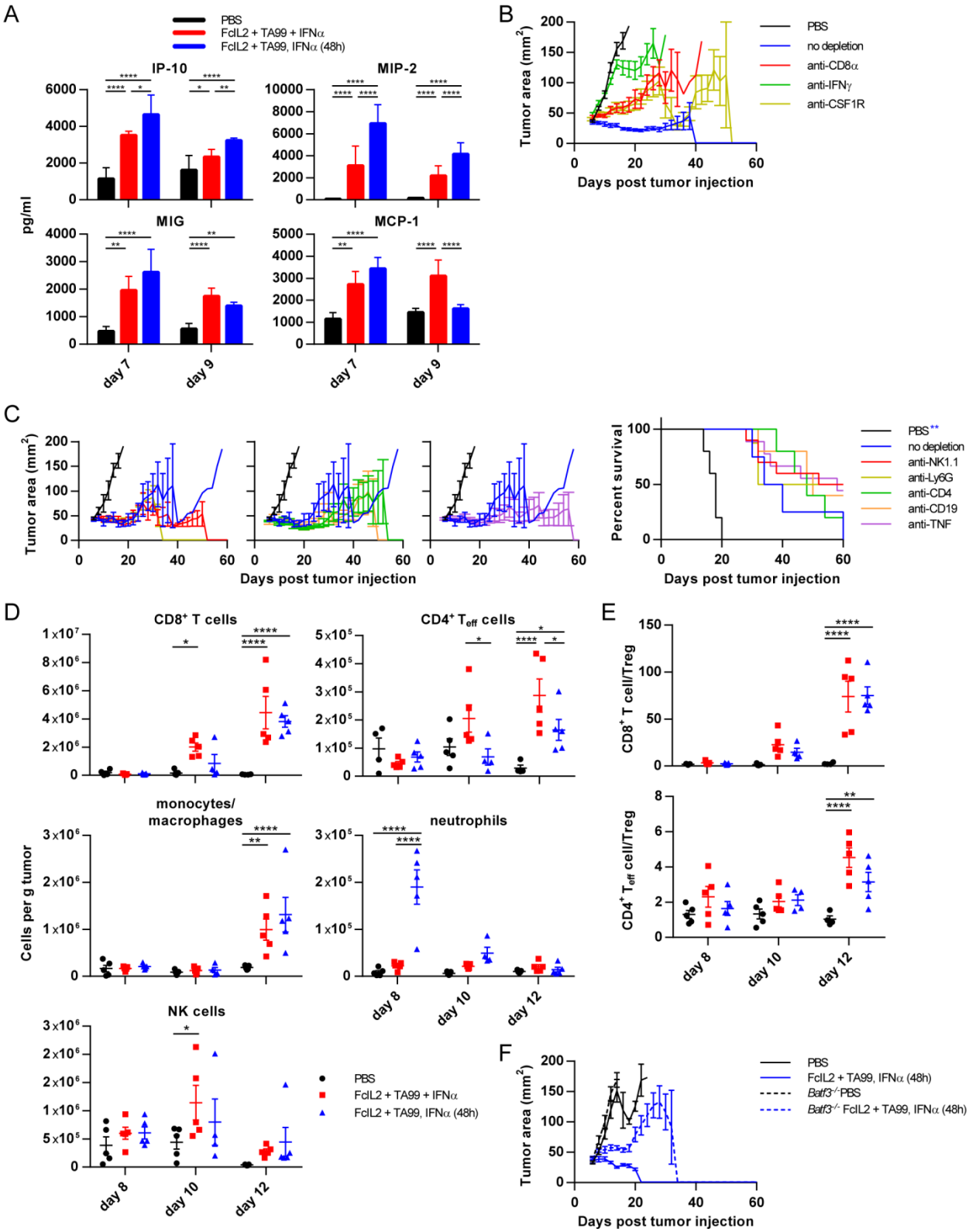
A
PBS
FcIL2 + TA99 + IFNα
FcIL2 + TA99, IFNα (48h)
IP-10
MIP-2
MIG
MCP-1
pg/ml
day 7
day 9
B
Tumor area (mm²)
Days post tumor injection
PBS
no depletion
anti-CD8α
anti-IFNγ
anti-CSF1R
C
Tumor area (mm²)
Days post tumor injection
Percent survival
PBS**
no depletion
anti-NK1.1
anti-Ly6G
anti-CD4
anti-CD19
anti-TNF
D
CD8⁺ T cells
CD4⁺ Teff cells
monocytes/ macrophages
neutrophils
NK cells
Cells per g tumor
day 8
day 10
day 12
PBS
FcIL2 + TA99 + IFNα
FcIL2 + TA99, IFNα (48h)
E
CD8⁺ T cell/Treg
CD4⁺ Teff cell/Treg
day 8
day 10
day 12
F
Tumor area (mm²)
Days post tumor injection
PBS
FcIL2 + TA99, IFNα (48h)
Batf3⁻/⁻ PBS
Batf3⁻/⁻ FcIL2 + TA99, IFNα (48h)

Figure S2. Combination Immunotherapy Induces Dynamic Chemokine Upregulation, Increases Tumor-Infiltrating Immune Cell Numbers, and Requires CD8α$^+$ Cells, CSF1R$^+$ Cells, and IFNγ, Related to Figure 1

(A) Intratumoral chemokine levels in mice injected s.c. with 10^6 B16F10 melanoma cells, then treated on day 6 with i.v. PBS or 25 μg FcIL2 + 100 μg TA99 as well as 50 μg IFNα administered simultaneously or 48 h later. On days 7 and 9, tumors were excised and homogenized for analysis using a bead-based multiplex assay. n = 4–5 per group.

(B and C) Tumor growth and Kaplan-Meier survival curves for mice injected s.c. with 10^6 B16F10 melanoma cells, then treated on days 6 and 12 with i.v. PBS or 25 μg FcIL2 + 100 μg TA99 and 50 μg IFNα 48 h later. Mice given immunotherapy were also injected i.p. with the indicated depleting or neutralizing antibodies. n = 8–15 per group for (B); n = 4–10 per group for (C).

(D) Tumors and draining lymph nodes were harvested from immunotherapy-treated, B16F10 tumor-bearing mice for the analysis of the indicated infiltrating immune cell populations by flow cytometry. Cell populations were defined as follows: $CD8^+$ T cells ($CD3^+CD8^+$); $CD4^+$ T_{eff} cells ($CD3^+CD4^+CD25^-FOXP3^-$); Tregs ($CD3^+CD4^+CD25^+FOXP3^+$); monocytes/macrophages ($CD3^-CD11b^+CD11c^{-/lo}NK1.1^-Ly6G^-SSC^{lo}$); neutrophils ($CD3^-CD11c^-CD11b^+Ly6G^+$); NK cells ($NK1.1^+CD3^-$). n = 4–5 per group.

(E) Intratumoral ratios of $CD8^+$ T cells or $CD4^+$ T_{eff} cells to Tregs for immunotherapy-treated mice bearing established s.c. B16F10 tumors. n = 4–5 per group.

(F) Tumor growth curves for mice treated as described in Figure 1D. n = 5–10 per group.

Data represent mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001 between the indicated pairs or versus the corresponding color group in the legend.

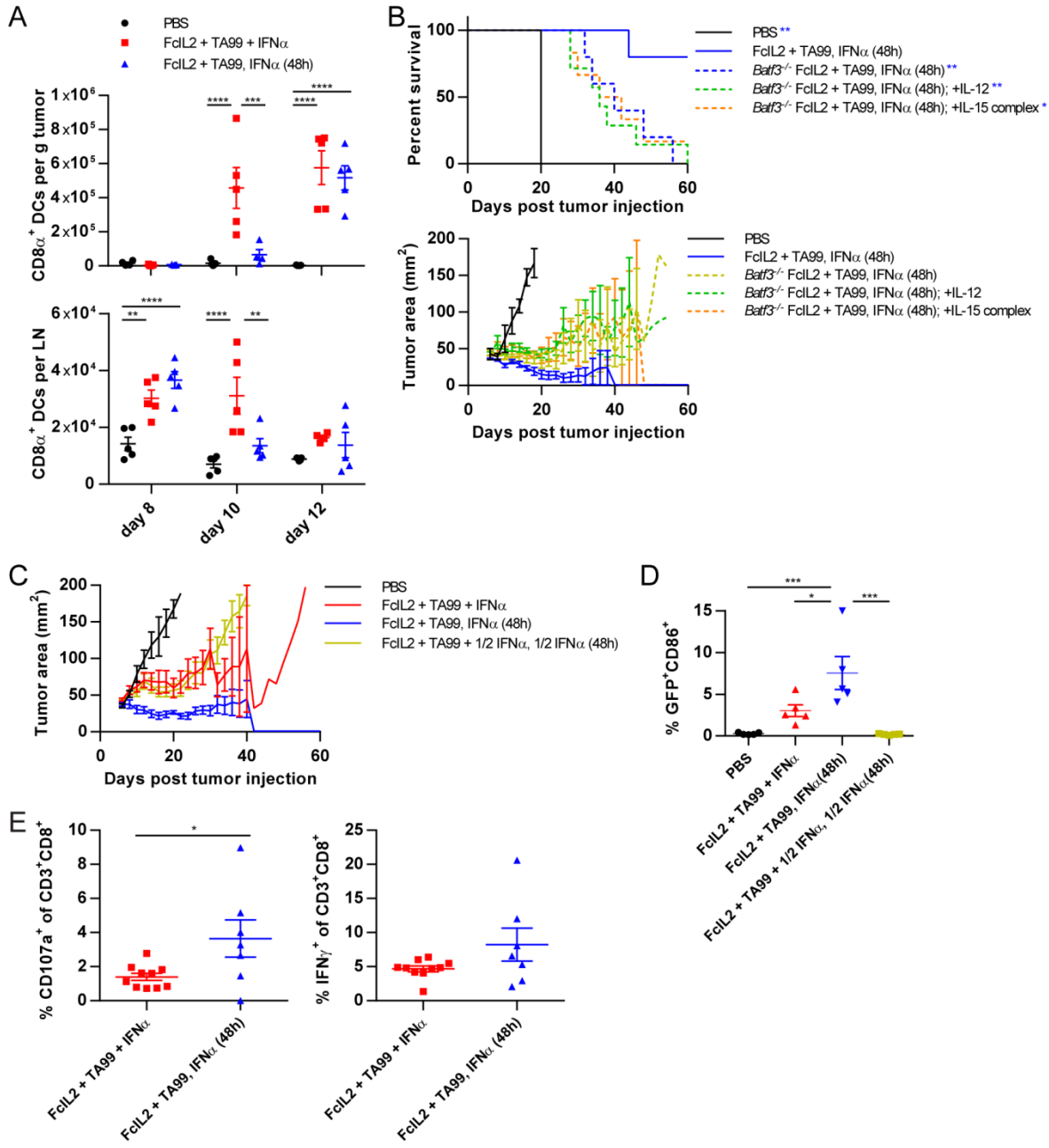

Figure S3. Absence or Premature Activation of CD8α$^+$ DCs Ablates Synergistic Antitumor Efficacy Mediated by Combination Immunotherapy and Cannot Be Rescued by IL-12 or IL-15, Related to Figure 2

(A) Tumor- or draining lymph node- infiltrating numbers of CD8α$^+$ DCs normalized to tumor mass or per lymph node for immunotherapy-treated mice bearing established s.c. B16F10 tumors. CD8α$^+$ DCs were defined as CD3$^-$CD11c^{hi}PDCA-1$^-$CD8α$^+$. n = 4–5 per group.

(B) Kaplan-Meier survival and tumor growth curves for wild-type or *Batf3*$^{-/-}$ mice injected s.c. with 10^6 B16F10 melanoma cells, then treated on days 6 and 12 with i.v. PBS or 25 μg FcIL2 + 100 μg TA99 and 50 μg IFNα 48 h later. Mice given immunotherapy were also injected i.t. with the indicated cytokines. n = 3–7 per group.

(C) Tumor growth curves for mice treated as described in Figure 2A. n = 5–10 per group.

(D) Percentages of GFP$^+$CD86$^+$ draining lymph node CD8α$^+$ DCs on day 10 from mice treated as described in Figure 2B. n = 5 per group.

(E) Percentages of intratumoral $CD8^+$ T cells expressing CD107a or IFNγ. On day 10, cells were isolated from immunotherapy-treated mice bearing established s.c. B16F10 tumors and incubated for 5 h in the presence of brefeldin A and monensin prior to flow cytometric analysis. n = 7–10 per group.
Data represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 between the indicated pairs or versus the corresponding color group in the legend.

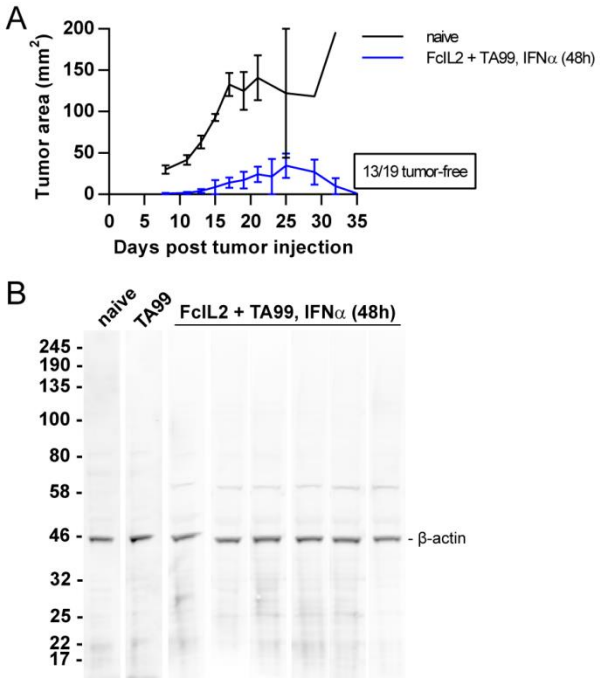

Figure S4. Characterization of Immune Memory Response Following Effective Combination Immunotherapy, Related to Figure 3

(A) Tumor growth curves for mice rechallenged as described in Figure 3A. n = 12–19 per group. Data represent mean ± SEM.

(B) β-actin loading controls for serum immunoblot shown in Figure 3D. 15 μg of B16F10 cell lysate protein was run in each lane.

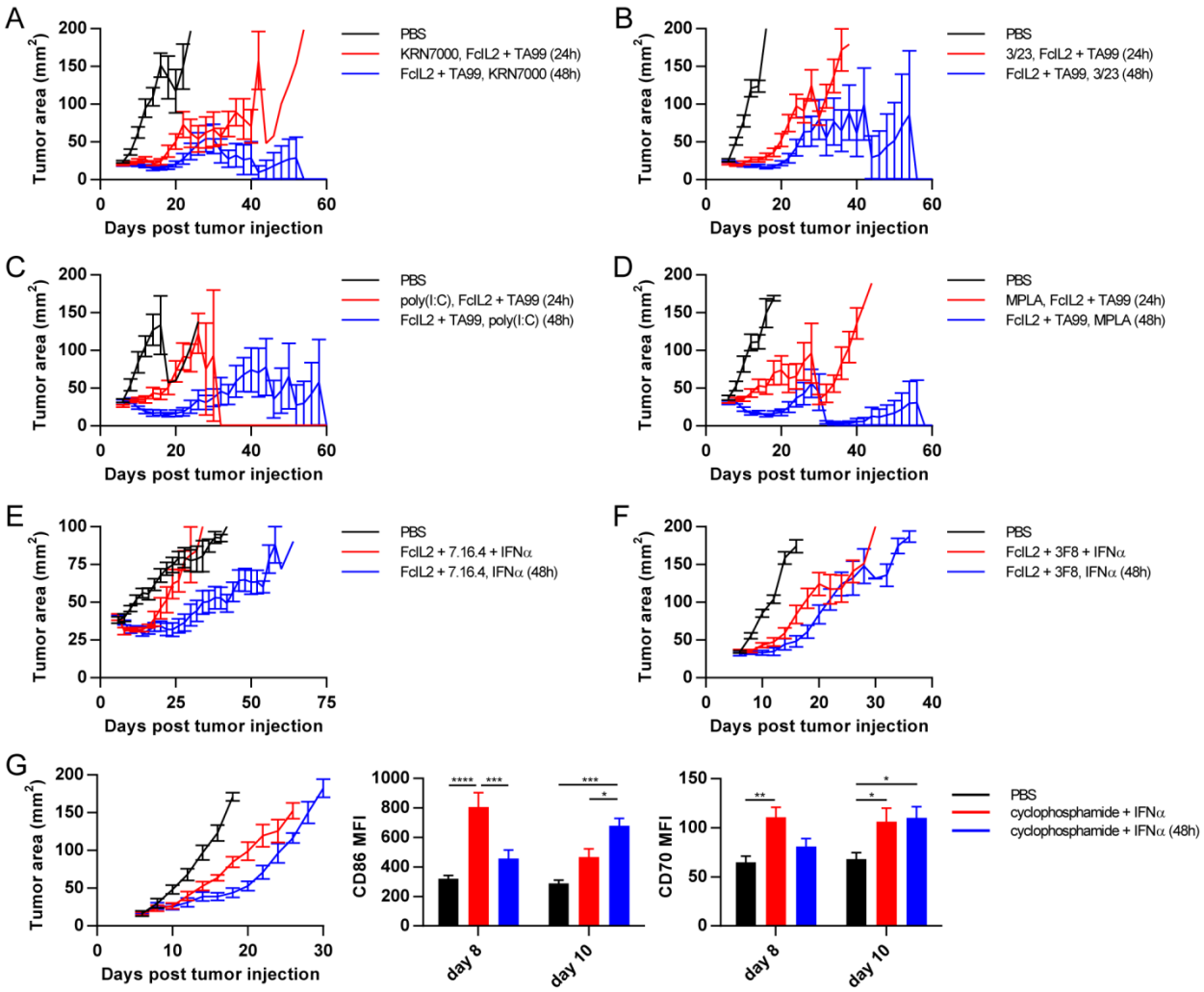

Figure S5. Effects of DC Activation Chronology on Antitumor Efficacy in Various Combination Immunotherapies, Related to Figure 4

(A–F) Tumor growth curves for mice treated as described in Figure 4. n = 5–16 per group.

(G) *Left:* Tumor growth curves for mice treated as described in Figure 4G. *Right:* MFI levels of CD86 and CD70 expression by draining lymph node CD8α$^+$ DCs from mice treated as described in Figure 4G. n = 5 per group.

Data represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 between the indicated pairs.

Supplemental Experimental Procedures

Cell Lines

B16F10 (ATCC) and HEK293-F (Life Technologies) cells were cultured according to the supplier's instructions. B16F10-GFP, DD-Her2/neu, RM9, and TC-1 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin (Life Technologies). MC38-CEA cells (Robbins et al., 1991), a gift from Dr. Jeffrey Schlom at the NCI, were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, and 0.5 mg/ml G418 (Life Technologies). The B16F10-GFP cell line was generated in house (Tzeng et al., 2015), and the RM9 prostate cancer cell line (Baley et al., 1995) was a gift from Dr. Timothy C. Thompson at the MD Anderson Cancer Center.

FcIL2 and Antibody Production

DNA inserts encoding the heavy or light chain variable regions of the anti-CD40 antibody 3/23 (White et al., 2011), a gift from Dr. Martin J. Glennie at the University of Southampton, were subcloned into a gWiz expression vector (Genlantis) containing the sequence for a murine IgG2a heavy chain constant region or a murine κ light chain constant region, respectively, to create gWiz-3/23-HC and gWiz-3/23-LC. The FcIL2 fusion protein and 3/23 antibody were expressed by transient transfection of HEK293-F cells using the FreeStyle 293 Expression System (Life Technologies). Briefly, cells were co-transfected with gWiz-Fc/IL2 and gWiz-Fc/FLAG (Zhu et al., 2015; FcIL2) or gWiz-3/23-HC and gWiz-3/23-LC (3/23) plasmids using polyethylenimine and OptiPRO (Life Technologies). The anti-TRP1 TA99 antibody was produced by a stable HEK293-F cell line. Cell culture supernatants were harvested 7–10 days post transfection (centrifugation at 15,000×*g* for 30 min at 4°C), passed through a 0.22-μm filter, and purified by protein A chromatography according to the manufacturer's instructions (Thermo Scientific). Purified proteins were quantified by measuring absorbance at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Molecular weights were confirmed by reducing and non-reducing SDS-PAGE using NuPAGE 4-12% Bis-Tris gels (Life Technologies) in MOPS buffer followed by Coomassie staining. All proteins were verified to contain minimal endotoxin (< 0.1 total EU/dose) using the QCL-1000 chromogenic LAL assay (Lonza).

IFNα Production

A DNA insert encoding murine IFNα A (Open Biosystems) was subcloned into the pE-SUMOpro expression vector (LifeSensors) using a modified *Pfu* polymerase-mediated site-directed mutagenesis protocol (Geiser et al., 2001). The resulting plasmid was transformed into Rosetta-gami 2 (DE3) competent cells (Novagen), and single colonies were inoculated into LB broth containing chloramphenicol, kanamycin, and tetracycline and cultured overnight at 37°C. After 1:300 dilution of the overnight culture in fresh LB broth containing 2 g/L glucose, cells were allowed to grow at 37°C until reaching an OD_{600} of 0.6–0.8. Subsequently, 0.5 mM IPTG was added, and cells were induced for 24 h at 20°C. Cultures were collected by centrifugation (15,000×*g* for 15 min at 4°C), resuspended in 50 mM sodium phosphate/300 mM NaCl/5% glycerol pH 7.0 with EDTA-free cOmplete protease inhibitor cocktail (Roche), and lysed by mild sonication. Clarified cell lysate (centrifugation at 75,000×*g* for 50 min at 4°C) was purified by IMAC using TALON metal affinity resin according to the manufacturer's instructions (Clontech Laboratories). Purified SUMO-IFNα protein was incubated with SUMO protease as detailed previously (Malakhov et al., 2004) and dialyzed against 20 mM Tris-HCl/150 mM NaCl pH 8.0 overnight at 4°C. The sample was then reapplied onto TALON resin to remove SUMO and SUMO protease, and the flowthrough fraction containing purified IFNα was quantified by measuring absorbance at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The molecular weight was confirmed by reducing and non-reducing SDS-PAGE using NuPAGE 4-12% Bis-Tris gels (Life Technologies) in MES buffer followed by Coomassie staining. Finally, the protein was passed through Detoxi-Gel endotoxin removal resin (Thermo Scientific) until endotoxin levels were below 0.1 total EU/dose as measured by the QCL-1000 chromogenic LAL assay (Lonza).

Tumor Cell Proliferation Assay

MC38-CEA, B16F10, or RM9 cells were seeded into flat-bottom 96-well plates at a density of 3,000 cells/well in 100 μl complete medium. Cells were incubated with varying concentrations of murine IFNα (made in house or purchased from R&D Systems) for 72 h, after which cell proliferation was determined using a WST-1-based

colorimetric assay according to manufacturer's instructions (Roche). 2 μg IFNα-neutralizing antibody (RMMA-1; PBL Interferon Source) was added to a corresponding set of wells to verify that antiproliferative effects were IFNα specific. To determine IC_{50} values, data were normalized to that of control cells cultured without IFNα and fit to a four-parameter logistic curve using GraphPad Prism 6 software.

Vaccination Assay

C57BL/6 mice were immunized with 250 μg endotoxin-free ovalbumin (Worthington Biochemical) subcutaneously at the flank and were treated with 50 μg IFNα either 24 h before or after immunization. 7 days later, T cell responses were quantified using tetramer staining. Briefly, PBMCs were isolated and incubated with PE-conjugated H-2 Kb SIINFEKL tetramer (MBL International) and Fc block (93; eBioscience) for 30 min at room temperature, then for an additional 10 min at 4°C with APC-conjugated anti-CD8α antibody (53-6.7; eBioscience). Cells were washed in buffer containing DAPI and analyzed on a BD FACSCanto flow cytometer. The percentage of live, tetramer-positive $CD8^+$ T cells was quantified using FlowJo v.10.0.7 software (Tree Star).

Antibody Depletions/Neutralizations and Tumor Treatment

Depleting or neutralizing antibodies (Bio X Cell) were administered intraperitoneally to mice at doses equal to or greater than those previously reported to deplete or neutralize their targets (Zhu et al., 2015). Specifically, 600 μg of anti-NK1.1 (PK136), anti-Ly6G (1A8), anti-CD8α (2.43), anti-CD4 (GK1.5), or anti-CD19 (1D3) antibody were injected on day 4 after tumor inoculation and every 3 days thereafter for a total of four doses. 600 μg of anti-CSF1R (AFS98) antibody were injected on day 4 after tumor inoculation and every 2 days thereafter for a total of six doses. 600 μg of anti-IFNγ (XMG1.2) or anti-TNF (XT3.11) antibody were injected on day 5 after tumor inoculation and every 2 days thereafter for a total of five doses. Supplemental cytokines were administered intratumorally to mice at the maximum tolerated dose. 10 ng of recombinant mouse IL-12 (Biolegend) or IL-15/IL-15R complex (eBioscience) were injected on day 8 after tumor inoculation and every 2 days thereafter for a total of five doses. For evaluating the efficacy of other combination immunotherapies, B16F10 tumors were induced as detailed previously and treated on days 6 and 12 post inoculation with the following regimens: 25 μg FcIL2 + 100 μg TA99 retroorbitally, and 25 ng KRN7000 (Cayman Chemical) retroorbitally either 24 h earlier or 48 h later; 25 μg FcIL2 + 100 μg TA99 retroorbitally, and 100 μg 3/23 retroorbitally either 24 h earlier or 48 h later; 25 μg FcIL2 + 100 μg TA99 retroorbitally, and 10 μg poly(I:C) (high molecular weight; InvivoGen) retroorbitally either 24 h earlier or 48 h later; 25 μg FcIL2 + 100 μg TA99 retroorbitally, and 2 μg MPLA (InvivoGen) retroorbitally either 24 h earlier or 48 h later; or 150 mg/kg cyclophosphamide (Sigma-Aldrich) intraperitoneally, and 50 μg IFNα retroorbitally either concurrently or 48 h later. For DD-Her2/neu tumor induction, 10^6 DD-Her2/neu breast cancer cells were injected subcutaneously into the flanks of BALB/c mice. Mice were treated retroorbitally on days 6 and 12 after tumor inoculation with 25 μg FcIL2 + 100 μg 7.16.4 (ATCC), and 50 μg IFNα retroorbitally either concurrently or 48 h later. For RM9 tumor induction, 2.5×10^5 RM9 prostate cancer cells were injected subcutaneously into the flanks of C57BL/6 mice. Mice were treated retroorbitally on days 6 and 12 after tumor inoculation with 25 μg FcIL2 + 100 μg 3F8 (Zhang et al., 1998; a gift from Dr. Nai-Kong V. Cheung at the Memorial Sloan Kettering Cancer Center), and 50 μg IFNα retroorbitally either concurrently or 48 h later.

Tumor Rechallenge

B16F10 tumor-bearing mice treated with FcIL2 + TA99, and IFNα 48 h later as detailed previously that survived the initial tumor challenge were injected with 10^5 B16F10 melanoma cells subcutaneously in the opposite flank 97–102 days following the primary challenge. As a control, naïve C57BL/6 mice received the same B16F10 cell inoculum. Tumor length and width were measured using calipers, and mice were euthanized when tumors reached 200 mm^2.

Flow Cytometry

B16F10 tumors were induced as detailed previously. On day 6 post inoculation, mice were injected retroorbitally with 25 μg FcIL2 + 100 μg TA99 or 150 mg/kg cyclophosphamide (Sigma-Aldrich), and 50 μg IFNα either concurrently or 48 h later. On days 8, 10, or 12, tumors and/or draining lymph nodes were harvested, weighed, mechanically dissociated between frosted glass slides, and rendered into single cell suspensions by filtration through 70-μm mesh screens. For dendritic cell analysis, samples were treated with 1 mg/ml collagenase D and 20 μg/ml DNase I (Roche) in RPMI-1640 with 2% FBS (Life Technologies) for 45 min at 37°C. Cells were incubated with

Zombie Aqua dye to exclude dead cells, followed by TruStain fcX (93) and the following fluorochrome-conjugated antibodies for 30 min at room temperature: anti-CD3ε (145-2C11), anti-CD4 (GK1.4), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD25 (PC61), anti-CD69 (H1.2F3), anti-CD70 (FR70), anti-CD86 (GL-1), anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-NK1.1 (PK136), anti-PDCA-1 (927), all from BioLegend. For intracellular Foxp3 staining, cells were fixed and permeabilized with the eBioscience Foxp3 staining buffer set according to the manufacturer's instructions and stained with PE-conjugated anti-Foxp3 (MF-14) or isotype control antibodies (BioLegend). Doublets were excluded based on FSC-W/FSC-H and SSC-W/SSC-H. Samples were analyzed using a BD LSR II flow cytometer, and data were evaluated using FlowJo v.10.0.7 software (Tree Star).

Intracellular Cytokine Staining

B16F10 tumors were induced as detailed previously. For intratumoral $CD8^+$ T cell analysis, tumors were harvested on day 10 post inoculation from mice treated as described above. Tumors were then weighed and rendered into single cell suspensions by mechanical dissociation through 70-µm mesh screens. Cells were incubated in complete RPMI-1640 containing 5 µg/ml each of brefeldin A and monensin (BD), as well as APC-conjugated anti-CD107a (1D4B) or isotype control antibodies (Biolegend), for 5 h at 37°C. For peripheral $CD8^+$ T cell analysis, blood was collected from treated or rechallenged mice by submandibular bleed into K_2EDTA-coated MiniCollect tubes (Greiner Bio-One) on day 12 post inoculation or day 8 post rechallenge. After treatment with ACK lysing buffer (Life Technologies), cells were incubated in complete RPMI-1640 containing 5 µg/ml each of brefeldin A and monensin (BD), with or without PMA/ionomycin Cell Stimulation Cocktail (eBioscience), for 6 h at 37°C. Following surface staining as described above, intratumoral and peripheral blood cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit according to the manufacturer's instructions and stained with PE-conjugated anti-IFNγ (XMG1.2) or isotype control antibodies (BioLegend). Doublets were excluded based on FSC-W/FSC-H and SSC-W/SSC-H. Samples were analyzed using a BD LSR II flow cytometer, and data were evaluated using FlowJo v.10.0.7 software (Tree Star).

DC Phagocytosis Assay

10^6 B16F10-GFP melanoma cells in 100 µl PBS were injected subcutaneously into the flanks of C57BL/6 mice. On day 6 post inoculation, mice were treated retroorbitally with 25 µg FcIL2 + 100 µg TA99, and 25 or 50 µg IFNα given concurrently and/or 48 h later. On days 8 or 10, draining lymph nodes were harvested, weighed, mechanically dissociated between frosted glass slides, and rendered into single cell suspensions by filtration through 70-µm mesh screens. Samples were treated with 1 mg/ml collagenase D and 20 µg/ml DNase I (Roche) in RPMI-1640 with 2% FBS (Life Technologies) for 45 min at 37°C. Subsequently, surface staining and flow cytometry analysis were performed as detailed above.

Multiplex Chemokine Assay

B16F10 tumors were induced as detailed previously. On day 6 post inoculation, mice were injected retroorbitally with 25 µg FcIL2 + 100 µg TA99, and 50 µg IFNα either concurrently or 48 h later. On days 7 or 9, tumors were harvested, immediately flash frozen in liquid nitrogen, and stored at −80°C until processing. Frozen samples were placed into chilled 2-ml homogenization tubes containing 3.0 mm zirconium beads (Benchmark Scientific). Cold PBS with cOmplete protease inhibitor cocktail (Roche) was added in a ratio of 4 µl/mg tissue, and the samples were homogenized using a Mini-Beadbeater-16 (BioSpec Products). After centrifugation (14,000×g for 15 min at 4°C), supernatant aliquots were taken for protein quantification using a BCA assay kit (Thermo Scientific). PBS with protease inhibitor was added to normalize the protein concentrations of all supernatant samples, and samples were flash frozen in liquid nitrogen and stored at −80°C until analysis. Samples were evaluated in triplicate for chemokine expression using a Luminex assay (Eve Technologies).

Serum Immunoblot

Three to five weeks post tumor rechallenge as described above, sera were collected in Microtainer tubes with serum separator (BD). Samples were flash frozen in liquid nitrogen and stored at −80°C until analysis as described previously (Beatty et al., 2014). Briefly, whole cellular lysate was prepared from B16F10 cells using Pierce IP lysis buffer (Thermo Scientific) according to the manufacturer's instructions, and protein content was determined using a BCA assay kit (Thermo Scientific). 15 µg/well of extracted protein were run on reducing NuPAGE 4-12% Bis-Tris

gels (Life Technologies) in MOPS buffer and transferred to nitrocellulose membranes (Bio-Rad) using a NuPAGE blot module (Life Technologies). After blocking with Odyssey blocking buffer (TBS; LI-COR Biosciences) for 1 h at room temperature, membranes were incubated with 1:1000 diluted serum and 1:500 rabbit anti-β-actin antibody (Biolegend) overnight at 4°C. 2 μg/ml TA99 antibody was used in place of serum as a positive control. Membranes were probed with 1:10,000 diluted IRDye 800CW-conjugated goat anti-mouse and IRDye 680RD-conjugated goat anti-rabbit antibodies (LI-COR Biosciences) for 1 h at room temperature and air-dried prior to imaging on a LI-COR Odyssey Infrared Imaging System.

IFNγ ELISPOT

B16F10 or TC-1 tumor cells were cultured overnight in the presence of 500 U/ml IFNγ and irradiated with 120 Gy. On day 6 post rechallenge as described above, spleens were harvested, rendered into single cell suspensions by mechanical dissociation through 70-μm mesh screens, and treated with ACK lysing buffer (Life Technologies). 10^6 splenocytes were incubated with 2.5×10^4 irradiated tumor cells for 24 h at 37°C prior to visualization using the Mouse IFNγ ELISPOT kit (BD) according to the manufacturer's instructions. Splenocytes, tumor cells, and media were also assessed individually. Spots were scanned and quantified on a C.T.L.-Immunospot S6 Macro Analyzer.

Graphical Illustration

The Graphical Abstract and Figure 5 were created using elements from Servier Medical Art: http://www.servier.fr/smart/banque-dimages-powerpoint.

Statistical Analysis

Univariate analysis of survival differences was performed using the Mantel-Cox method. Comparisons among flow cytometry, DC phagocytosis, and multiplex chemokine assay data from different treatment groups were made using two-way ANOVA with post-hoc Tukey test if multiple time points were analyzed or one-way ANOVA with post-hoc Tukey test if a single time point was analyzed. Intracellular cytokine staining results were evaluated using unpaired two-tailed t tests for comparisons between two groups and one-way ANOVA with post-hoc Tukey test for comparisons among three or more groups. Significance was determined for ELISPOT data using two-way ANOVA with post-hoc Sidak test.

Supplemental References

Baley, P.A., Yoshida, K., Qian, W., Sehgal, I., and Thompson, T.C. (1995). Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Bio *52*, 403-413.

Beatty, G.L., Haas, A.R., Maus, M.V., Torigian, D.A., Soulen, M.C., Plesa, G., Chew, A., Zhao, Y., Levine, B.L., Albelda, S.M., *et al.* (2014). Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce antitumor activity in solid malignancies. Cancer Immunol Res *2*, 112-120.

Geiser, M., Cebe, R., Drewello, D., and Schmitz, R. (2001). Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. Biotechniques *31*, 88-92.

Malakhov, M.P., Mattern, M.R., Malakhova, O.A., Drinker, M., Weeks, S.D., and Butt, T.R. (2004). SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. J Struct Func Genomics *5*, 75-86.

Robbins, P.F., Kantor, J.A., Salgaller, M., Hand, P.H., Fernsten, P.D., and Schlom, J. (1991). Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line. Cancer Res *51*, 3657-3662.

Tzeng, A., Kwan, B.H., Opel, C.F., Navaratna, T., and Wittrup, K.D. (2015). Antigen specificity can be irrelevant to immunocytokine efficacy and biodistribution. Proc Natl Acad Sci U S A *112*, 3320-3325.

Zhang, H., Zhang, S., Cheung, N.K., Ragupathi, G., and Livingston, P.O. (1998). Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases. Cancer Res *58*, 2844-2849.