Supplemental Information

Supplemental Figure Legends

Figure S1. Kaplan-Meier plots of overall survival from the S1320 trial stratified by prior exposure to ICT, Related to Figure 1. Results from univariate Cox regression model.

Figure S2. Impacts of treatment regimens containing anti-PD-L1 and/or MEKi on tumor cells, CD45$^+$ cells, CD45$^+$ sub-populations, and CD8$^+$ T-cell sub-populations, Related to Figure 2.

(A) Immunofluorescence of p-ERK levels in tumors of indicated tumor models, with or without trametinib treatment (3 days). DAPI, nuclear stain. Scale bar, 50 μm. Representative images shown for two of five tumors analyzed per group.

(B) Tumor volumes of mSK-Mel254 treated with indicated regimens and on the timelines in Figure 2A. Trametinib at 3 mg/kg/d. Anti-PD-L1 (200 μg/mouse) and anti-CTLA-4 (200 μg/mouse), both twice per week IP. N = 8 tumors/group. Data are means ± SEMs (P-values, Student t-test).

(C, D, E, G) Frequencies of CD45$^+$ population in the total live cells (C), immune cell types in the CD45$^+$ population (D), CD4$^+$ Th1-like T-cells in the CD45$^+$ population (E), and CD8$^+$ subsets in the CD8$^+$ population (G) of three syngeneic tumor models. Mean± SEMs. Pairwise comparisons were performed in (i) vehicle *vs.* two doses of anti-PD-L1, (ii) MEKi starting d7 *vs.* anti-PD-L1 d0-d7, MEKi starting d7, (iii) MEKi+anti-PD-L1 starting d7 *vs.*

anti-PD-L1 d0-d7, anti-PD-L1+MEKi starting d7. P-value, Student's *t* test. *p < 0.05, **p < 0.01 and ***p < 0.001. See Figure 2A.

(F) Heatmaps showing the expression values of immune phenotypic protein markers across multiple subsets in tumor-infiltrating $CD8^+$ cells analyzed by CyTOF in three indicated syngeneic SC tumor models. The expression values of each marker were normalized to the maximum mean value across subsets. T_{CM} (central memory): $CD62L^+CD44^+GZMB^-$; T_{EM} (effector memory): $CD62L^-CD44^+GZMB^-$; T_C (cytotoxic): $GZMB^+$; T_{TD} (terminally differentiated): $CD62L^-CD44^-GZMB^-$.

Figure S3. Coupled scRNA-seq/scTCR-seq analysis of mSK-Mel254 tumors from mice undergoing anti-PD-L1 and/or MEKi treatment regimens, Related to Figure 3.

(A, B) UMAP in Figure 3A colored by single-cell expression patterns of indicated cell lineage markers (A) and TCR (B).

(C to E) Frequencies of immune subpopulations in the $CD45^+$ population (C), Mo/MΦ subpopulations among $CD45^+$ cells (D), subpopulations among $CD4^+$ T-cells (E, top) and $CD8^+$ T-cells (E, bottom). See Figure 2A.

(F) Boxplots of *Tcf1*$^+$ stem-like gene signature scores in $CD8^+$ T-cell subsets. Pairwise comparisons were performed in (i) vehicle *vs*. anti-PD-L1 d0-d7, (ii) MEKi d7 *vs*. anti-PD-L1 d0-d7, MEKi d7, (iii) anti-PD-L1+MEKi d7 *vs*. anti-PD-L1 d0-d7, anti-PD-L1+MEKi d7. P-value, Wilcoxon test. *p < 0.05, ***p < 0.001. See Figure 2A.

(G) Heatmap displaying the scaled mean expression levels of highlighted genes (rows) in each treatment regimen group and time point (column) for T_C, T_{REG}, T_{H1}/T_{H2}, NKT and

$T_{\gamma\delta}$ subpopulations. See Figure 2A. Gene expression levels were row-scaled across $CD8^+$ T_C, T_{REG}, T_{H1}/T_{H2}, NKT, $T_{\gamma\delta}$, and Ki-$67^{hi}CD8^+$ subpopulations.

Figure S4. Anti-PD-L1 lead-in before MAPKi co-treatment optimizes suppression of extracranial and intracranial metastatic progression and augments T-cell clonal expansion, Related to Figures 5 and 6.

(A) *In vivo* BLI of representative mice bearing metastatic YUMM1.7ER cells from the untreated group on indicated timepoints. All images were adjusted to the same radiance scale.

(B) *Ex vivo* photo (left) and BLI (right) at necropsy of a mouse from the untreated group on day 23 post-IC injection. All images were adjusted to the same radiance scale.

(C, D) Temporal BLI quantification (radiance, photons/sec) based on the dosing timeline in Figure 5A of ventral extracranial (C) or intracranial (D) tumor burden. Data are mean ± SD based on the indicated numbers of mice in the untreated and treatment regimen groups. Pairwise comparisons (mixed model framework with Bonferronic correction for multiple testing) of the group MEKi d0, anti-PD-L1 d-4 *vs*. the group denoted by the specific color of symbol #. # $p = 0.05\text{-}0.001$, ## $p < 0.001$. *Indicates death of mouse or mice, resulting in drops in mean BLI values. True complete responses or CRs defined in STAR Methods.

(E) Flowchart showing the status of surviving mice from experiment in Figure 5G as they were monitored and grouped beyond 10 weeks (post MAPKi treatment initiation). Specific

mice (number and color designations in F) and their final status at the end of long-term follow-ups are shown.

(F) Schematic of the individual natural histories of long-term surviving mice from experiment in Figure 5G (treatment regimens coded by orange and red colors) and their disease, treatment, and vital status.

(G) Tumor cell-involved brain and ovarian tissues were collected from mice at time points and in groups as indicated in Figure 5A (brain, n = 2 mice per group, except the no treatment group; ovary, n = 1 mouse per group; two geographically distinct regions of each organ site were sampled for TCR-seq analysis). Dot plot showing the diversity and gini clonality indices for α or β chain in brain or ovarian tissues (red dots, average values). Pairwise comparison was performed between MEKi d0, anti-PD-L1 d-4 and each of the other treatment groups with Student's *t* test. P-values, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(H) The total sizes of large TCR clones (≥ 8%) for the α or β chain in tumor-involved brain or ovarian tissues (red dots, average values). Pairwise comparisons were performed as in E.

(I) The total sizes of largest, top 5, and top 10 TCR clones (≥ 8%) for the α or β chain in tumor-involved brain or ovarian tissues (red dots, average values). Pairwise comparisons were performed as in E.

(J) Venn plots showing the overlapping fractions of TCR clones (α or β chain) between brain and ovarian tissues in each individual.

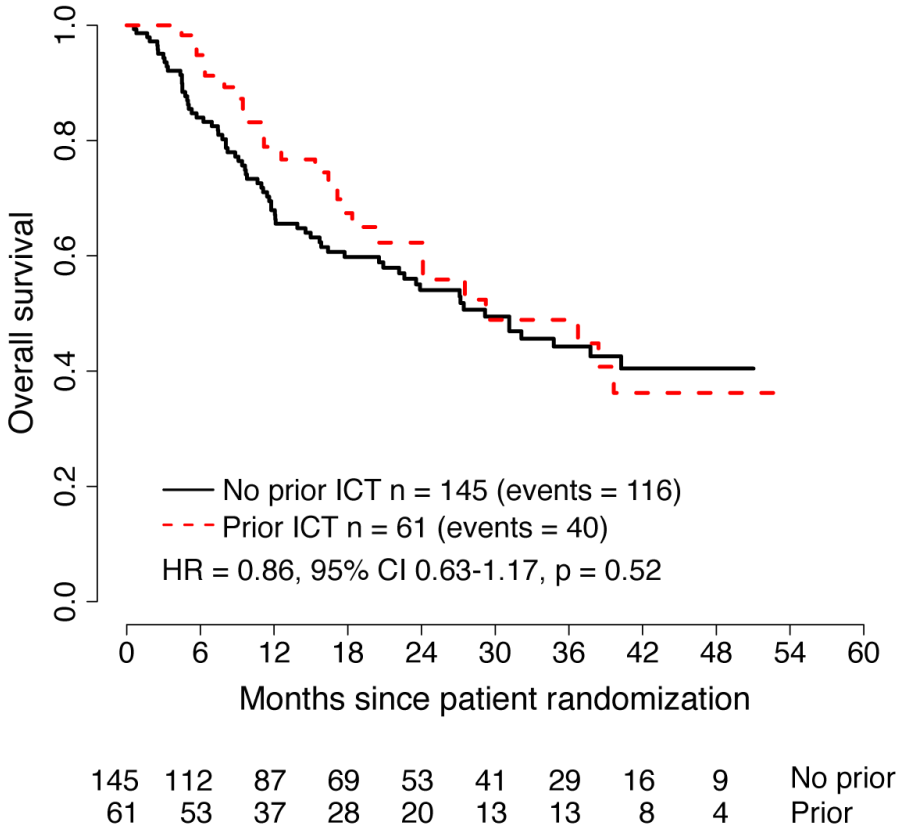
Overall survival
0.0
0.2
0.4
0.6
0.8
1.0
No prior ICT n = 145 (events = 116)
Prior ICT n = 61 (events = 40)
HR = 0.86, 95% CI 0.63-1.17, p = 0.52
0 6 12 18 24 30 36 42 48 54 60
Months since patient randomization
145 112 87 69 53 41 29 16 9 No prior
61 53 37 28 20 13 13 8 4 Prior

Figure S1

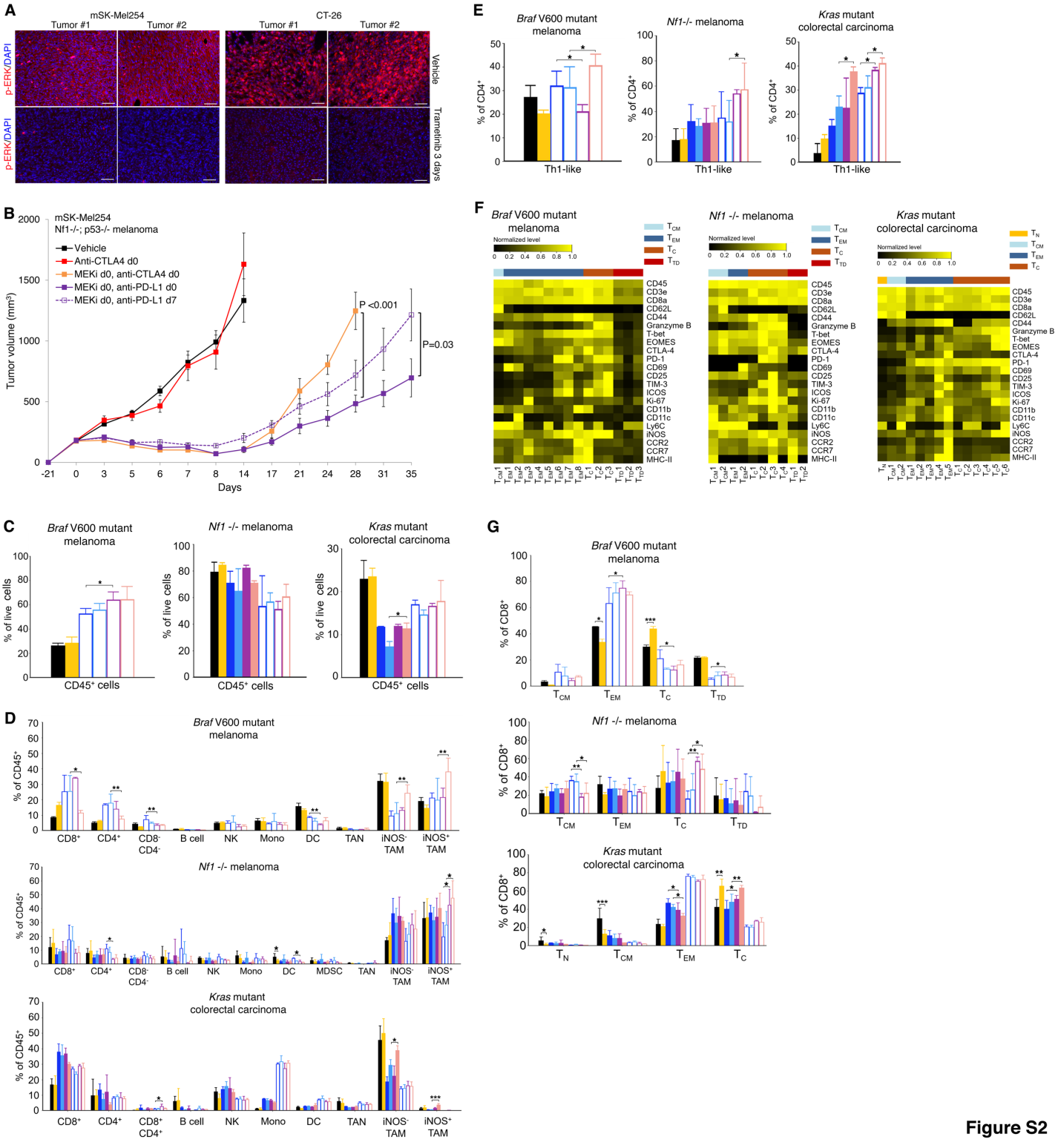
A
mSK-Mel254
CT-26
Tumor #1
Tumor #2
Tumor #1
Tumor #2
p-ERK/DAPI
p-ERK/DAPI
Vehicle
Trametinib 3 days
B
mSK-Mel254
Nf1-/-; p53-/- melanoma
Vehicle
Anti-CTLA4 d0
MEKi d0, anti-CTLA4 d0
MEKi d0, anti-PD-L1 d0
MEKi d0, anti-PD-L1 d7
Tumor volume (mm³)
Days
P <0.001
P=0.03
C
Braf V600 mutant melanoma
Nf1 -/- melanoma
Kras mutant colorectal carcinoma
% of live cells
CD45⁺ cells
D
Braf V600 mutant melanoma
Nf1 -/- melanoma
Kras mutant colorectal carcinoma
% of CD45⁺
CD8⁺
CD4⁺
CD8⁻ CD4⁻
B cell
NK
Mono
DC
MDSC
TAN
iNOS⁻ TAM
iNOS⁺ TAM
E
Braf V600 mutant melanoma
Nf1-/- melanoma
Kras mutant colorectal carcinoma
% of CD4⁺
Th1-like
F
Braf V600 mutant melanoma
Nf1 -/- melanoma
Kras mutant colorectal carcinoma
Normalized level
CD45
CD3e
CD8a
CD62L
CD44
Granzyme B
T-bet
EOMES
CTLA-4
PD-1
CD69
CD25
TIM-3
ICOS
Ki-67
CD11b
CD11c
Ly6C
iNOS
CCR2
CCR7
MHC-II
G
Braf V600 mutant melanoma
Nf1 -/- melanoma
Kras mutant colorectal carcinoma
% of CD8⁺

Figure S2

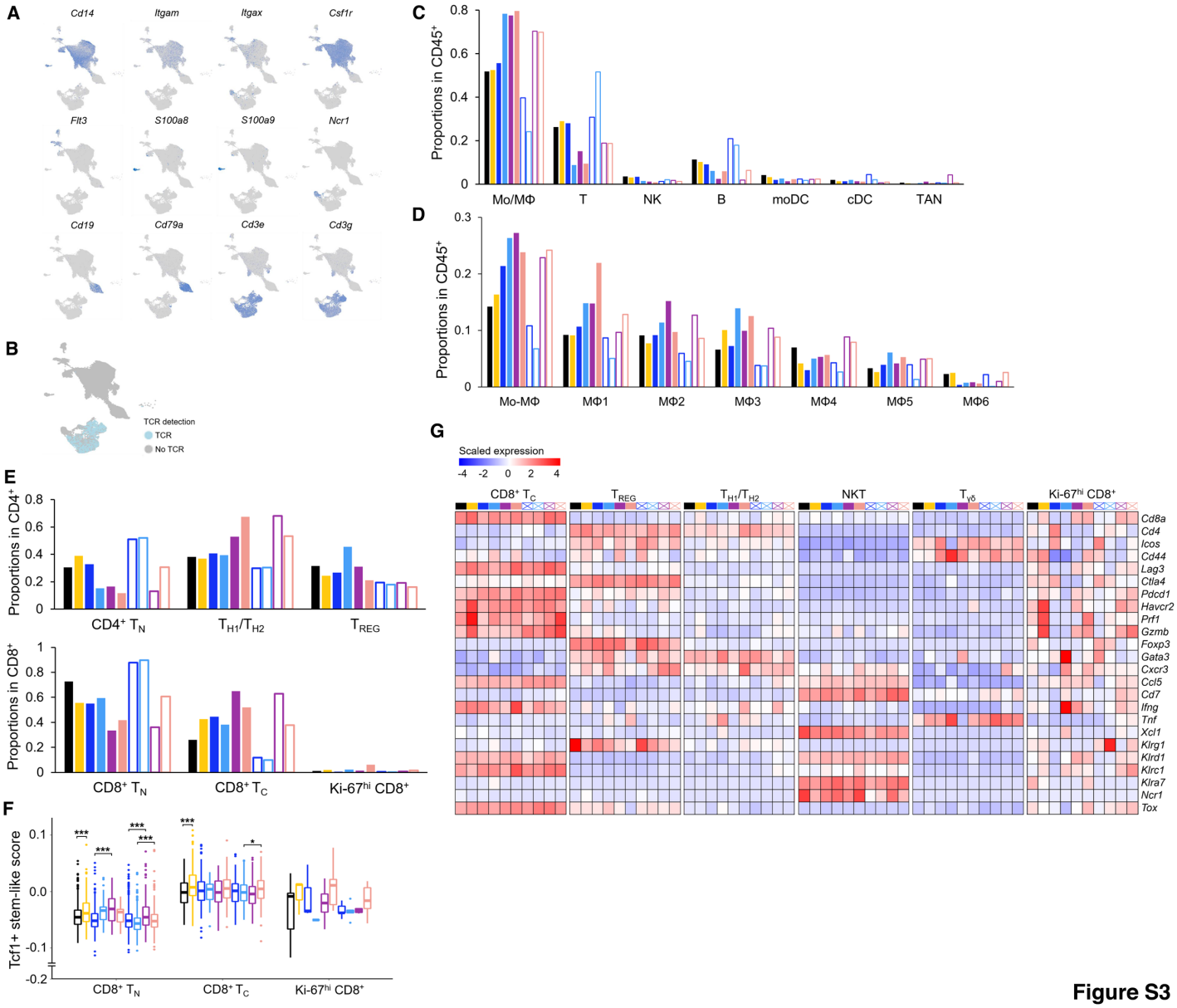
A
Cd14
Itgam
Itgax
Csf1r
Flt3
S100a8
S100a9
Ncr1
Cd19
Cd79a
Cd3e
Cd3g
B
TCR detection
TCR
No TCR
C
Proportions in CD45+
Mo/MΦ
T
NK
B
moDC
cDC
TAN
D
Proportions in CD45+
Mo-MΦ
MΦ1
MΦ2
MΦ3
MΦ4
MΦ5
MΦ6
E
Proportions in CD4+
CD4+ TN
TH1/TH2
TREG
Proportions in CD8+
CD8+ TN
CD8+ TC
Ki-67hi CD8+
F
Tcf1+ stem-like score
CD8+ TN
CD8+ TC
Ki-67hi CD8+
G
Scaled expression
CD8+ TC
TREG
TH1/TH2
NKT
Tγδ
Ki-67hi CD8+
Cd8a
Cd4
Icos
Cd44
Lag3
Ctla4
Pdcd1
Havcr2
Prf1
Gzmb
Foxp3
Gata3
Cxcr3
Ccl5
Cd7
Ifng
Tnf
Xcl1
Klrg1
Klrd1
Klrc1
Klra7
Ncr1
Tox

Figure S3

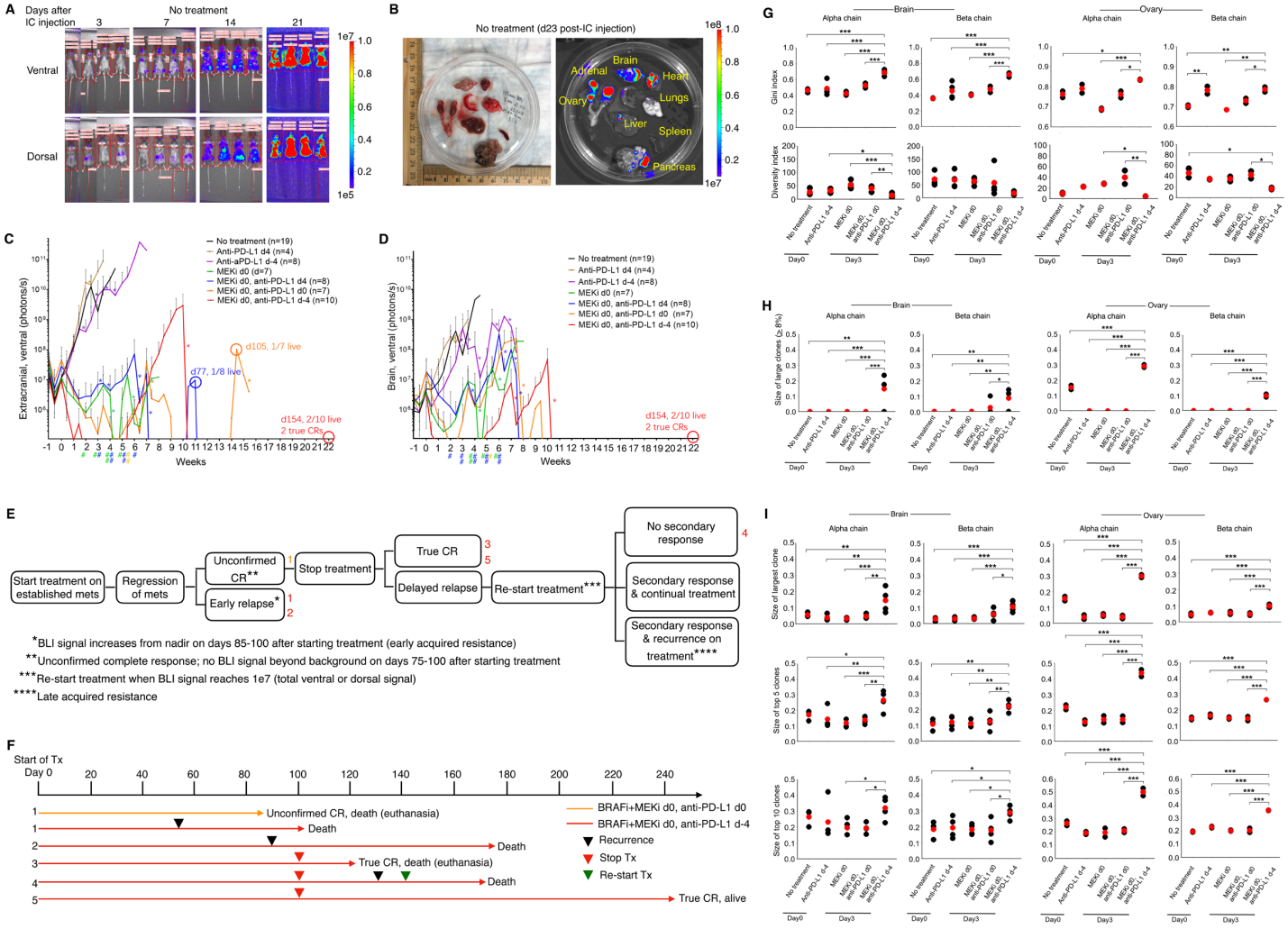
A
Days after IC injection
No treatment
3
7
14
21
Ventral
Dorsal
1e7
1.0
0.8
0.6
0.4
0.2
1e5
B
No treatment (d23 post-IC injection)
Brain
Adrenal
Heart
Ovary
Lungs
Liver
Spleen
Pancreas
1e8
1e7
C
No treatment (n=19)
Anti-PD-L1 d4 (n=4)
Anti-aPD-L1 d-4 (n=8)
MEKi d0 (d=7)
MEKi d0, anti-PD-L1 d4 (n=8)
MEKi d0, anti-PD-L1 d0 (n=7)
MEKi d0, anti-PD-L1 d-4 (n=10)
Extracranial, ventral (photons/s)
d105, 1/7 live
d77, 1/8 live
d154, 2/10 live
2 true CRs
Weeks
D
No treatment (n=19)
Anti-PD-L1 d4 (n=4)
Anti-PD-L1 d-4 (n=8)
MEKi d0 (n=7)
MEKi d0, anti-PD-L1 d4 (n=8)
MEKi d0, anti-PD-L1 d0 (n=7)
MEKi d0, anti-PD-L1 d-4 (n=10)
Brain, ventral (photons/s)
d154, 2/10 live
2 true CRs
Weeks
E
Start treatment on established mets
Regression of mets
Unconfirmed CR**
Early relapse*
Stop treatment
True CR
Delayed relapse
Re-start treatment***
No secondary response
Secondary response & continual treatment
Secondary response & recurrence on treatment****
*BLI signal increases from nadir on days 85-100 after starting treatment (early acquired resistance)
**Unconfirmed complete response; no BLI signal beyond background on days 75-100 after starting treatment
***Re-start treatment when BLI signal reaches 1e7 (total ventral or dorsal signal)
****Late acquired resistance
F
Start of Tx
Day 0
Unconfirmed CR, death (euthanasia)
Death
True CR, death (euthanasia)
True CR, alive
BRAFi+MEKi d0, anti-PD-L1 d0
BRAFi+MEKi d0, anti-PD-L1 d-4
Recurrence
Stop Tx
Re-start Tx
G
Brain
Ovary
Alpha chain
Beta chain
Gini index
Diversity index
H
Size of large clones (> 8%)
I
Size of largest clone
Size of top 5 clones
Size of top 10 clones
J
Day0
Day3
No treatment
Anti-PD-L1 d-4
MEKi d0
MEKi d0, anti-PD-L1 d0
MEKi d0, anti-PD-L1 d-4
Alpha
Beta
Ovary
Brain
3437 (94.2%)
112 (3.07%)
98 (2.69%)
4145 (94.1%)
131 (2.97%)
128 (2.91%)
2041 (82.4%)
204 (8.24%)
232 (9.37%)
2291 (83.8%)
454 (12.6%)
855 (23.8%)
1545 (44.4%)
534 (15.4%)
1397 (40.2%)
4200 (94.1%)
100 (2.24%)
163 (3.65%)
5635 (94.7%)
130 (2.18%)
188 (3.16%)
3443 (87.9%)
162 (4.14%)
312 (7.97%)
3679 (72.6%)
373 (6.98%)
1090 (20.4%)
2464 (55.3%)
452 (10.1%)
1539 (34.5%)

Figure S4

Supplemental Table Legends

Table S1. For S1320 trial, associations between baseline patient characteristics and prior ICT, Related to Figure 1. Median (range) or N (%) reported.

Table S2. Multivariable Cox regression models for PFS (A) and OS (B), Related to Figure 1.

Table S3. Expansion indices as well as top 5 and 10 clone sizes of CD8$^+$ T_C, Ki-67^{hi} CD8$^+$ T and T_{REG} cells in each treatment regimen group and time point, Related to Figure 3. See Figure 2A.

Supplemental Table S1

Factor	No prior exposure (N = 145)	Prior checkpoint therapy (N = 61)	P-value
Randomized arm			
Continuous Dosing	74 (51)	31 (51)	1
Intermittent Dosing	71 (49)	30 (49)	
Age at randomization (years)	62 (52, 69)	58 (47, 67)	0.16
Age at randomization (years)			
Younger than 45	24 (17)	15 (25)	0.18
45 and older	121 (83)	46 (75)	
Gender			
Female	51 (35)	23 (38)	0.75
Male	94 (65)	38 (62)	
Race			
White	142 (98)	59 (97)	0.6
Asian and white	1 (1)	0 (0)	
Native American	0 (0)	1 (2)	
Unknown race	2 (1)	1 (2)	
Ethnicity			
Hispanic	4 (3)	2 (3)	1
Not hispanic	141 (97)	59 (97)	
PS			
0	82 (57)	35 (58)	0.95
1	60 (42)	24 (40)	
2	2 (1)	1 (2)	
LDH at randomization			
Elevated LDH	53 (37)	24 (39)	0.75
Normal LDH	92 (63)	37 (61)	
Primary			
Cutaneous primary	112 (78)	52 (88)	0.12
Unknown primary	32 (22)	7 (12)	
Stage			
M0	14 (10)	11 (18)	0.41
M1A	25 (17)	11 (18)	
M1B	32 (22)	12 (20)	
M1C	74 (51)	27 (44)	
Response			
CR	13 (9)	9 (15)	0.43
Unconfirmed CR	3 (2)	0 (0)	
PR	76 (54)	38 (64)	
Unconfirmed PR	22 (16)	6 (10)	
Stable	22 (16)	5 (8)	
Increasing disease	2 (1)	0 (0)	
Inadequate assessment	3 (2)	1 (2)	

Supplemental Table 2A

Covariate	HR	80% CI	P-value
Prior checkpoint therapy (ref = No prior exposure)	0.6	(0.47, 0.77)	0.009
Intermittent Dosing (ref = Continuous Dosing)	1.57	(1.27, 1.96)	0.0073
Age at randomization (years)	0.99	(0.98, 1)	0.17
Male (ref = Female)	0.93	(0.73, 1.18)	0.69
PS 1 (ref = PS 0)	1.3	(1.04, 1.62)	0.13
PS 2 (ref = PS 0)	3.2	(1.23, 8.34)	0.12
Normal LDH (ref = Elevated LDH)	0.63	(0.5, 0.79)	0.01
Unknown primary (ref = Cutaneous primary)	0.71	(0.53, 0.95)	0.13
M1A (ref = M0)	1.24	(0.79, 1.95)	0.53
M1B (ref = M0)	1.6	(1.04, 2.46)	0.16
M1C (ref = M0)	1.9	(1.27, 2.82)	0.039

Supplemental Table 2B

Covariate	HR	80% CI	P-value
Prior checkpoint therapy (ref = No prior exposure)	0.86	(0.63, 1.17)	0.52
Intermittent Dosing (ref = Continuous Dosing)	1.13	(0.85, 1.49)	0.58
Age at randomization (years)	1	(0.99, 1.01)	0.81
Male (ref = Female)	0.63	(0.47, 0.85)	0.051
PS 1 (ref = PS 0)	2.16	(1.64, 2.85)	<0.001
PS 2 (ref = PS 0)	12.71	(4.48, 36.06)	0.0018
Normal LDH (ref = Elevated LDH)	0.54	(0.41, 0.73)	0.0069
Unknown primary (ref = Cutaneous primary)	0.65	(0.44, 0.95)	0.15
M1A (ref = M0)	1.67	(0.79, 3.5)	0.38
M1B (ref = M0)	3.01	(1.48, 6.13)	0.047
M1C (ref = M0)	3.61	(1.84, 7.09)	0.015

Supplemental Table 3

	Expansion index			Size of top5 clones			Size of top10 clones		
	$CD8^+$ T_C	Ki-67^{hi} $CD8^+$	T_{REG}	$CD8^+$ T_C	Ki-67^{hi} $CD8^+$	T_{REG}	$CD8^+$ T_C	Ki-67^{hi} $CD8^+$	T_{REG}
Day7	0.0630	0.0000	0.0086	0.0465	0.0159	0.0171	0.0734	0.0159	0.0282
Day7	0.1796	0.0204	0.0000	0.1478	0.1063	0.1113	0.1777	0.1096	0.1213
Day10	0.1852	0.0000	0.0074	0.1022	0.0424	0.0849	0.1283	0.0424	0.0945
Day10	0.0193	0.0000	0.0000	0.1129	0.0000	0.0484	0.1532	0.0000	0.0887
Day10	0.1392	0.0817	0.0000	0.2160	0.0488	0.0418	0.3031	0.0488	0.0592
Day10	0.1416	0.0817	0.0000	0.1203	0.0253	0.0190	0.1582	0.0253	0.0190
Day14	0.0067	0.0000	0.0067	0.0516	0.0299	0.0387	0.0616	0.0299	0.0464
Day14	0.1175	0.0000	0.0066	0.0235	0.0063	0.0093	0.0334	0.0066	0.0136
Day14	0.2392	0.0000	0.0334	0.1800	0.0284	0.1411	0.2141	0.0284	0.1492
Day14	0.2210	0.0245	0.0000	0.1018	0.0583	0.0317	0.1372	0.0590	0.0398