Supplementary Methods

Tumor Models

EMT6 and A20: Six- to eight-week-old female BALB/c mice (Charles River/Envigo) were utilized for in vivo studies in a fully accredited AAALAC facility. Tumors were implanted using $1x10^5$ EMT6 mouse mammary carcinoma (ATCC: CRL-2755) or $1x10^6$ A20 murine lymphoma cells (ATCC: TIB-208) inoculated subcutaneously into the flank. Palpable tumors were measured using a calliper with tumor volume calculated using (L x W x H)/2 or 0.52 x Length x $Width^2$. Mice (n=7-13/treatment group) were randomized when tumors reached approximately 100-150 mm^3 and treated with the following antibodies; anti-mOX40 rIgG1 (clone OX86, BE0031BioXCell) or isotype control antibodies, rIgG1 (clone HRPN, BioXCell BE0088).

CT26: CT26 (ATCC: CRL-2638) mouse colon carcinoma cells were inoculated subcutaneously into the flank as described in main methods. Sequential vs concurrent dosing was performed as follows: dosing with anti-OX40 and anti-PD-1;OX40>PD-1 sequential mice were dosed with anti-OX40 and isotype control for doses 1, 2 and 3, and then received anti-PD-1 and isotype control for doses 4, 5, and 6. PD-1>OX40 sequential mice were dosed with anti-PD-1 and isotype control for doses 1, 2 and 3, and then received anti-OX40 and isotype control for doses 4, 5, and 6. Concurrent dosing: mice were given the same treatment throughout the 6 doses. Total amount of antibody in each group was the same. Tumor measurements of > either 2,000 or 4000 mm^3 (depending on site where study was conducted) resulted in mice being removed from study. Mice were also removed due to weight loss (>20%), ulceration or tumor necrosis, or any other obvious inhibition of normal mouse activity.

LLC and B16-F10: cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS. Cells were grown in monolayer culture at 37°C in 5% CO_2. Female C57BL/6 mice (8 to 10 weeks old) were housed at the CCSG Animal Facility Core at

South Campus Vivarium, M.D. Anderson Cancer Center. All experiments were conducted in accordance with Institutional IACUC and National Institutes of Health (NIH) guidelines. LLC (1 x10^5 cells/200 µl of PBS), and B16F10 cells (2.5 x 10^4/100 µl of PBS mixed with an equal volume of Matrigel) were implanted on the right flank using the subcutaneous route with 27 gauge needles. Treatment started when tumors reached 150 mm^3, typically 15 days after tumor inoculation. The tumor growth was measured twice a week using a caliper, and values were expressed as volume in mm^3 using the formula (L x W x H)/2. Mice were sacrificed when tumor size reached 2 cm or 4,000 mm^3 or when tumor sites were ulcerated.

EG.7-Ova: E.G7-OVA cells were injected subcutaneously into the flank as detailed in main methods. Staggered vs concurrent dosing study were performed as follows; for concurrent dosing, as in supplemental Fig. 5, anti-OX40hIgG1 (100 µg) and anti-PD-1 (250 µg) were given IP concurrently starting when tumors reached between 5x5-10x10 mm and then treated every 3rd day. Staggered dosing was performed as detailed in main Fig. 5F. In both cases, mice were culled when they reached humane end point or end of the experiment if long term survivors.

Nanostring® Analysis:

In monotherapy studies CD4+ and CD8+ T cells were isolated from blood, spleen, lymph node and tumor following treatment with either isotype control antibodies or anti-OX40. In combination studies the whole tumor was processed for evaluation of gene expression. RNA was extracted from the cells or tumor and 100 ng of total RNA was used for hybridization per reaction using the NanoString® Mouse PanCancer_Immune profiling panel. The RNA was analysed following the manufacturer's instructions. Raw counts were extracted from the RCC file with nSolver version 2.0 using the custom-made library file (RLF). Samples with very low expression counts and significantly lower correlation with other replicates were

removed. The data were filtered removing genes with very low expression and normalized with TMM normalization methodology implemented in edgeR Bioconductor package. A total of 366 genes and 170 samples were left in the final matrix. Negative binomial distribution was assumed for identification of differential expression and a 10% false discovery rate cut off was used to identify differentially expressed genes.

Cytokine analysis in CT26 combination studies

Following challenge with $5x10^4$ CT26 tumor cells, mice were randomized into five groups receiving vehicle, isotype control antibodies (rIgG1 100 μg + rIgG2a 200 μg), anti-mOX40 (OX86 100 μg +rIgG2a 200 μg), anti-PD-1 (PD-1 200 μg + rIgG1 100 μg) or anti-mOX40 and anti-PD-1 (OX86 100 μg +PD-1 200 μg). The mice were dosed twice a week on Day 0, 3 and 7 and blood and tumor tissues harvested on Day 3, 7 and 10 following the first dose equating to 1, 2 and 3 doses, respectively. Serum was collected for cytokine analysis and analysed by Meso-Scale Discovery mouse V-Plex customized kit.

In vitro studies:

PBMCs from healthy human donors were cultured with anti-CD3/anti-CD28 beads at beads:cells ratio of 1:20, IL-2 and MCSF in AIM-V media for 48 hours. The cells were then restimulated with anti-CD3 beads at beads:cells ratio of 1:1, antibody anti-OX40 hIgG1 (clone 106-222), pembrolizumab (anti-PD-1) or both at 10 μg/ml for 72 hours. Cell supernatants were collected and analyzed for IFNγ and TNFα by Meso Scale Discovery.