Supporting Information

Christiansen et al. 10.1073/pnas.1011037108

SI Materials and Methods

Tumor Lines. The BALB/c-derived mammary carcinoma line 4T1.2 was maintained in α-MEM supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, and 2 mM penicillin/streptomycin at 37 °C with a 10% CO_2 atmosphere. The BALB/c-derived renal carcinoma, Renca, were maintained in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, and 2 mM penicillin/streptomycin at 37 °C with a 5% CO_2 atmosphere. The C57BL/6-derived colon adenocarcinoma MC38, the retrovirally transduced lines MC38/MSCV, MC38/Bcl-2, and MC38/c-FLIPL, and the RM1 prostate adenocarcinoma line were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, and 2 mM penicillin/streptomycin at 37 °C with a 10% CO_2 atmosphere.

Mice and in Vivo Experiments. All mice used in experiments were 6–12 wk of age and were housed under specific pathogen-free conditions with food and water freely available according to the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee Guidelines. BALB/c and C57BL/6 mice were purchased from the Walter and Eliza Hall Medical Research Institute and TRAIL$^{-/-}$, perforin$^{-/-}$ Rag1$^{-/-}$, and Rag2$^{-/-}$c-γ-chain$^{-/-}$ mice were bred in-house at the Peter MacCallum Cancer Centre.

Vorinostat was administered i.p. at 150 or 100 mg·kg^{-1}·d^{-1}. Panobinostat was administered at either 10 mg/kg (CT-26, RM1) or 5 mg/kg (4T1.2 experiment) every day for 5 d, followed by a 2 d break, then again for 5 d. Therapeutic antibodies (anti-CD40 and anti-CD137) were administered i.p. at the following doses unless otherwise stated in the figure legend. MD5-1 at 50 μg, administered every 4 d for 4 doses; BimAb (25 μg anti-CD40, 100 μg anti-CD137) administered every 4 d for 4 doses; α-c-GC was synthesized as described (1, 2) and mice administered 500 ng every 4 d as per therapeutic antibody schedule (3). The depletion antibodies anti-CD4 (clone GK1.5) anti-CD8 (clone 53–6.7), and anti-asialo GM1 were administered at 100 μg/dose the day before V/bimAb therapy commenced, the day of therapy and every 4 d until completion of the experiment. Depletion of cell subsets was confirmed via assessment of the peripheral blood of treated mice via flow cytometry.

Retroviral Transduction of MC38 Cells. Retrovirus-containing supernatants were produced by transiently transfecting HEK-293T packaging cell line with 10 μg of MSCV/empty vector, MSCV/c-FLIPL, or MSCV/Bcl-2 plasmid DNA (all combined with 10 μg amphotrophic helper plasmid DNA) by standard calcium phosphate transfection techniques. Forty-eight hours after transfection, supernatants containing the retrovirus were collected, filtered through a 0.45-μm filter, aliquoted, and stored at −80 °C. MC38 colon adenocarcinoma cells (1×10^5) in complete media were plated into a single well of a six-well tissue culture plate. Cells were incubated (37 °C, 5% CO_2) for at least 4 h to allow cells to adhere before complete media was removed and replaced with 1.5 mL of retrovirus-containing supernatants and polybrene (4 μg/mL). Supernatants were replaced every 12 h with exposure of tumor cells to a total of six retroviral preparations. Following further culture and expansion, GFP-positive cells were then isolated by flow cytometry-mediated cell sorting, further cultured, and then resorted at least twice more to enrich the GFP-positive population of cells.

Western Blotting. Immunoblotting was performed as per standard established Western blotting techniques. Thirty micrograms of whole cell tumor lysates were separated by PAGE using 15% polyacrylamide gels in SDS running buffer (25 mM Tris·HCl, 192 mM glycine, 0.1% SDS in H_2O). Proteins were transferred to Immobilon-P PVDF membrane (Millipore) by electroblotting in Western transfer buffer (25 mM Tris·HCl, pH 8.3, 192 mM glycine, 20% vol/vol methanol) in a wet transfer apparatus. Bcl-2 or c-FLIPL were detected on the membrane using anti-Bcl-2 (Clone 3F11; BD Biosciences) or anti-c-FLIP (Clone Dave-2; Alexis), respectively.

Statistical Analysis. Statistical significance was assessed using Prism or Microsoft Excel software. For comparisons of means, Student *t* test (parametric data) or Mann-Whitney rank sum test (nonparametric data) were used. In all cases, $P < 0.05$ was considered significant.

1. Chen G, Chien M, Tsuji M, Franck RW (2006) E and Z alpha-C-galactosylceramides by Julia-Lythgoe-Kocienski chemistry: a test of the receptor-binding model for glycolipid immunostimulants. *ChemBioChem* 7:1017–1022.
2. Yu KO, et al. (2005) Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci USA* 102:3383–3388.
3. Teng MW, et al. (2007) Combined natural killer T-cell based immunotherapy eradicates established tumors in mice. *Cancer Res* 67:7495–7504.

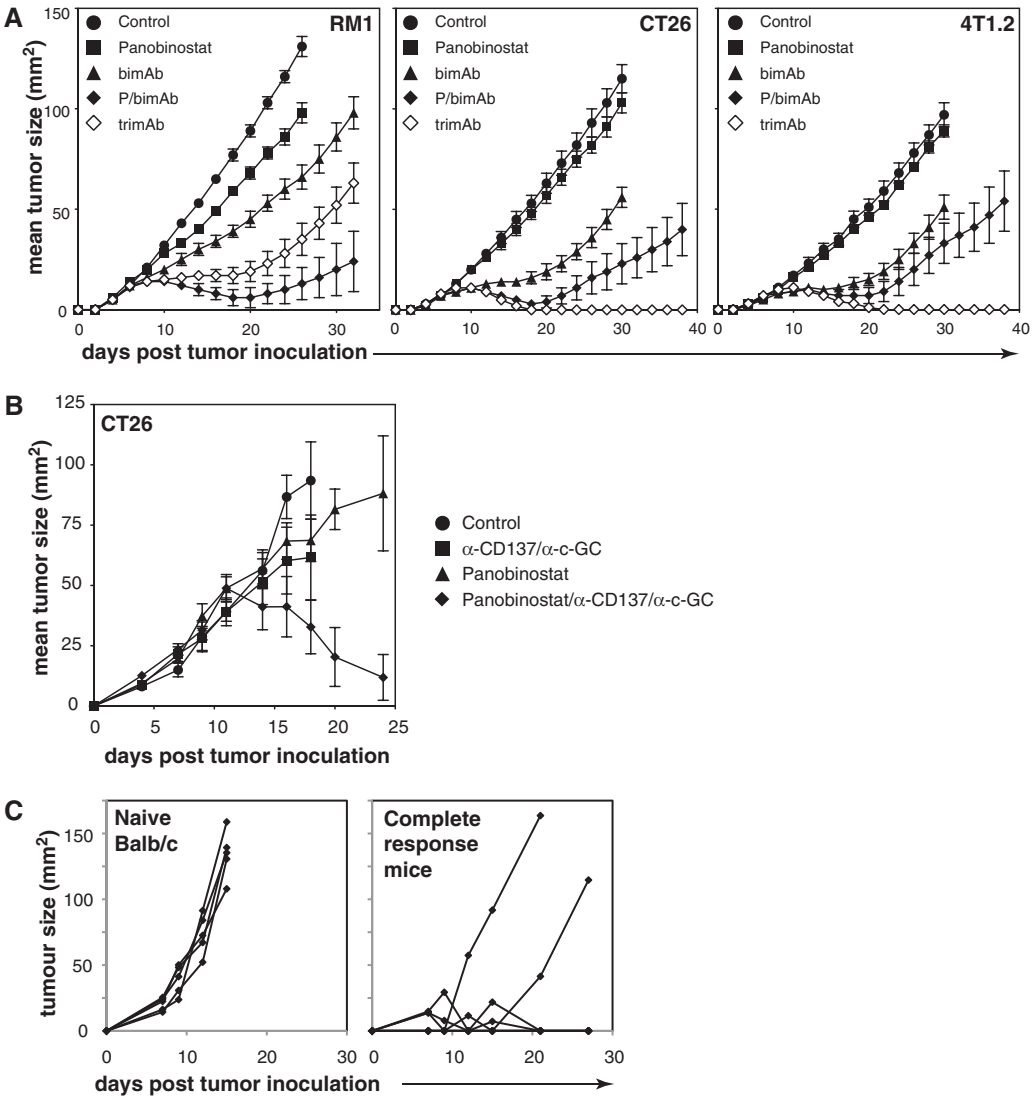

Fig. S1. P/bimAb and P/α-CD137/α-c-GC therapy are efficacious against established carcinomas. (*A*) Cohorts of mice with established (>9 mm^2) s.c. carcinoma (RM1, CT26, or 4T1.2) were treated i.p. with 10 mg/kg (RM1 and CT26) or 5 mg/kg (4T1.2) panobinostat for 5 d followed by a 2-d break. BimAb (100 μg α-CD137, 25 μg α-CD40) or trimAb (50 μg MD5-1, 100 μg α-CD137, 25 μg α-CD40) was administered every 4 d for 4 doses. Tumor growth was assessed every 2 d; mean tumor size ± SEM are shown and are representative of two independent experiments. Complete tumor regressions were observed in 60, 40, and 20% of P/bimAb-treated mice bearing RM1, CT26, or 4T1.2 tumors, respectively. (*B*) Mice with established CT26 (>9 mm^2) were treated with panobinostat (7.5 mg/kg) every 5 d followed by a 2-d break, α-CD137 and α-c-GC (100 μg and 500 ng, respectively) every 4 d, or a combination of all three reagents. Tumor growth was assessed every 2–3 d; mean tumor size ± SEM are shown and are representative of two independent experiments (*C*). Mice that achieved complete tumor regressions (CT26, from *B*) when treated with P/bimAb were rechallenged on the opposite hind flank with 2×10^5 CT26 carcinoma cells, and tumor growth was assessed compared with naïve BALB/c mice. Tumors spontaneously regressed in 7/9 rechallenge mice, demonstrating P/bimAb therapy facilitates the generation of long-term immunological memory.

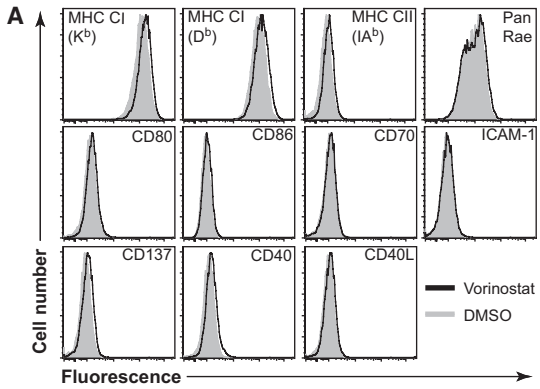

Fig. S2. Vorinostat does not alter the immunogenicity of MC38 tumors. MC38 tumors were treated in vitro with 5 μM vorinostat for 16 h and then assessed via flow cytometry for alterations in the expression of surface receptors or ligands as shown. Control DMSO-treated cells are shown in shaded gray, vorinostat-treated are open black histograms. Fluorescence is represented on a logarithmic scale. No significant changes in MHC, adhesion, costimulatory, or NK activatory molecules were observed. Data shown are representative of three independent experiments.

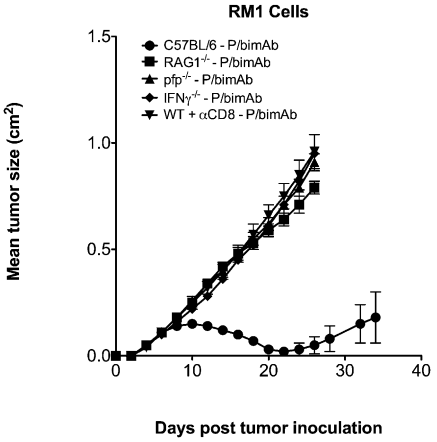

Fig. S3. Role of immune-specific effector cells and regulatory proteins in mediating P/bimAb therapy. Cohorts of wild-type C57BL/6 mice, RAG1$^{-/-}$ mice, wild-type mice treated with anti-CD8 (53-6.7) to deplete CD8$^+$ cells, or knockout mice deficient in IFNγ (IFN-γ$^{-/-}$) or perforin (pfp$^{-/-}$) with established (>9 mm^2) s.c. RM1 tumors were used to test the efficacy of P/bimAb therapy. Mice were treated i.p. with 10 mg/kg panobinostat for 5 d followed by a 2-d break. BimAb (100 μg α-CD137, 25 μg α-CD40) was administered every 4 d for 4 doses. Tumor growth was assessed every 2–3 d and tumor size in individual mice (cm^2) is shown. Data shown are representative of two independent experiments.

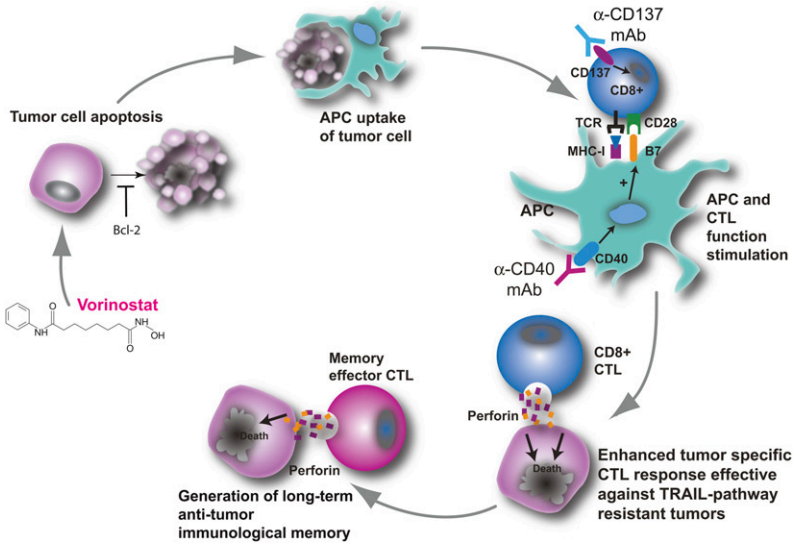

Fig. S4. Proposed mechanism of V/bimAb therapy. V/bimAb therapy induces a potent antitumor response capable of the eradication of established carcinomas of diverse tissue origins. Vorinostat-induced apoptosis is critical for efficacy of V/bimAb therapy. Vorinostat-induced apoptosis facilitates uptake and processing of tumor cells by APCs. The immunostimulating agonistic antibodies anti-CD40 and anti-CD137 promote APC activation and CTL proliferation and survival such that a potent antitumor CTL response capable of the eradication of established solid tumors. Tumor-specific CTLs critically rely on the membrane-disrupting protein perforin for tumor eradication. Importantly, V/bimAb therapy also induces the generation of long-term antitumor immunological memory capable of the spontaneous eradication of tumors upon secondary challenge.