# COSTIMULATION AS A PLATFORM FOR THE DEVELOPMENT OF VACCINES: A PEPTIDE-BASED VACCINE CONTAINING A NOVEL FORM OF 4-1BBL ERADICATES ESTABLISHED TUMORS

**Rajesh K. Sharma *et. al.***

## SUPPLEMENTARY MATERIALS

**Including:**

**Supplementary Figures 1-5; Supplementary Table 1.**

**Reagents, Expanded Methods and Related References**

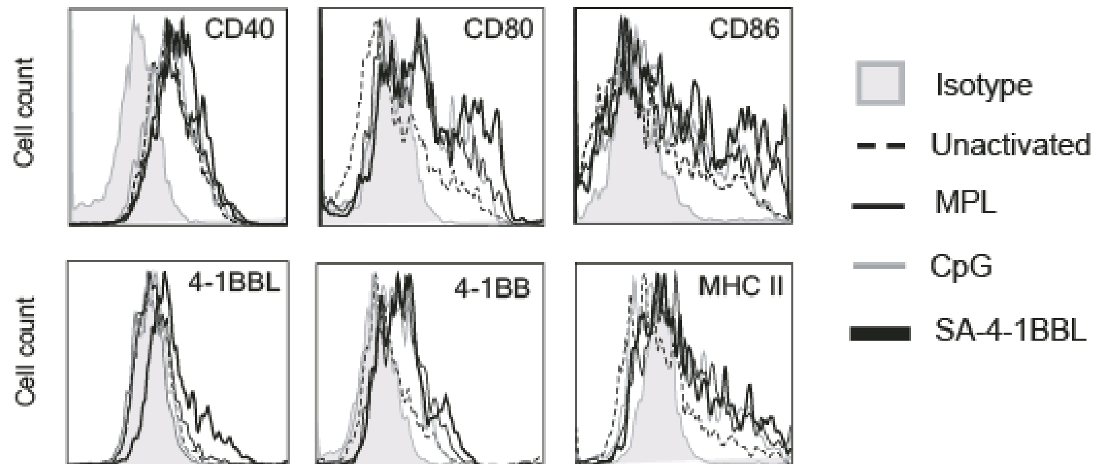


**Figure S1**. **Comparison of the effects of SA-4-1BBL, MPL, and CpG on the activation of BM-DCs.**

BM-DCs were cultured with SA-4-1BBL (5 μg/ml), MPL (5 μg/ml), CpG (5 μg/ml), or left untreated for 24 hrs. Activation was assessed by examining the cell surface expression levels of CD40, CD80, CD86, 4-1BBL, 4-1BB, and MHC class II molecules using flow cytometry. Data are representative of a minimum of two independent experiments for each panel.

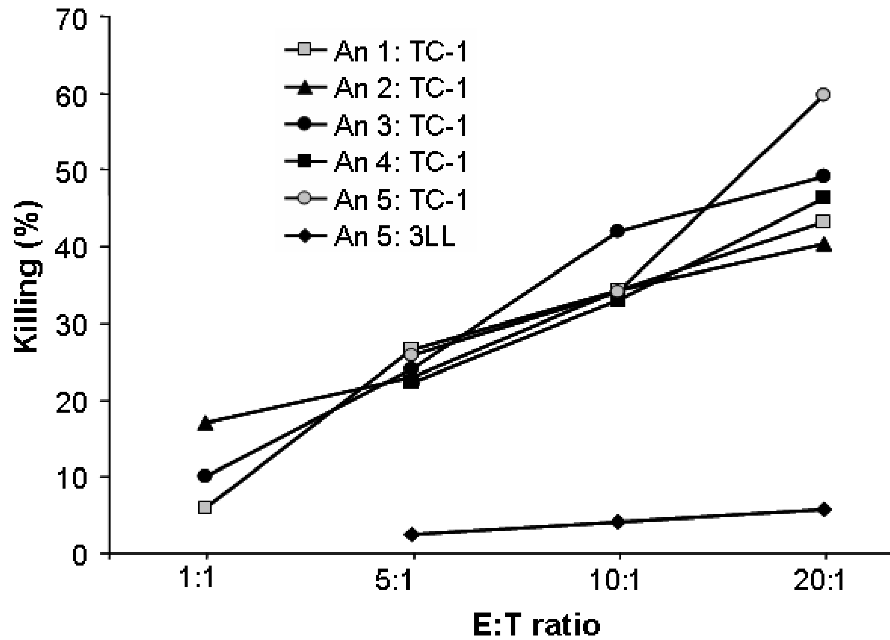


**Figure S2**. **Vaccination with SA-4-1BBL and $E7_{49\text{-}57}$ peptide induces an effective CTL response against TC-1 cells.**

C57BL/6 mice were immunized s.c. with 50 µg $E7_{49\text{-}57}$ peptide in combination with 25 µg SA-4-1BBL. Splenocytes from vaccinated mice were harvested 7 days later and cultured in the presence of $E7_{49\text{-}57}$ peptide and 50 IU/ml rIL-2 for 5 days. Effector cells were harvested using Ficoll gradients and co-cultured for 6 hrs with thymidine labeled E7 expressing TC-1 tumor targets at the indicated effector:target ratios (E:T). The 3LL lung carcinoma cell line was used as irrelevant target for killing. The in vitro killing was calculated as described previously for JAM assay. The data were obtained from two independent experiments involving 5 mice.

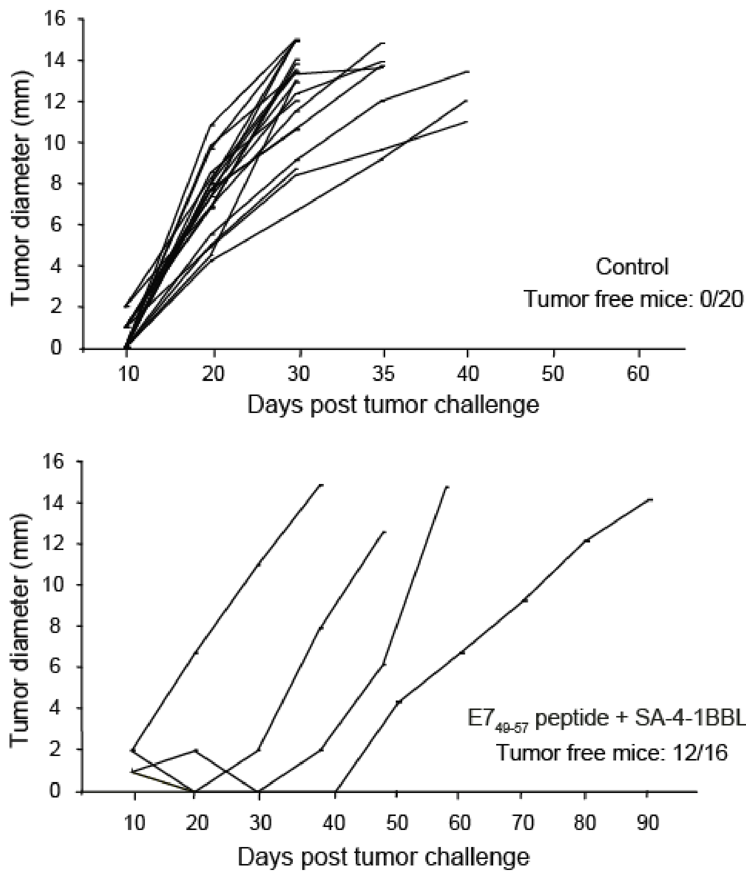


**Figure S3**. **Vaccination with SA-4-1BBL is effective in eradicating established tumors.**

Tumor growth kinetics for the animals shown in Figure 2B presented as the mean mm in diameter. Animals were euthanized due to experimental end-point (tumor size 15 mm in diameter), ulceration of tumor, or poor health conditions.

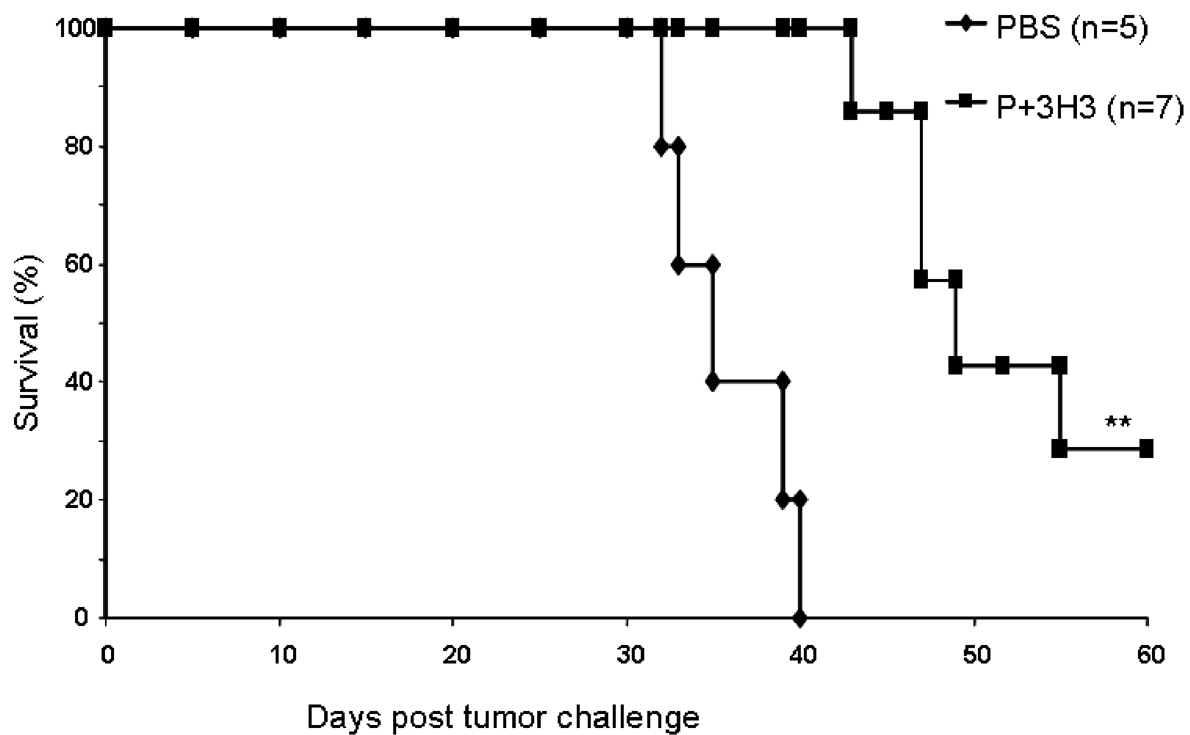


**Figure S4**. **Vaccination with an agonistic Ab to 4-1BB has moderate efficacy in eradicating established tumors.**

C57BL/6 mice were challenged with live TC-1 cells ($1x10^5$) and vaccinated once s.c. 10 days later with 50 μg $E7_{49-57}$ peptide in combination with 100 μg of 3H3 agonistic Ab to 4-1BB receptor or PBS. Mice were followed for tumor incidence and survival. **$p<0.0001$ for P+3H3 *vs*. control.

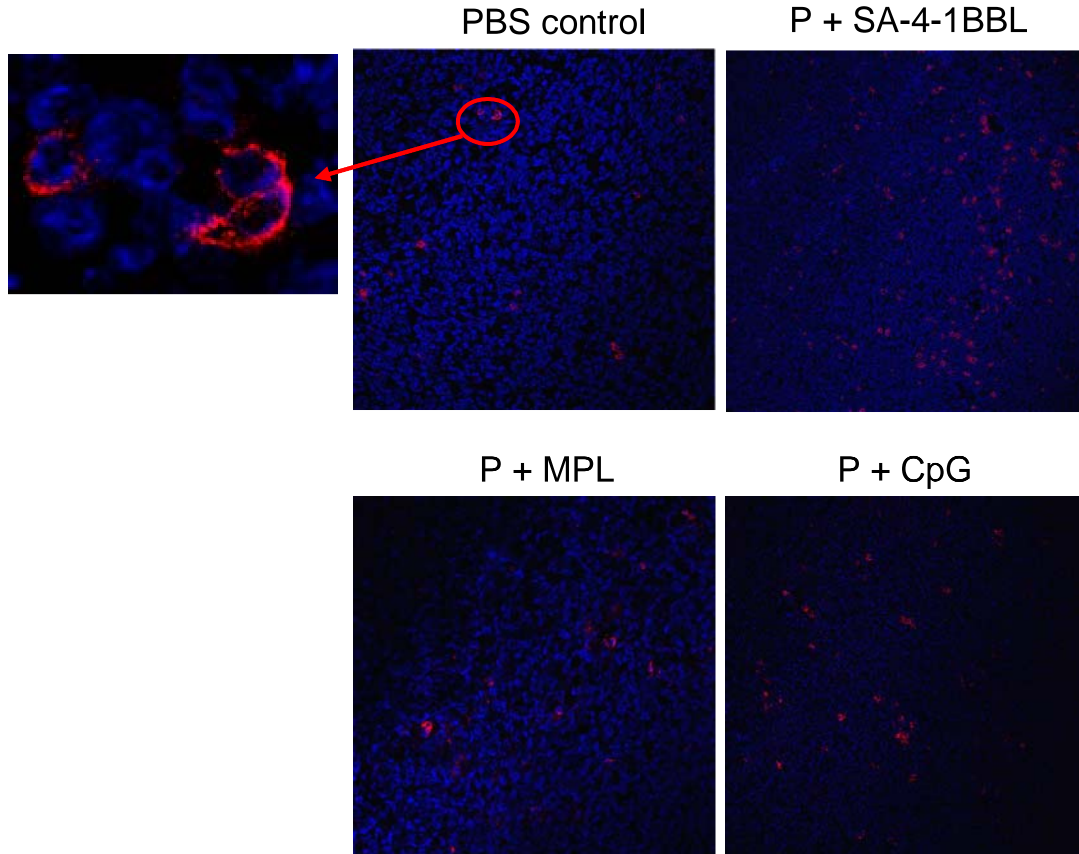


**Figure S5**. **Vaccination with SA-4-1BBL augments infiltration of CD8$^{+}$ T cells into the tumor.**

Mice with 3 mm in diameter TC-1 tumors (n=3) were injected s.c. with 50 µg $E7_{49-57}$ peptide in combination with 25 µg SA-4-1BBL, 25 µg MPL, or 10 µg CpG. Tumors were harvested 7 days later, stained with CD8-PerCp Ab to CD8+ T cells (red), and counterstained with Hoechst 33342 (blue). Images were obtained using confocal microscopy. Magnifications are 28X for P+4-1BBL, 20X for PBS, P+MPL, and P+CpG panels. The upper left panel is 120X zoom of the area in red circle to show the specificity of staining.

**TABLE S1.** Percent expression and mean fluorescent intensities of activation markers on BM-DCs shown in **Figure S1**.

| Treatment | CD80 | | CD86 | | CD40 | | MHC II | | 4-1BB | | 4-1BBL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | MFI | % | MFI | % | MFI | % | MFI | % | MFI | % | MFI |
| Untreated | 16.0 | 19.8 | 15.6 | 22.8 | 41.0 | 75.1 | 15.3 | 39.6 | 10.7 | 20.0 | 3.2 | 37.2 |
| SA-4-1BBL | **41.5** | **68.1** | **33.8** | **79.0** | **59.9** | **111** | **34.6** | **111** | **24.1** | **37.6** | **30.3** | **88.2** |
| CpG | 34.6 | 46.7 | 25.2 | 39.6 | 43.5 | 77.4 | 29.0 | 76.3 | 11.1 | 21.3 | 1.0 | 34.7 |
| MPL | 31.6 | 41 | 21.6 | 48.2 | 50.4 | 98.9 | 35.2 | 105 | 21.7 | 33.3 | 8.4 | 41.7 |

**EXPANDED METHODS**

**Reagents**. Fluorochrome-conjugated Abs (anti-CD4-APC, anti-CD25-PE, anti-CD19-APC, anti-I-A/I-E-PE, anti-CD80-FITC, anti-CD40-FITC, anti-CD86-APC, anti-4-1BB-bio, anti-4-1BBL-PE, anti-CD45.1-APC, anti-CD45.2-PE, and SA-PerCP) and isotype controls were purchased from BD PharMingen and eBioscience.

***In vitro* DC activation.** Bone marrow-derived DCs (BM-DC) were generated as described previously (1). Briefly, bone marrow cells were depleted for T and B cells by first incubating with a panel of Abs to T and B cells for 30 min at 4°C, and then rabbit complement for 30 min at 37°C. Cells were washed extensively and cultured in 6-well plates at a concentration of $10^6$ cells/ml in complete RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin and streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1 µg/ml indomethacine, and 50 µM N-methyl-L-arginine (Sigma). After an overnight culture, nonadherent cells were collected and cultured for 5 more days in the presence of IL-4 (5 ng/ml) and GM-CSF (5 ng/ml). Immature BM-DCs or JAWS II cells were activated with SA-4-1BBL (5 µg/ml), LPS (5 µg/ml), MPL (5 µg/ml), CpG (5 µg/ml), or SA (2 µg/ml). Cells were harvested at various time points, blocked for Fc-receptors, and then stained with appropriate fluorochrome labeled Abs against CD80, CD86, MHC class II, CD40, 4-1BB, and 4-1BBL, and analyzed using multiparameter flow cytometry.

**JAM cytotoxicity assay.** Splenocytes were harvested from mice 7 days post vaccination using 50 μg $E7_{49-57}$ peptide in combination with 25 μg SA-4-1BBL and cultured (5 x $10^6$ cells/ml) complete MLR medium supplemented with 10 μg $E7_{49-57}$ peptide/ml and 50 IU/ml rIL-2 for 5 days as described previously (2). Live lymphocytes were recovered by centrifugation over a Percoll gradient and used as effectors against [$^3$H]-thymidine labeled TC-1 cells at various E:T ratios for 6 hrs as previously described (3).

LLC cells labeled with [$^3$H]-thymidine used as antigen specificity control.

**Methods for confocal staining.** For immunofluorescence analysis, tumors from vaccinated mice described above were dissected, washed, embedded in OCT, snap-frozen, and 6.0-μm sections were cut with a cryostat. Sections were incubated with PBS supplemented with 1% BSA and 5% goat serum for 30 min at room temperature to block any nonspecific binding. To assess the presence of tumor infiltrating $CD8^+$ T cells, sections were next incubated with a 1/20 dilution of a rat anti-mouse CD8 Ab (31.25 μg/ml; clone clone Ly-2; PharMingen) for 1 hr at room temperature. After extensive washing with PBS, sections were incubated with 1/400 dilution of goat anti-rat Alexa 488 (2 mg/ml, Invitrogen), counterstained with Hoechst 33342, and analyzed using confocal microscopy (Leica TCS SP5). A minimum of 5 fields for each tumor section were analyzed to assess the infiltration of $CD8^+$ T cells.

## References


1. Kilinc MO, Mukundan L, Yolcu ES, Singh NP, Suttles J, Shirwan H. Generation of a multimeric form of CD40L with potent immunostimulatory activity using streptavidin as a chaperon. Exp Mol Pathol 2006;80:252-61.
2. Matzinger P. The JAM test. A simple assay for DNA fragmentation and cell death. J Immunol Methods 1991;145:185-92.
3. Singh NP, Yolcu ES, Taylor DD, et al. A novel approach to cancer immunotherapy: tumor cells decorated with CD80 generate effective antitumor immunity. Can Res 2003;63:4067-73.