## Antitumor Reactive T-Cell Responses Are Enhanced In Vivo by DAMP Prothymosin Alpha and Its C-Terminal Decapeptide

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**Figure S1.** Schematic presentation of the melanoma antigen extract preparation (acid wash extract; AWE) used as tumor-associated peptide vaccine. B16.F1 cells were washed with HBSS and incubated for 5 min in citrate buffer pH 3.0. After centrifugation to remove cell debris, the supernatant containing the detached peptides was immediately processed on a pre-conditioned (with 10 mL ethanol and 20 mL d. H<sub>2</sub>O) SepPak C18 cartridge. The peptide-bound material was eluted from the column with 5 mL 80% acetonitrile, lyophilized to dry and reconstituted in 1 mL HBSS. To recover peptides with MW < 10 kDa, the peptide mixture was filtered through a Centricon filter and the flow through material was used as peptide vaccine. Each dose being administered to mice contained peptides that were eluted from  $2 \times 10^7$  B16.F1 cells.



**Figure S2.** In vivo titration studies of B16.F1 cells injected s.c. to C57BL/6 mice. Tumor growth was recorded every 2 to 3 days by measuring the major and minor axes of the masses via a digital caliper. Tumor volumes were calculated using the formula: tumor volume (cm<sup>3</sup>) = major axis × minor axis<sup>2</sup> × 0.5. Mean volumes from 4 animals/B16.F1 dose are shown. SDs are omitted for clarity. For the

establishment of the in vivo melanoma mouse model used herein, we selected to inject mice with  $1 \times 10^5$  melanoma cells, a dose showing the desirable tumor-growth rate profile.



Figure S3. Assessment of cytokine and chemokine levels in mice serum. The sera used were collected: for the PBS group on day 34; for AWE, GM-CSF and scrambled groups on day 48; for proT $\alpha$  and proT $\alpha$ (100-109) groups on day 54. (A) Sera were assessed in duplicate with ELISAs specific for the cytokines IFN- $\gamma$  (Th1) and IL-4 (Th2), performed according to the manufacturers' instructions. Mean concentrations  $\pm$  SD from 4-5 animals are shown. Based on the levels of IFN- $\gamma$ , the in vivo predominance of Th1-type immune responses seems likely. (B) The same sera were assessed with a 20-plex Luminex assay. Samples were diluted 1:2 in assay diluent; 25 µL of 1× antibody beads were added into each well of a 96-well flat bottom plate; wells were washed with 200 µL of 1× wash solution; 50 µL incubation buffer was added; 100 µL of diluted standards, blank and diluted samples were added in the relevant wells; the plate was covered and incubated on an orbital plate shaker (2 h; room temperature); wells were washed twice with 200 µL 1× wash solution; 1× biotinylated detector antibody (100  $\mu$ L biotin diluent and 10  $\mu$ L 10× biotinylated antibody/well) was added; the plate was incubated on an orbital shaker (1 h; room temperature); wells were washed twice with 200 µL 1× wash solution; 100 µL 1× streptavidin-RPE solution (100 µL RPE-diluent and 10 µL 10× streptavidin-RPE/well) was added; the plate was incubated on an orbital shaker (30 min; room temperature); wells were washed thrice with 200 µL 1× wash solution; 150 µL 1× wash solution was added to each well; the plate was placed on a shaker for 2 to 3 min and then analyzed into a Luminex<sup>®</sup> 100/200<sup>™</sup> instrument. Cytokine and chemokine concentrations were determined from the standard curve using curve fitting software. Mean values from sera of 4-5 animals were calculated and expressed in a heatmap (MATLAB software, by MathWorks®) as fold-increase compared to the levels detected in the PBS-treated group defined as 0. The colors of the heatmap were mapped linearly as in the color key shown (low concentration in brown; high concentration in green). The levels of proinflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-12) and chemokines (MCP-1, MIP-1 $\alpha$ ) as well as Th1-type cytokines (IL-2, IFN- $\gamma$ ) were found increased in the serum of proT $\alpha$ /AWE- and proT $\alpha$ (100-109)/AWE-treated animals.



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