

The novel complex combination of alum, CpG ODN and HH2 as adjuvant in cancer vaccine effectively suppresses tumor growth *in vivo*

Supplementary Materials

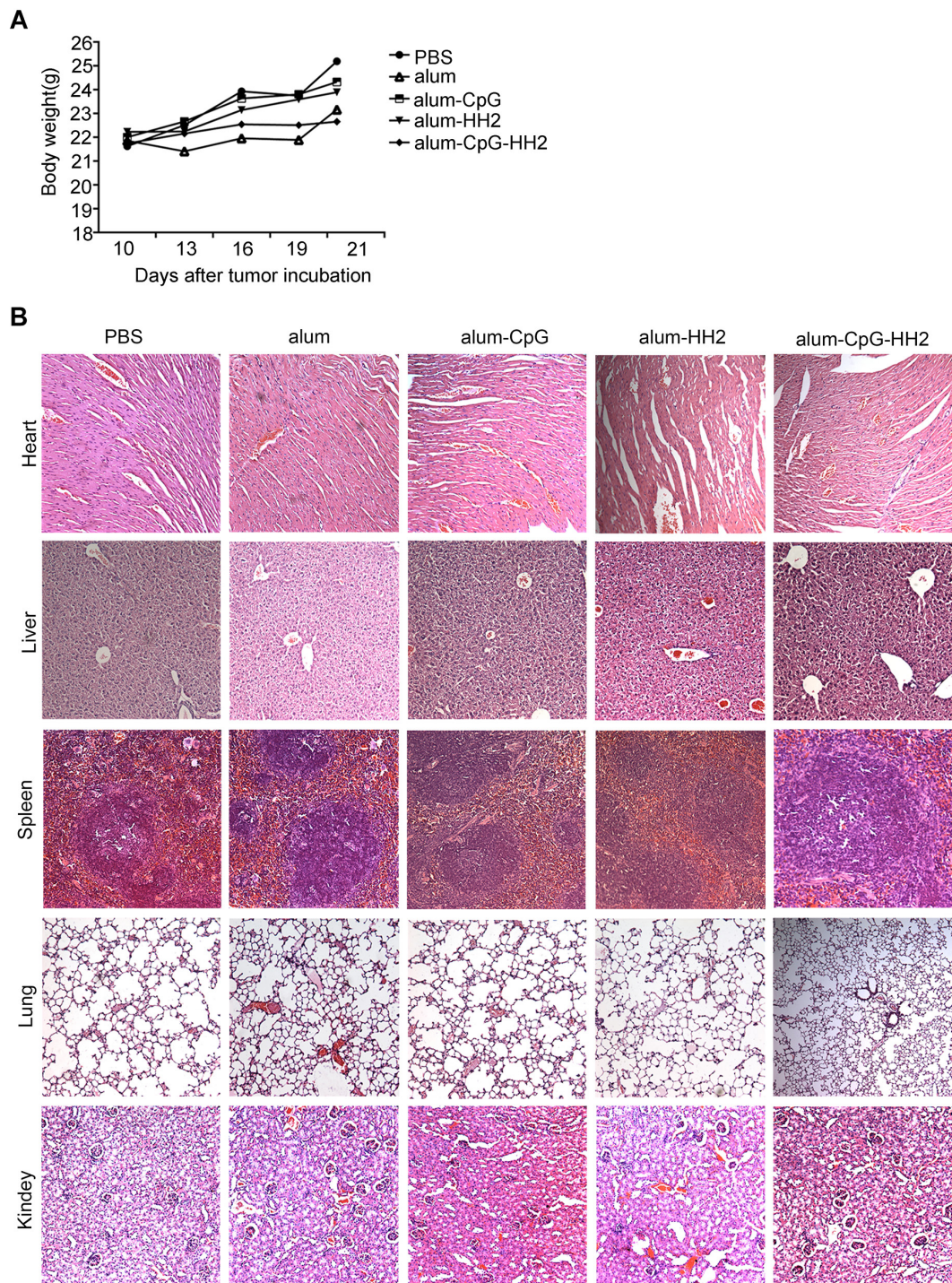
MATERIALS AND METHODS

Preparation of NY-ESO-1 protein and vaccines

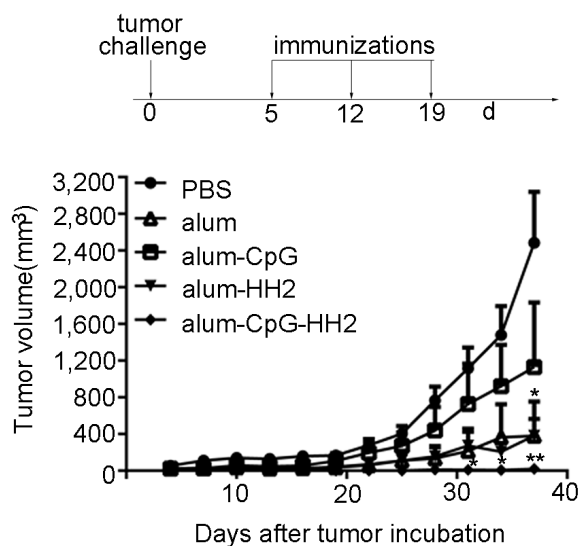
NY-ESO-1 protein (18 kDa) was prepared according to the protocols from a previous study [16]. Finally, the quality of NY-ESO-1 protein was detected by SDS-PAGE and Western blotting (Supplementary Figure 4).

To prepare the vaccines, 20 μ g CpG 1826 (Thermo Fisher Scientific, San Jose, CA, USA) was incubated with 40 μ g HH2 (Ketai Biotechnology Company, Shanghai,

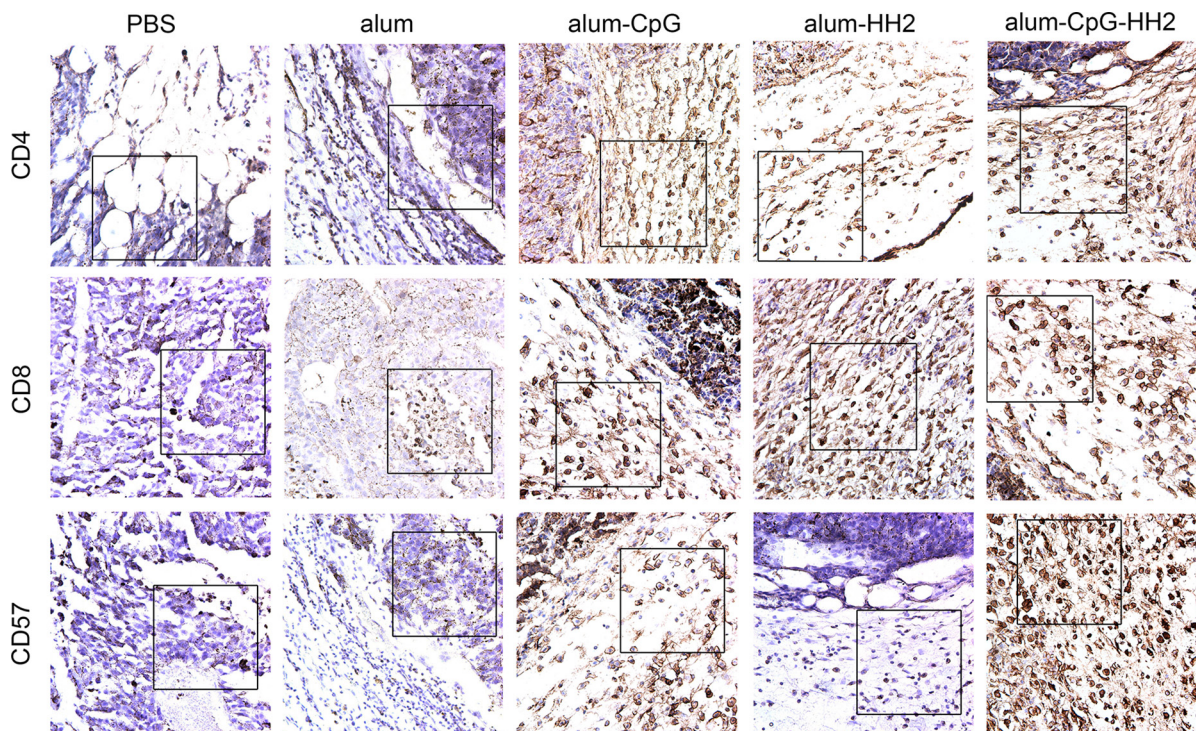
China) to form stable CpG-HH2 complex and then mixed with 125 μ g alum (Brenntag Biosector, Frederikssund, Denmark) at 37°C for 10 min, followed by the addition of 5 μ g recombinant NY-ESO-1 protein. All vaccine formulations were in a total volume of 100 μ L with phosphate-buffered saline (PBS, pH = 7.4). Mice in each tumor model reported here were divided into five groups. The clear representation of vaccine formulations in each group was shown in Supplementary Table 1.



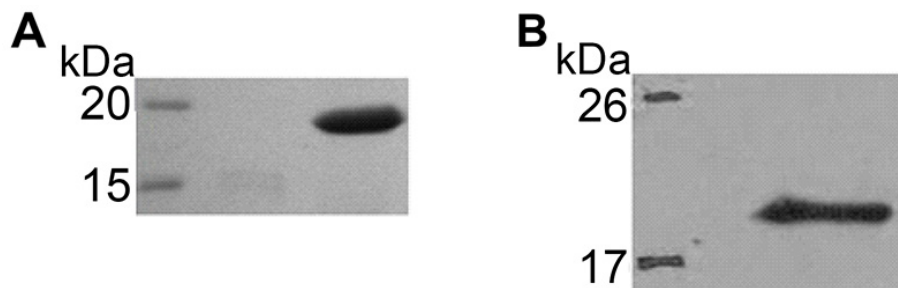
Supplementary Figure 1: Body weight and histological analyses of all major organs. (A) Body weight was determined every 3 days during the delivery period. (B) Histological analyses of heart, liver, spleen and kidney were performed by H&E staining (200×magnification).



Supplementary Figure 2: Alum-CpG-HH2-NY vaccine inhibits tumor growth in therapeutic hepatoma models. Mice were inoculated with 1×10^7 NY-ESO-1⁺ Hepa1-6 cells and treated with indicated vaccines on days 5, 12 and 19. Error bars represent mean + SEM. * $p < 0.05$, ** $p < 0.01$.



Supplementary Figure 3: The representative IHC staining for CD4⁺, CD8⁺, and CD57⁺ lymphocytes in tumor tissue from each group. A square portion of the whole image was selected and enlarged for a better observing of the positive lymphocytes.



Supplementary Figure 4: The identification of NY-ESO-1 protein. (A) SDS-PAGE analysis of purified NY-ESO-1 protein. (B) Western blot analysis of purified NY-ESO-1 protein, showing that NY-ESO-1 specifically binds to anti-human NY-ESO-1 antibody.

Supplementary Table 1: The formulations of vaccines used in animal experiments

Vaccines or groups	Vaccine formulations			
	Recombinant NY-ESO-1 protein (NY)	CpG	HH2	alum
alum-NY	5 µg	/	/	125 µg
alum-CpG-NY	5 µg	20 µg	/	125 µg
alum-HH2-NY	5 µg	/	40 µg	125 µg
alum-CpG-HH2-NY	5 µg	20 µg	40 µg	125 µg

Note: All vaccines were prepared to a total volume of 100 µL with PBS (pH = 7.4).