Supporting Information

Specific inhibition of tumor growth by T cell receptor-drug conjugates targeting intracellular cancer-testis antigen NY-ESO-1/LAGE-1

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Supplementary Information

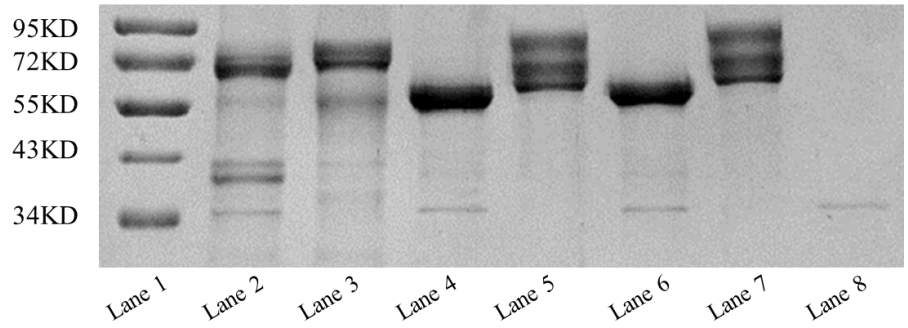

Fig. S1. Purified NY-TCRs and deglycosylated NY-TCRs separated on reduced SDS-PAGE gel stained with Coomassie brilliant blue. The NY-TCRs expressed in HEK-293 cells are subject to glycosylation, resulting in molecular sizes higher than the theoretical value. Lane 1: protein marker; Lane 2: deglycosylated NY-TCR-1 (the sizes of the two chains are approximately 38 kDa and 67.4 kDa); Lane 3: NY-TCR-1; Lane 4: deglycosylated NY-TCR-2 (the sizes of the two chains are approximately 53 kDa and 57.4 kDa); Lane 5: NY-TCR-2; deglycosylated Lane 6: NY-TCR-3 (the sizes of the two chains are approximately 53 kDa and 57.4 kDa); Lane 7: NY-TCR-3; Lane 8: Glycosylase.

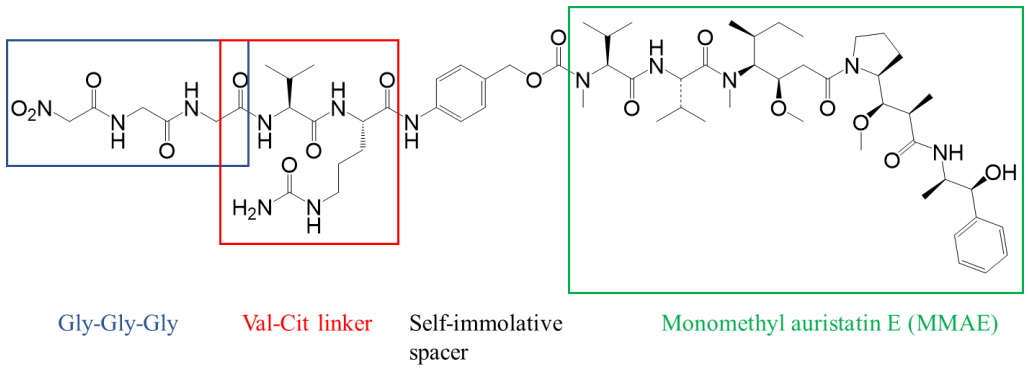

Fig. S2. Structure of triple glycine-modified MMAE toxin. The Gly-Gly-Gly motif was added to be recognized by sortase A and to form a covalent bond between the antibody and toxin. The Val-Cit linker is a cleavage site for cathepsin enzyme that was introduced to facilitate MMAE release after internalization. The self-immolative spacer will fall off automatically after enzyme cleavage. MMAE is an anti-mitotic toxin that can prevent chromosome migration and block the cell cycle by inhibiting the function of tubulin.

Supplementary Table 1. Expression of NY-ESO-$1_{157\text{-}165}$, HLA-A*02 and NY-TCR-2 binding ratio on different tumor cell lines

Cell lines	NY-ESO-$1_{157\text{-}165}$	HLA-A*02	NY-TCR-2 binding ratio
K562	Negative	Negative	Negative
K562-A2	Negative	Positive	Negative

K562-A2-NY	Positive	Positive	4.67
K562-A2-NY-pMHC	Positive	Positive	59.70
A375	Positive	Positive	Not tested
A375-NY	Positive	Positive	10.17

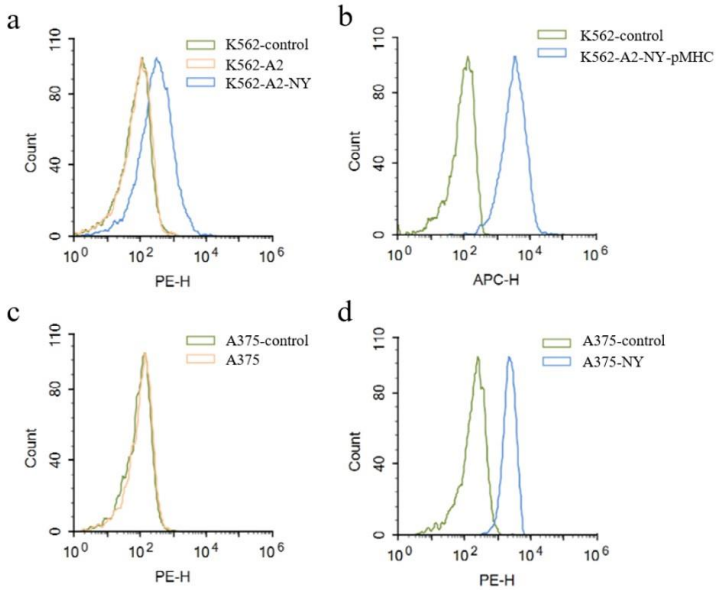

Fig. S3. NY-TCR-2 binding ability on different tumor cell lines. The extent of peak shifts to the right indicates the expression level of HLA-A2/NY-ESO-$1_{157\text{-}165}$. (a, c, d) The cells were incubated with NY-TCR-2 and then washed, immunostained with PE-labeled goat anti-human IgG (H+L), and analyzed by flow cytometry in an ACEA NovoCyteTM flow cytometer. (b) The cells were incubated with NY-TCR-2 and then washed, immunostained with Cy5-labeled goat anti-human IgG (H+L), and analyzed by flow cytometry in an ACEA NovoCyteTM flow cytometer.

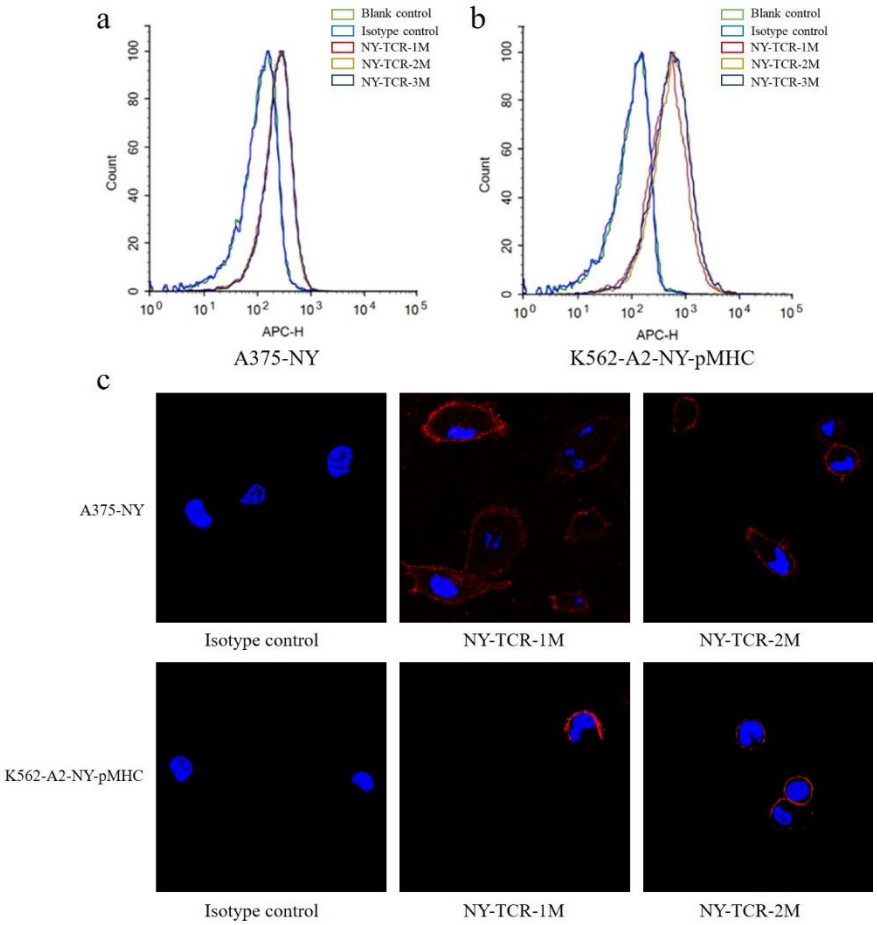

Fig. S4. NY-TCR-vcMMAEs can bind to target cells without non-specific binding caused by Fc. (a) NY-TCR-vcMMAEs specifically bind on A375-NY cells. The isotype control was an uncorrelated antibody. No peak shift to the right in isotype control group, indicating the binding specificity of NY-TCR-vcMMAEs. (b) NY-TCR-vcMMAEs specifically bind on K562-A2-NY-pMHC cells. The isotype control was an uncorrelated antibody. No peak shift to the right in isotype control group, indicating the binding specificity of NY-TCR-vcMMAEs. (c) The binding of NY-TCR-vcMMAEs on A375-NY cells and K562-A2-NY-pMHC cells using fluorescence confocal microscope. Cells were seeded on slides and treated with 10 μg/mL NY-TCR-1M, NY-TCR-2M and uncorrelated antibody. Then the cells were gently washed with PBS and incubated with Cy3-labeled goat anti-rabbit IgG (H+L) polyclonal antibody (Beyotime, China), and then incubated with DAPI for 5 min at RT. Finally, the cells were imaged with an Olympus confocal laser scanning microscope at the same parameter settings. Blue fluorescence: DAPI, red fluorescence: Cy3. No red fluorescence was observed in isotype control group, indicating the binding specificity of NY-TCR-vcMMAEs.

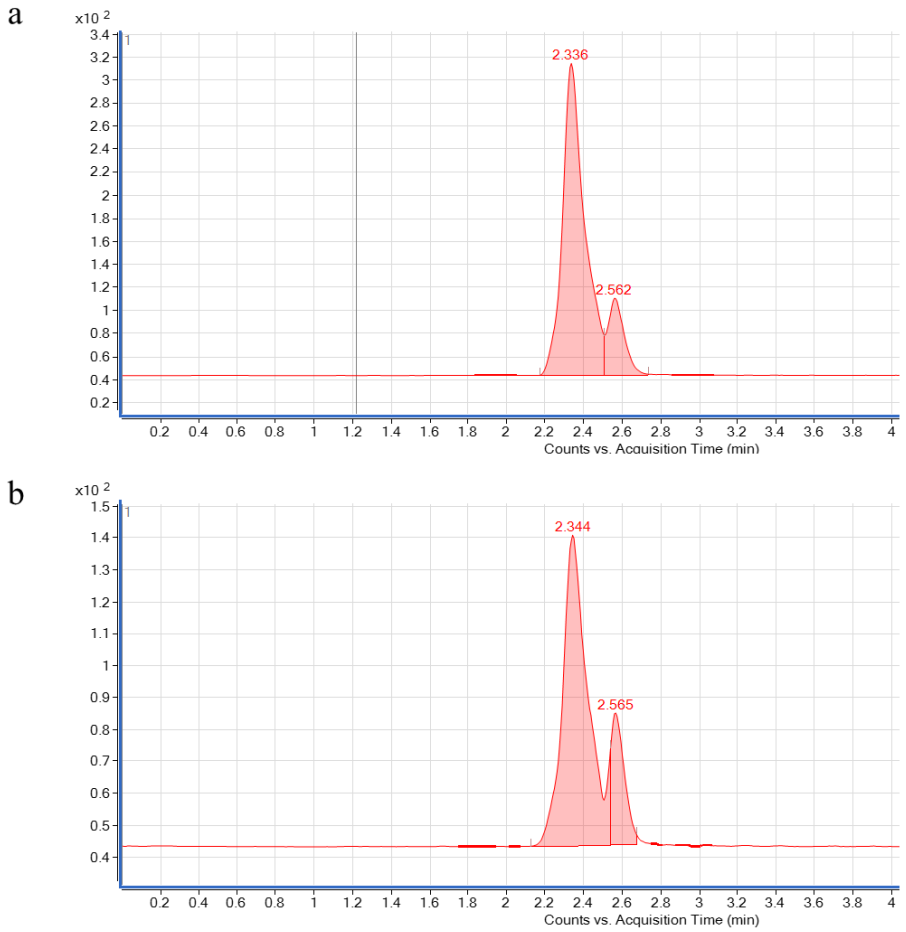

Fig. S5. Analysis of free MMAE by LC-MS/MS. An Agilent ZORBAX SB-C18（3.5 μm, 2.1×50 mm）column was used, and the chromatographic conditions were as follows: 20% B phase for 1 min, 20%-70% B phase for 1 min, 70% B phase for 2 min, and 20% B phase for 1.5 min (solvent A: 0.1% methanoic acid in water; solvent B: 1% methanoic acid in acetonitrile); column temperature: 10 ℃; flow rate: 0.300 mL/min. Agilent Technologies 6460 Triple Quad LC/MS equipped with an ESI and a Qualitative Analysis B.07.00 and QQQ Quantitative Analysis data processing system was used, and the mass spectrometry parameters were as follows: ESI: positive ionization; gas temperature: 350 ℃; gas flow: 5 L/min; nebulizer: 50 psi; sheath gas flow: 11 L/min; capillary: 4000 V; fragmentor: 159 (MMAE) and 140 (anti-protonazole as an internal standard); cell accelerator voltage: 8 V; Collision energy: 29 V (MMAE) and 35 V (internal standard); scanning mode: multi-response monitoring (MRM); ion reactions used for quantitative analysis: m/z 718.5→152.2 (MMAE) and m/z 280.2→237 (internal standard). (a) The standard free MMAE. (b) Free MMAE from the cytoplasm of K562-A2-NY-pMHC cells after incubation with NY-TCR-2M for 48 hours.

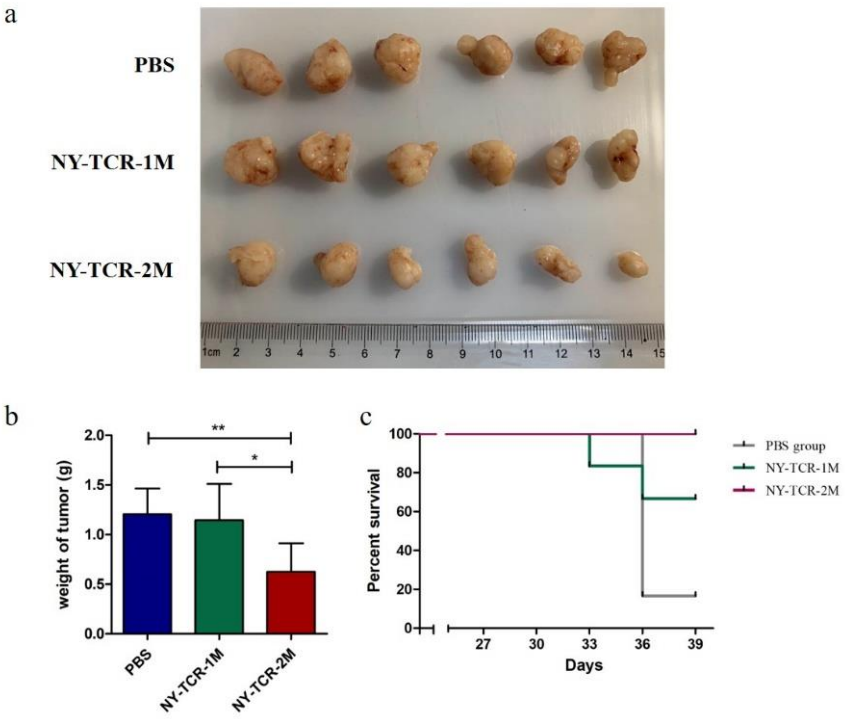

Fig. S6. *In vivo* activity of NY-TCR-1M and NY-TCR-2M in A375-NY xenograft mouse models. (a) The tumors were peeled from the mice 35 days after injection. (b) The weight of tumors 35 days after injection. The data were analyzed by t-test, and the p-value is indicated as follows: ** $p < 0.01$, * $p < 0.1$. (c) The overall survival analysis based on the antitumor activity.