# Tuning Hapten Density on Protein Conjugates Programs Vaccine Immunity via Carrier Protein Processing

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# Supplementary Tables and Figures

## 1. Buffers

The common buffer solutions used in the experiment are listed:

(1) Phosphate buffer solution (PBS buffer, 0.01mol $L^{-1}$, pH 7.4);

(2) Coating buffer (0.05 mol $L^{-1}$carbonate buffer, pH 9.6);

(3) Blocking buffer (2% skim milk powder (w/v));

(4) Washing buffer (PBST, 0.01mol $L^{-1}$ PBS buffer with 0.05% Tween-20 (v/v), pH 7.2);

(5) Goat anti-mouse IgG (HRP labeled) dilution buffer (0.01mol $L^{-1}$ PBS buffer containing 5% albumin (w/v));

(6) Stopping reagent (2 mol/L $H_2SO_4$).

## 2. Determination of antibody titer

The antibody titers of the mouse antisera were measured by using nc-ELISA: 96-well microplates were coated overnight at 4 °C with 100 μL/well coating antigens (BSA, C4 or AMDH-OVA) at 2 μg/mL in coating buffer. To eliminate non-specific binding interference, the plate was blocked with 150 μL/well blocking buffer for 2 hours at 37 ℃ and washed three times with washing buffer. Then, 100 μL/well of antisera serially diluted 2-fold was added and incubated at 37 ℃ for 1 h. After washing three times with washing buffer to remove unbound antibodies, 100 μL/well of HRP-labeled goat anti-mouse IgG (diluted at 1:5000) was added and incubated at 37 ℃ for 30 min, followed by four washes. Subsequently, 100 μL/well of substrate/chromogen solution was added to each well and incubated at 37 ℃ for 15 min. The reaction was stopped by adding 50 μL/well of 2 M $H_2SO_4$. BSA-specific,

AMDH-BSA-specific, and AMDH-specific antibody titers were defined as the reciprocal of the highest serum dilution yielding an $OD_{450nm}$ value greater than the mean plus three standard deviations (mean + 3SD) of the control wells

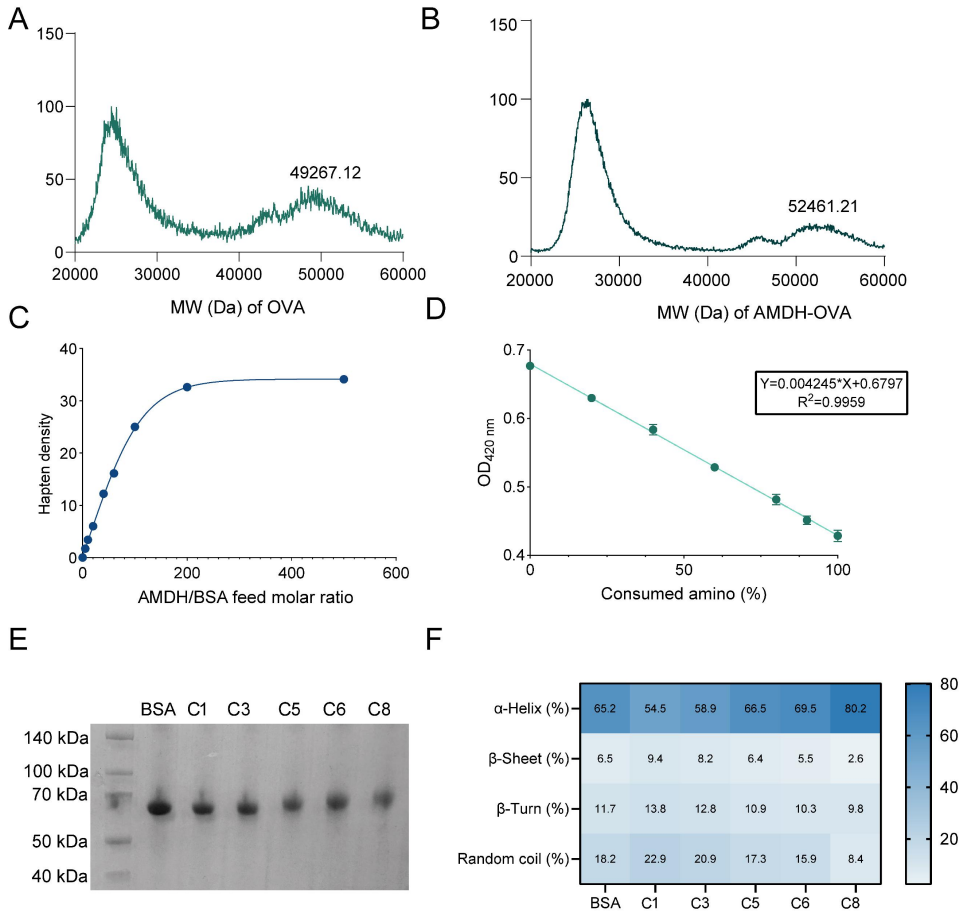
**3. Determination of antibody subclass**

Antibody subclasses in mouse antisera were determined using a ncELISA. Briefly, 96-well microplates were coated overnight at 4 ℃ for 16 h with 100 μL/well of coating antigen (AMDH-OVA and BSA) at 2 μg/mL in coating buffer. Plates were blocked with 150 μL/well blocking buffer for 2 h at 37 ℃and washed three times with washing buffer. Mouse antisera from all immunization groups were uniformly diluted to 1:1600, and 100 μL/well was added and incubated at 37 °C for 1 h. After washing three times, 100 μL/well of HRP-conjugated, isotype-specific goat anti-mouse secondary antibodies (IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA; all diluted 1:2000) were added and incubated at 37°C for 1 h followed by four washes. Subsequently, 100 μL/well of substrate/chromogen solution was added and incubated at 37 °C for 15 min, and the reaction was stopped with 50 μL/well of 2 M $H_2SO_4$. The $OD_{450nm}$ values were used to quantify the relative levels of each antibody subclass in the antisera.

**4. Determination of antibody affinity**

Antibody affinity of the mouse antisera was measured by using ic-ELISA: The 96-well microplates were coated with 100 μL/well of AMDH-OVA in coating buffer at 1 μg/mL or 0.2 μg/mL and incubated overnight at 4 °C for 16 h. After blocking, 50 μL/well of AMD standard at 100 μg/L and serially diluted antisera were added and incubated at 37 °C for 30 min. Plates were washed, followed by the addition of 100

μL/well of HRP-conjugated goat anti-mouse IgG (1:5000) and incubated at 37 °C for 30 min. After discarding wash buffer, the substrate/chromogen was added and incubated for 15 min. Then, the reaction was stopped by 50 μL/well of $H_2SO_4$, and the $OD_{450}$ were measured. Serum dilutions yielding $OD_{450}$ values within 1.5-2.0 were selected to calculate inhibition rates for antibody affinity evaluation. The lowest inhibition rate obtained for each antisera under its optimal coating antigen concentration and antisera dilution was used for subsequent statistical analyses.




Supplementary Fig. 1 Characterization of hapten density and protein structure of AMDH-BSA and AMDH-OVA. (A and B) Molecular weight characterization of OVA and AMDH-OVA conjugates by MALDI-TOF/MS, with the corresponding hapten densities summarized in Table 1. (C) Correlation between hapten density calculated by MOLDI-TOF/MS result and feed ratio used during synthesis. Data are fitted to a linear regression ($R^2 = 0.9959$). (D) The standard curve of amino groups occupation in natural BSA was detected by the TNBS method, plotted as $OD_{420}$ versus percentage of consumed amino groups. (E) SDS-PAGE detection of AMDH-BSA conjugates C1, C3, C5, C6, and C8 alongside BSA and molecular weight markers. (F) Comparisons of secondary structure of AMDH-BSA conjugates C1, C3, C5, C6, and C8 by CD spectrum, showing α-helix, β-sheet, β-turn, and random coil percentages.

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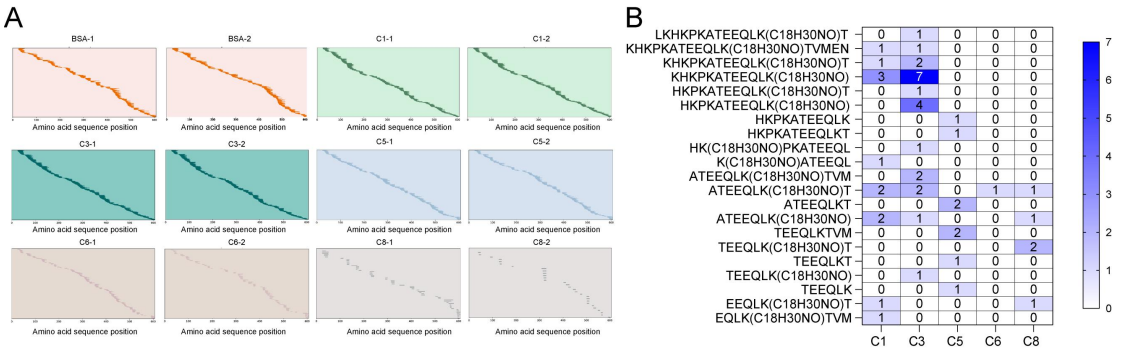
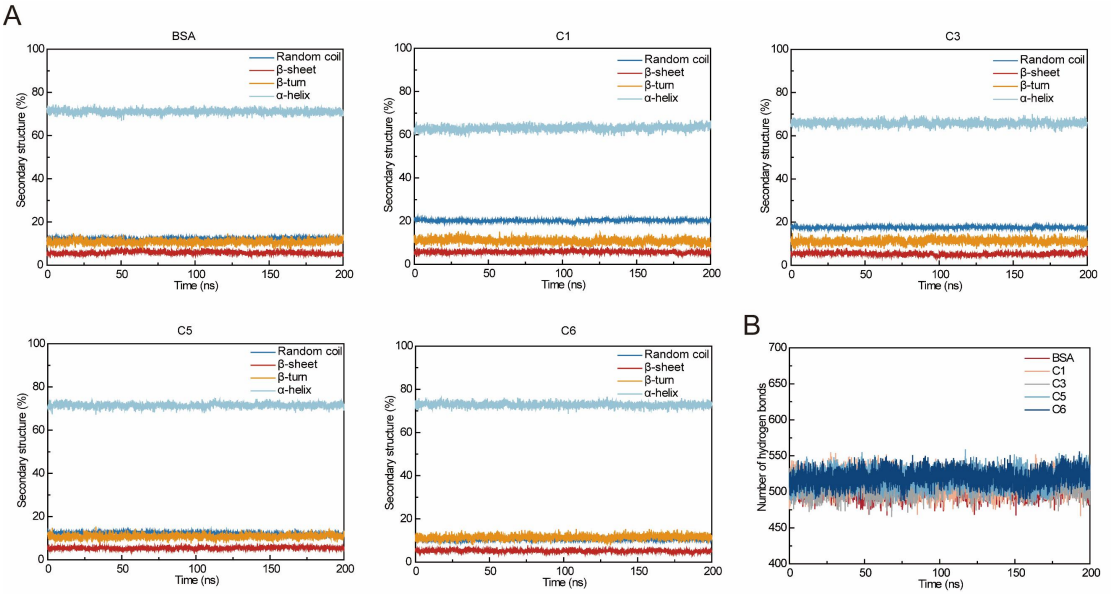
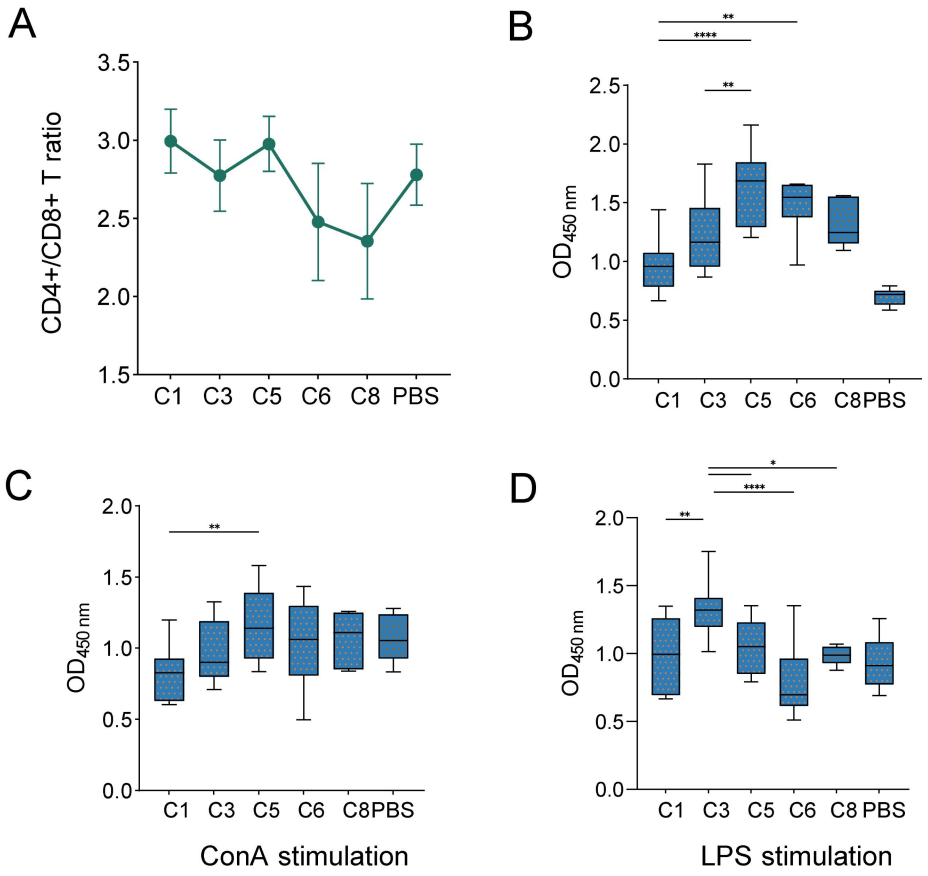



Supplementary Fig. 2 Influence of AMDH-BSA conjugates with varied hapten densities on cathepsin S-mediated BSA digestion *in vitro*. (A) Peptide coverage maps of BSA and AMDH-BSA conjugates C1, C3, C5, C6, and C8 after enzymatic degradation with cathepsin S. Each identified peptide is represented as a horizontal line mapped onto the BSA amino acid sequence. The y-axis height varies with the abundance of detected peptides, while the x-axis corresponds to amino acid positions in BSA. (B) Heat map of PSMs differences for the LKH556-571 peptide cluster of BSA. The peptide sequence shown corresponds to the AMDH-modified peptide containing the modification (C18H30NO) on the lysine residue. PSMs were quantified with posterior error probability (PEP) values of <0.01. Two independent experiments are labeled as "-1" and "-2" after each sample name.




Supplementary Fig. 3 Effect of hapten density on the secondary structure and hydrogen bonding interactions of BSA by 200 ns MD simulations. (A) Secondary structure composition of BSA and AMDH-BSA conjugates C1, C3, C5, C6 over the 200 ns simulation trajectory, showing percentages of α-helix, β-sheet, β-turn, and random coil at 0, 50, 100, 150, and 200 ns. (B) Number of hydrogen bonds in BSA and AMDH-BSA conjugates C1, C3, C5, and C6 over the 200 ns simulation

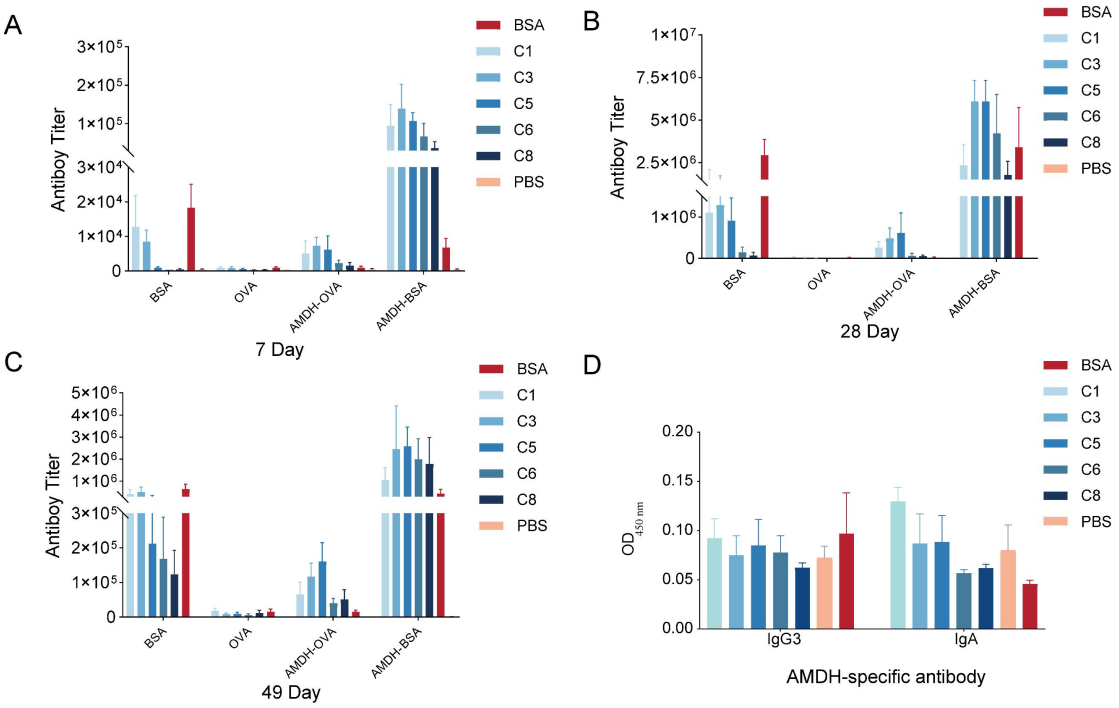
trajectory.



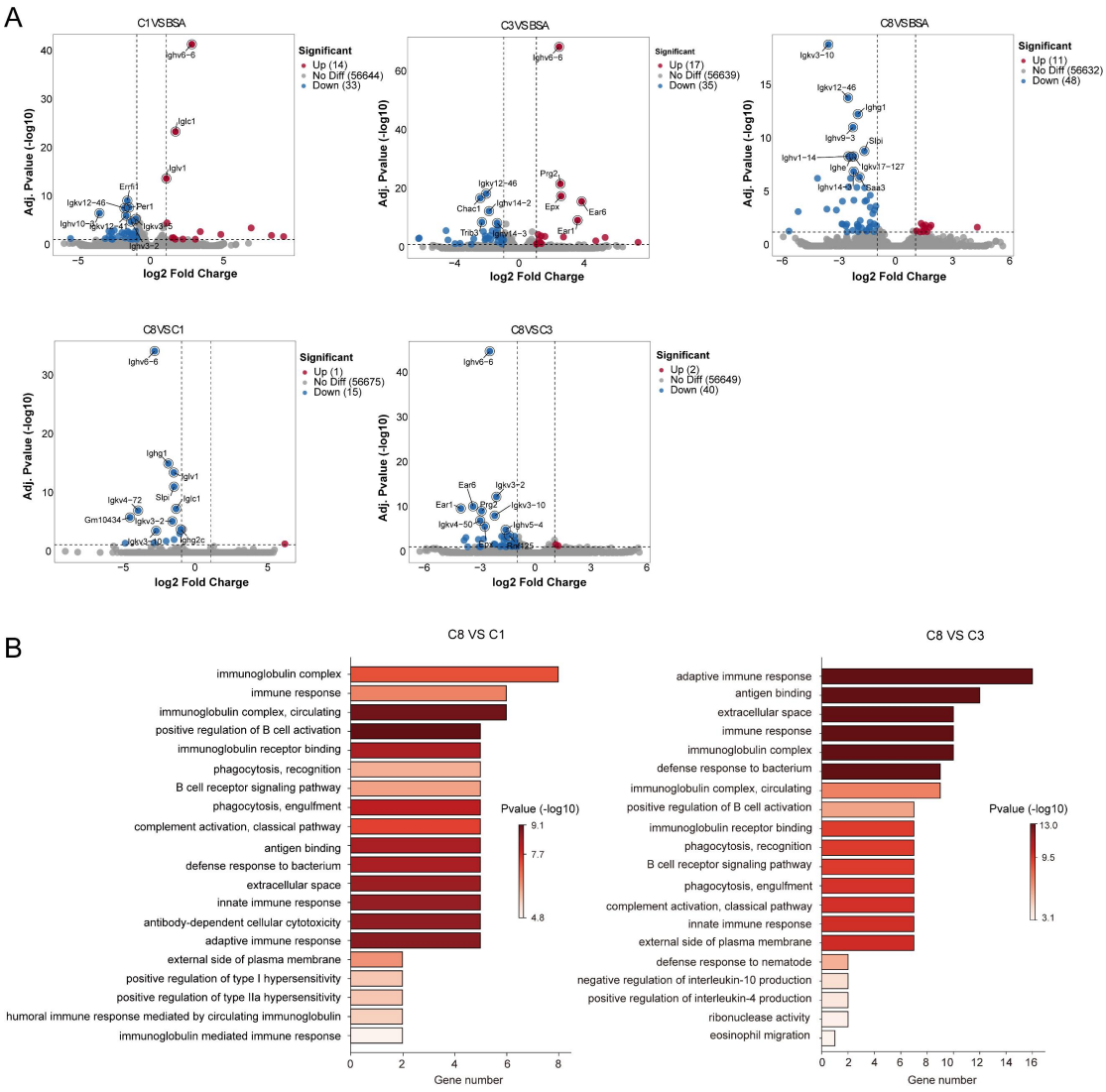

Supplementary Fig. 4 Influence of AMDH-BSA conjugates with varied hapten densities on $CD4^+/CD8^+$ T cells ratio and splenocytes proliferation.(A) $CD4^+/CD8^+$ T cells ratio in splenic lymphocytes from mice immunized with C1, C3, C5, C6, C8, or PBS. (B) $OD_{450}$ of splenocytes proliferation after treatment with 50 μg/mL AMDH-BSA conjugates C1, C3, C5, C6, C8, or PBS for 72 h; (C) $OD_{450}$ of splenocyte proliferation after treatment with AMDH-BSA conjugates in the presence of 10 μg/mL ConA (T cell stimulant). (D) $OD_{450}$ of splenocyte proliferation after treatment with AMDH-BSA conjugates in the presence of 12.5 μg/mL LPS (B cell stimulant). * $p < 0.05$, * * $p < 0.005$, * * * $p < 0.001$, * * * *$p < 0.0001$. Means and standard deviations are displayed (n=3 mice/group). Statistical significance was determined by ANOVA with multiple comparison testing among all groups except the

PBS

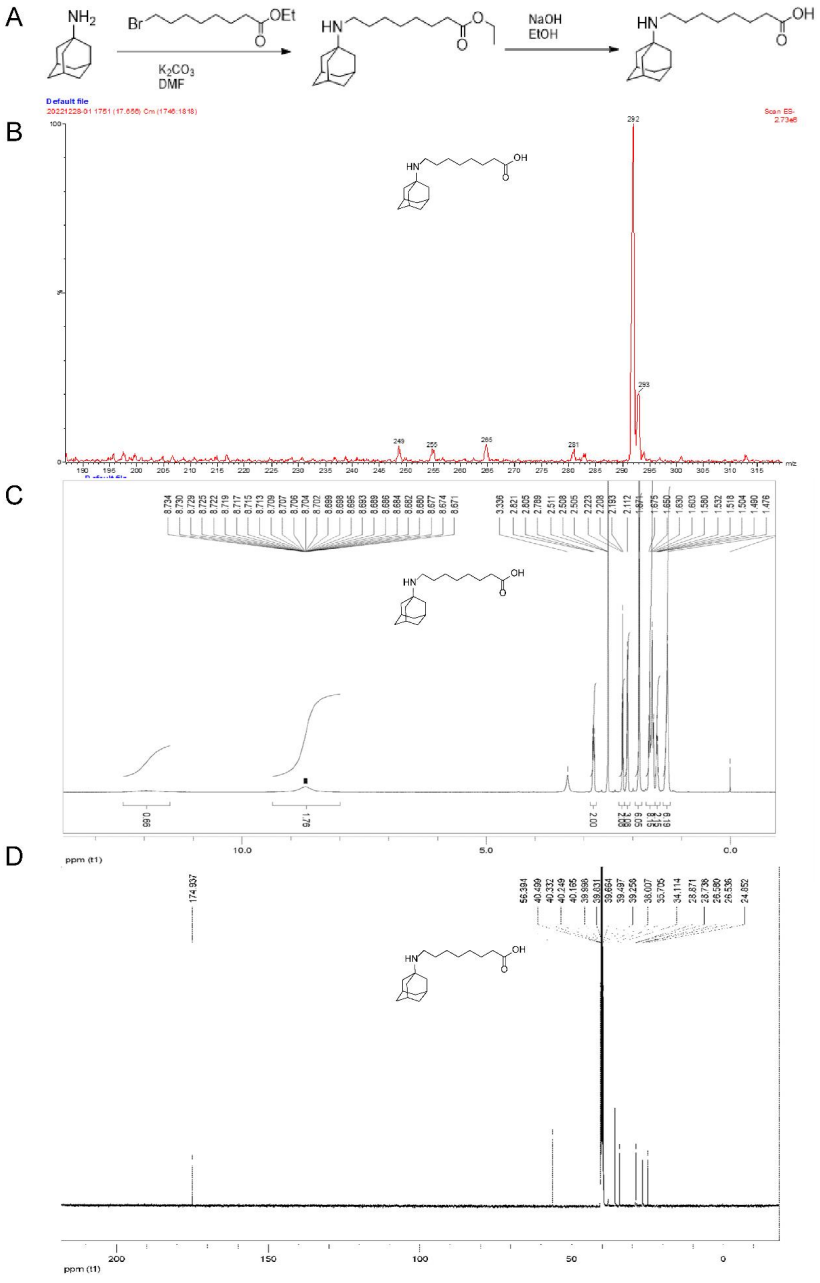
group.




Supplementary Fig. 5 AMDH-BSA-specific, BSA-specific, and AMDH-specific antibody titers of antisera from vaccinated mice on (A) day 7, (B) day 21 and (C) day 49. (D) AMDH-specific IgG3 and IgA subclasses $OD_{450}$ values in antisera from immunized mice on day 28, determined by nc-ELISA. Means and standard deviations are displayed (n=6-8 mice/group).




Supplementary Fig. 6 Influence of hapten density on immune repertoire and transcriptional programs in bulk RNA-seq. (A) Volcano plots of differential expression analysis for C1 versus BSA, C3 versus BSA, C8 versus BSA, C8 versus C1, C8 versus C3. Significantly upregulated and downregulated genes are shown in red and blue, respectively (n=3 mice per group). (B) Top 20 GO analysis for C8 versus C1 (left) and C8 versus C3 (right).




Supplementary Fig. 7 Synthesis and characterization of hapten AMDH. (A) Synthetic routes of AMDH. Reaction conditions: (i) ethyl bromoacetate, $K_2CO_3$, DMF, 60 °C, 16 h; (ii) NaOH, EtOH/$H_2O$, 1 h, then HCl to pH 5. (B) The mass spectrum of AMDH. The observed molecular ion $[M-H]^-$ at m/z 292 is consistent with the calculated mass ($C_{18}H_{30}NO_2^-$, 292). (C) The $^1H$ NMR spectrum of AMDH in DMSO-$d_6$. (D) The $^{13}C$ NMR spectrum of AMDH in DMSO-$d_6$.

Supplementary Table 1. Molecular descriptors of AMD and AMDH

| Analytes | Molecular Properties | | | | | | |
|---|---|---|---|---|---|---|---|
| | MW[a] (g/mol) | $V_m$[b] (Å$^3$) | E[c] (kcal/mol) | μ[d] (D) | SA[e] (Å$^2$) | MPI[f] (kcal/mol) | PSA[g] (Å$^2$) |
| AMD | 151.25 | 213.65 | -446.03 | 1.30 | 308.21 | 5.82 | 25.68 (13.56 %) |
| AMDH | 293.45 | 410.72 | -909.74 | 2.23 | 574.45 | 7.25 | 72.83 (19.92%) |

[a] MW: molecular weight; [b] Vm:Molar volume; [c] Single Point Energy; [d] Dipole Moment; [e] SA: Solvent Accessible Surface Area; [f] Molecular Polarizability Index; [g] Polar Surface Area.

Supplementary Table 2. Analysis of hapten density in AMDH-BSA conjugates by TNBS method

| Conjugates | AMDH-BSA mole ratios | $OD_{420}$ | TNBs | |
|---|---|---|---|---|
| | | | amount of consumed amino (%) | Hapten density[a] (moles/BSA molecule) |
| BSA | 0;1 | 0.68 | -0.3 | 0 |
| C1 | 5:1 | 0.678 | 0.5 | 0.2 |
| C3 | 20:1 | 0.648 | 12.3 | 7.5 |
| C5 | 60:1 | 0.632 | 18.8 | 11.5 |
| C6 | 100:1 | 0.561 | 45.4 | 27.7 |
| C8 | 500:1 | 0.513 | 66.6 | 40.6 |

[a] Hapten density is calculated by percentage of consumed amino multiplying the 61 free amino acids of BSA.

Supplementary Table 3. The information of immunization procedures.

| Immunizations | Adjuvants | Dose/mice | Immunization routes | Interval time | Blood collection | Spleen harvest |
|---|---|---|---|---|---|---|
| 1st | Freund's complete adjuvant | 100 μg | Subcutaneous multiple injections on the neck and back | 21 days | After 7 days | - |
| 2rd | Freund's incomplete adjuvant | 100 μg | Subcutaneous multiple injections on the neck and back | 21 days | After 7 days | After 7 days |
| 3st | Freund's incomplete adjuvant | 100 μg | Subcutaneous multiple injections on the neck and back | 21 days | After 7 days | After 7 days |

- No spleen collection.

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