

1 **Tuning Hapten Density on Protein Conjugates Programs**

2 **Vaccine Immunity via Carrier Protein Processing**

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Abstract

Hapten-protein conjugates are widely used in vaccines against tumors and addictive drugs, antidotes to toxins, and diagnostic antibodies for hazard detection. However, how hapten density of conjugates governs the ensuing immune response remains poorly defined, hindering rational hapten-based vaccine design. Here, we systematically investigate the relationship between hapten density and immunogenicity using a model system of AMDH-BSA conjugates with precisely controlled and well-characterized hapten densities spanning 1.8 to 36.3. We demonstrate that low-to-moderate hapten densities (1.8-17.2) elicit robust humoral immunity, characterized by potent germinal center B cell responses, CD4⁺ T cell activation, and high-affinity antibody production. In contrast, excessive hapten loading (26.7 to 36.3) compromises immune responses through a chemically defined mechanism whereby the carrier protein is stabilized into a rigid, α -helix-rich conformation that sterically hinders proteolytic cleavage, thereby reducing the diversity of processed peptides. This impaired antigen processing leads to defective CD4⁺ T cell activation, attenuated germinal center reactions, and ultimately compromised humoral immunity. Bulk and single-cell transcriptomics corroborate this mechanism, revealing global downregulation of antigen-processing and B cell receptor signaling pathways, while TCR/BCR repertoire sequencing shows that higher hapten density conjugates altered V(D)J usage and fail to drive effective clonal

44 expansion. Our findings establish hapten density as a tunable chemical parameter for
45 programming vaccine immunity, providing a quantitative strategy for the rational
46 design of next-generation hapten-based immunogens.

47 **Key Words:** Hapten density; Antigen processing; Conformational rigidity; Chemical
48 immunology; Vaccine design

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Introduction

Small molecules with single B cell epitope such as hormones, polysaccharides, drugs of abuse, pesticides, antibiotics, etc. are usually not capable of eliciting an immune response by themselves. Therefore, structural analogs of target molecules, named haptens with active groups, are chemically conjugated to larger molecules with T cell epitope, called carriers such as proteins, inducing specific immune response and antibodies against small molecules¹⁻³. Since Karl Landsteiner's pioneering work in the 1920s, the implementation of hapten-protein conjugates has proven to be a versatile chemical strategy in vaccines against tumors and drugs, antidotes to toxins, and immunoassays for compounds⁴⁻⁷. The utility of these conjugates depends on their immunogenicity that is the ability to elicit high-titer, high-affinity antibodies against the target hapten. The immunogenicity of hapten-protein conjugates have been widely attributed to the physicochemical characteristics of each component in a given conjugate⁸, including hapten structure^{9,10}, spacer linker^{11,12}, carrier protein type^{13,14}, and chemical conjugation method¹⁵. In addition to the above, hapten density, the average number of haptens conjugated to each carrier protein molecule, is also a key determinant of immunogenicity, as it directly impacts antigen processing, B cell activation, and antibody responses^{16,17}. Given that hapten density is a readily tunable chemical parameter, understanding how it programs vaccine immunity is essential. Despite its importance, the specific role of hapten density remains poorly understood and often overlooked, and no clear consensus exists on the optimal hapten density for achieving both high antibody titers and affinity maturation^{16,18,19}.

Evidence from protein antigens indicates that multivalency enhances B-cell activation, class switching, and affinity maturation^{20,21}. Similarly, studies on Pfs25-based circumsporozoite protein conjugate vaccines have shown that optimizing repeat valency and the peptide-to-carrier ratio is critical for eliciting potent and durable anti-sporozoite antibody responses²². However, it remains unclear to what extent principles learned from protein or peptide epitope valency can be directly applied to hapten epitopes. Recently, several studies have reported that increasing hapten density can lead to higher antibody titers; however, other publications have shown that excessively high hapten density can instead reduce antibody performance^{1,23–25}. The basis for these discrepancies is unknown. These apparent contradictions have limited the application of hapten-protein conjugates in vaccine development and antibody discovery.

From the available studies, it is difficult to accurately define the relationship between the immunogenicity and hapten density of conjugates because: (1) hapten density of conjugates is often not measured or reported or fully characterized; (2) haptens, carrier proteins, and employed adjuvants, immune procedures and evaluation timelines are often proprietary and thus not readily available for direct comparative analyses; (3) the extent to which hapten modification alters carrier protein conformation and processing, and how this in turn shapes T-cell epitope presentation and vaccine immunity, remains largely unknown; (4) immunogenicity of hapten-protein conjugates is only evaluated by antibody titer or/and antibody affinity, with little exploration of antigen processing and presentation, B/T cell activation, germinal

center (GC) responses, transcriptome and TCR/BCR repertoires changes. Thus, a thorough understanding of how the hapten density of conjugates affect the strength and quality of immune responses to hapten epitope is highly demanded. This requires precisely controlled conjugate preparation within a suitable range and full characterization from multiple perspectives including molecular weight, structural conformation and hapten conjugation sites and in-depth immunological analysis including antigen processing, antigen processing cell (APC) and T cell activation, GC response and antibody production.

To address these gaps, we designed a model system using amantadine (AMD) as the target small molecule. AMD was approved by the US Food and Drug Administration in 1968 as a prophylactic agent for influenza and Parkinson's disease. It also served as potential therapeutics for COVID-19 and several further indications^{26,27}. However, AMD has been illegally used in poultry production, leading to the 2012 “Doping Chickens”, which is one of the largest food safety incidents in China and raised significant food safety concerns. AMD possesses a defined and stable “lipophilic bullet” with free hydrophilic amino, facilitating spacer linker introduction, making it an ideal hapten epitope model. In this work, we introduced an optimum length spacer linker with 8 carbon atoms (8.8 Å) as AMD hapten named AMDH^{11,28}. Bovine serum albumin (BSA) was mostly used as the carrier protein, which is widely recognized as a standard model protein for *in vivo* carrier studies, with its crystal structures and potential epitopes having been extensively investigated²⁹. We precisely prepared and fully characterized AMDH-BSA conjugates

with varied hapten densities ranging from 1.8 to 36.3 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and three complementary techniques based on distinct analytical principles. Using this well-defined conjugate library, we combined in vitro antigen processing assays, in vivo immunization, multi-omics profiling, and immune repertoire sequencing to achieve two questions: what density range elicits optimal antibody responses, and by what chemical mechanism does density modulate immunogenicity? We hypothesized that tuning hapten density on protein conjugates programs vaccine immunity not simply through valency effects on BCR crosslinking, but more fundamentally through altering the structural properties of the carrier protein, thereby affecting its susceptibility to proteolytic processing and subsequent T-cell help.

Results

1. Rational Design and Validation of Hapten AMDH

Our previous study demonstrated that haptens with a spacer linker of 6-8 carbon atoms (6.3-8.8 Å in length) more effectively elicit strong antibody responses against small molecules¹¹. In this study, we designed a model hapten of AMD, named AMDH, by introducing an 8-methylene linear spacer linker with 8.8 Å to the amino group of AMD. This derivatization results in a terminal carboxyl group of AMDH that serves as the bioconjugation site (Fig. 1A and Supplementary Fig.1A).

To assess the rationality of this design prior to chemical synthesis, we performed ESP mapping, a widely accepted tool for evaluating electronic properties of small molecules³⁰. As shown in Fig. 1A, the ESP map of the lowest-energy conformations of AMDH closely resembled that of AMD, with neutral regions (white) dominating the van der Waals surface and an ESP distribution ranging primarily from 10 to -10 kcal/mol. Superposition of the lowest-energy conformations of AMD and AMDH yielded an RMSD of only 0.0048 Å, indicating that AMDH retains the electronic characteristics and conformation of the parent drug. Additional molecular descriptors, such as dipole moment (μ), molecular polarizability index (MPI), and polar surface area (PSA), were also concordant between AMDH and AMD (Supplementary Table 1). These results confirm the structural and electronic similarity of AMD and AMDH, guarantee the rationality of the hapten AMDH design, and support its suitability as a hapten for subsequent immunogenicity studies. Having established the chemical validity of AMDH, we selected BSA as the carrier protein. BSA is structurally well-

characterized, with a known crystal structure and extensively mapped epitopes, making it a standard model for carrier studies *in vivo*²⁹. This well-defined system allowed us to precisely control and characterize hapten density on the conjugate, as described below.

2. Preparation and Characterization of AMDH-BSA Conjugates with Various Hapten Densities

To investigate the influence of hapten density of conjugates on immunogenicity, we conjugated AMDH to BSA as an immunogen and to OVA as a coating antigen using the active ester method. By systematically varying the molar ratio of AMDH to BSA from 5:1 to 500:1, we generated a series of AMDH-BSA conjugates and designated them as C1 through C8 in ascending order of hapten density (Fig. 1B). The conjugation of AMDH and BSA or OVA involved forming amide bonds between the carboxyl group of AMDH and the ϵ -amino groups of lysine residues (LYS) of BSA or OVA. Then, we characterized the hapten density of AMDH-BSA conjugates using multiple complementary analytical methods.

We first measured hapten densities using MALDI-TOF/MS, a precise technique for quantifying conjugate composition¹⁹. As shown in Fig. 1C and Table 1, the calculated hapten densities ranged from 1.8 to 36.3, successfully covering a near-theoretical maximum range on BSA. We also prepared an AMDH-OVA conjugate as a coating antigen, with a hapten density of 10.1 (Table 1 and Supplementary Fig.1A and 1B). The relationship between the feed molar ratio of hapten to BSA and the calculated

hapten density was correlated but not linear. The hapten density gradually increased with a rising molar ratio of AMDH to BSA and plateaued when the molar ratios exceeded 200:1 (Supplementary Fig. 1C). From this series, we selected five conjugates, C1, C3, C5, C6, and C8, as immunogens for subsequent immunogenicity study due to their significantly distinct hapten densities. To further validate these density assignments, we performed TNBS assay for quantitative analysis (Supplementary Fig. 1D and Supplementary Table 2) and SDS-PAGE for qualitative characterization (Supplementary Fig. 1E). Although the calculated hapten density by TNBS assay ranging from 0.2 to 40.6 differed slightly from values obtained via MALDI-TOF/MS, the overall trend of increasing hapten density with higher feed molar ratio of AMDH to BSA remained consistent across methods (Fig. 1C and Supplementary Fig. 1E).

We next asked whether varying hapten density induced conformational changes in the carrier protein, as such changes could influence immune cell recognition and activation^{31,32}. To address this, we used fluorescence spectroscopy and CD spectra to characterize structural alterations of native BSA and the AMDH-BSA conjugates with varied hapten densities. Fluorescence spectroscopy, conducted at an excitation wavelength of 290 nm, selectively targeted tryptophan residues, allowing to assess changes in their local microenvironment after hapten conjugation³³. It was observed in Fig. 1D that the emission wavelength of free hapten AMDH was 290-310 nm which was out of the emission wavelength of BSA between 310-450 nm, implying that AMDH did not interfere with the intrinsic fluorescence of BSA. Increasing hapten

density from C1 to C8 progressively quenched fluorescence of BSA, indicating additional amide bonds formation between AMDH and BSA, and alterations in the local microenvironment of carrier BSA tryptophan residues. A blue shift in the maximum wavelength further suggested significant conformational changes in the structure of conjugates³⁴⁻³⁶. CD spectroscopy revealed hapten conjugation altered the secondary structure composition of BSA. Even at the lowest density (C1, 1.8), conjugation induced significant changes in the secondary structure, with decreased α -helical content from 65.2 to 54.5% and increased random coil content from 18.2 to 22.9% (Fig.1E, 1F and Supplementary Fig. 2F). This indicates that even a small amount of hapten modification can disrupt native BSA conformation, particularly its α -helices structure. Previous studies have suggested that newly formed amide bonds may disrupt the α -helix by breaking hydrogen bonding networks of BSA and altering the local chemical environment^{37,38}. However, as hapten density increased from 1.8 to 36.3, we observed a progressive increase in α -helix content by 25.7 percentage points, accompanied by decreases in β -sheet, β -turn and random coil by 6.8, 4.0 and 9.8 percentage points, respectively. This trend suggests that conjugates with high hapten densities could promote α -helix formation and allow the conjugates to partially refold into a more ordered structure³⁹. The structural basis for this density-dependent refolding was further investigated through molecular dynamics simulations (see below).

In summary, we successfully prepared and extensively characterized AMDH-BSA conjugates with hapten densities of 1.8-36.3. Specifically, BSA contains 61 primary

amino groups from 60 lysine and an N-terminus amino group, of which 30-35 are solvent-accessible and thus available for conjugation, forming amide bonds between hapten and protein⁴⁰. Thus, these conjugates with hapten density almost covers theoretically the lowest of 1.8 and the highest hapten densities of 36.3, providing a well-defined model system for studying how hapten density affects immune responses to small molecule.

3. Hapten Density Regulates Macrophage Uptake and Activation

Antigen uptake by antigen-presenting cells (APCs), such as macrophages and dendritic cells, is the initial step in eliciting immune responses⁴¹. FITC-labeled AMDH-BSA with various hapten densities were incubated *in vitro* with RAW264.7 macrophages and MFI values were measured at 30 min and 2 h. As shown in Fig. 2A and 2B, the MFI of RAW264.7 cells increased markedly upon exposure to conjugates with higher hapten densities of 26.7-36.3 at 30 min ($p < 0.01$). The MFI increased significantly in a stepwise manner with increased hapten density, i.e., C8 > C6 > C5 > C3 > C1 > BSA. This trend became more pronounced after 2 h of incubation; notably, C8 with the highest hapten density displayed more than a 10-fold increase in MFI compared to C1 and bare BSA, highlighting a strong correlation between hapten density and macrophage internalization. CLSM further confirmed the hapten density-dependent uptake of AMDH-BSA conjugates by macrophages (Fig. 2C).

We next asked whether this increased uptake translates into enhanced macrophage activation. Activated macrophages upregulate cell proliferation and surface expression

of MHC-II and costimulatory molecules such as CD40, CD80, and CD86, which then facilitate T cell activation⁴². CCK8 assays indicated that C5, C6 and C8 with higher hapten density of 17.2-36.3 significantly decreased RAW264.7 cell proliferation in a dose-dependent manner, particularly at 60 µg/mL and 30 µg/mL ($p < 0.0001$) (Fig. 2D). In contrast, C1 and C3 with lower hapten density of 1.8 and 6.4 exhibited pro-proliferative effects at these concentrations; C1 even exceeded the positive control of LPS at 60 µg/mL. Thus, higher hapten densities of conjugates above 17.2 inhibited macrophage proliferation, showing an opposite trend to that observed for macrophage uptake.

We then quantified surface expression of activation markers by flow cytometry. C1 and C3 modestly increased MHC-II and costimulatory molecule expression. C5 induced the highest level of MHC-II upregulation but did not further augment CD40, CD80, and CD86. In the case of C6 and C8 with higher hapten density (26.7-36.3), expression of MHC-II, CD40, CD80, and CD86 all sharply declined (Fig. 2E-2F). Together, these data reveal a clear hapten density-dependent functional dichotomy, with low hapten densities enhancing macrophage proliferation and activation, whereas very high densities (>26.7 in the study) suppress these activation hallmarks despite substantially increased uptake. This phenomenon of high uptake with low activation is attributed to the high hapten density of the conjugate acting on the endosome-lysosome degradation pathway, thereby impairing antigen processing efficiency and resulting in low activation⁴³. We therefore investigated the effect of hapten density on proteolytic degradation, as described below.

4. Hapten Density Modulates Antigen Processing via Enzymatic Degradation

Building on the discovery of the paradoxical "high uptake with low activation" phenomenon in APCs induced by high hapten density conjugates (26.7-36.3), we established an LC-MS/MS based *in vitro* enzymatic degradation platform to systematically evaluate the impact of hapten density on conjugate proteolysis⁴⁴. AMDH-BSA conjugates with varied hapten densities were treated with cathepsin S, a key protease for antigen processing, to simulate this process in APCs⁴⁵. We first analyzed peptide-spectrum matches (PSMs) for hapten-modified lysine (K) residues. All detected hapten-modified lysine residues are marked in red in Fig. 3A. We ranked the identified PSMs for each conjugate and selected the top-ranked lysine modification sites to infer the most possible modification sites. Mapping of these sites revealed distinct hapten density-dependent conjugation preferences. The speculated hapten conjugation sites of BSA for each conjugate are marked by yellow, green, red, and purple stars for AMDH-BSA conjugates C1, C3, C5, C6, and C8, respectively. Conjugates C1 and C3 with lower hapten densities of 1.8 and 6.4 primarily modified surface-exposed lysine residues such as Lys36 and Lys420, whereas conjugates C6 and C8 with higher hapten density exhibited modification extending to internal lysine residues of BSA, including Lys412 and Lys597. This shift from protein surface to interior modification with increasing hapten density indicates progressive structural alteration of the carrier protein, consistent with the conformational changes observed by CD spectroscopy (Fig. 1E-F).

We next assessed the overall location of the degraded peptides generated from enzymatic digestion of the BSA and hapten-BSA conjugates. As shown in Fig. 3B and Supplementary Fig. 2A, C6 and C8 with higher hapten density of 26.7-36.3 obviously narrowed the diversity of detectable peptide species, representing by obviously reduced numbers of peptides observed in the y-axis plotted at a fixed height. Peptides generated from C1, C3 and C5 with low hapten densities *in vitro* enzymatic processing could be classified into 20-22 major peptide clusters of T cell epitopes that were visualized as distinct color segments on a donut plot, a reduced epitope diversity of only 8-17 clusters was observed for C6 and C8 with high hapten densities (Fig. 3C). In addition, the total number of peptides derived from C8 plummeted to 28 peptides that was a dramatic 98% reduction compared to the 1,356 peptides from C3 with hapten density of 6.4, showing a marked decline in enzymatic degradation efficiency. Of particular interest, hapten-preferred peptide clusters such as and YST393-413 and LKH556-571 yielded far fewer AMDH-modified peptides in the high-density conjugates C6 and C8 than in lower-density conjugates (Fig. 3D and Supplementary Fig. 2B), suggesting that high hapten density interferes with their proteolytic release. Thus, AMDH-BSA conjugates with high hapten densities of 26.7-36.3 let to narrowed peptide diversity, reduced enzymatic degradation efficiency, and decreased peptide abundance.

The impaired enzymatic degradation observed with high hapten density conjugates provides a mechanistic explanation for the dampened macrophage activation noted earlier. By limiting antigen processing, conjugates with high hapten

density may reduce the number and diversity of degraded peptides available for MHC-II presentation, thereby inhibiting antigen presentation and subsequent T-cell help. Moreover, this reduction in T cell epitope diversity and abundance may negatively impact CD4⁺ T cell activation and downstream immune cascade, potentially weakening the overall immune response to hapten epitope. These findings point to a structural basis for this processing impairment: excessive hapten modification may physically and conformationally limit protease accessibility. We next used molecular dynamics simulations to investigate this structural basis.

5. Structural Basis of Hapten Density Induced Antigen Processing Inhibition

To elucidate the structural basis for the observed processing impairment, we conducted 200 ns MD simulations on BSA and conjugates C1, C3, C5, and C6. Conjugate C8 was excluded due to extremely poor enzymatic digestibility that limiting peptide detection and accurate structural modeling. We first evaluated the stability, as antigen stability is a key determinant of antigen proteolysis, antigen processing, and T cell epitopes expression⁴⁶. As shown in Fig. 4A, all systems exhibited initial fluctuations in root mean square deviation (RMSD). Conjugates C1 and C3 with low hapten density displayed pronounced instability, with C1 fluctuating substantially within the first 50 ns and C3 destabilizing between 100-150 ns. In contrast, C5 and C6 with high density of 17.2 and 26.7 maintained stable RMSD trajectories throughout the simulation, indicating increased structural stability of

happen protein conjugates at higher hapten densities. Root mean square fluctuation (RMSF) and Δ RMSF analysis in Fig. 4B further proofed high residue-level flexibility of C1 and C3, and suppressed residue fluctuations in C5 and C6 showing the highest degree of structural rigidity.

Radius of gyration (R_g) values generally was stable across all systems of BSA and conjugates within 200 ns, indicating that hapten density did not induce large-scale expansion or collapse of the BSA backbone. Thus, conjugation predominantly causes local rather than global structural differences (Fig. 4C). Solvent-accessible surface area (SASA) analysis showed that the values of all systems decreased during the initial stage of simulation, particularly for C5 and C6, possibly reflecting early rearrangements driven by solvent interactions with densely hapten modified surface regions (Fig. 4D). Secondary structure analysis demonstrated substantial α -helix loss (e.g., 63.3% of C1 and 65.9% of C3 vs 71.13% of BSA) and increased random coil content (20.4% of C1 and 17.6% of C3 vs 12.35% of BSA) in C1 and C3, indicating local structural loosening (Fig. 4E and Supplementary Fig. 3A). Conversely, C5 and C6 with higher hapten density displayed increased α -helix content (71.6% and 72.8%) and reduced random coil proportions (12.0% and 10.6%), aligning with results from CD spectroscopy (Fig. 1F), suggesting that higher hapten density enhances structural stability of conjugates by promoting α -helix formation.

Hydrogen bond analyses revealed comparable total hydrogen bond numbers across all systems (Supplementary Fig. 3B), with C5 and C6 displaying slightly elevated values, i.e., 506.4 ± 10.3 of BSA, 510.6 ± 11.3 of C1, 510.9 ± 11.1 of C3, $519.6 \pm$

11.6 of C5, 519.7 ± 10.7 of C6, respectively. This suggested that hapten conjugation introduced additional stabilization via hydrogen bond formation. Given that AMDH contains carbonyl groups ($-C=O$) that can act as hydrogen bond acceptors and form exogenous hydrogen bonds with surrounding amino acids, such as TYR400 and GLN521 in the case of C1, ARG435 and LYS187 in the case of C5, ARG347, ASN482, and LYS221 in the case of C6 (Fig. 4F). These interactions likely contribute to α -helix stabilization and restricted side-chain motion, reinforcing overall protein structure rigidity and stability⁴⁷.

We next analyzed the masking effect of hapten density on protease cleavage site of cathepsin S for BSA (Val/Ile/Leu/Met site)⁴⁸. In C1 and C3, most cleavage sites of BSA remained solvent-exposed, consistent with their efficient proteolytic degradation. In contrast, C5 and C6 with high hapten density exhibited extensive hapten masking these sites (Fig. 4G), suggesting steric blocking of critical protease cleavage sites.

Together, the MD results indicate that high hapten density promotes α -helix formation, increase local structural rigidity, and reduces residue-level flexibility by additional hydrogen bonding interactions, thereby reducing structural flexibility required for proteolytic processing. Additionally, high hapten density physically obstructs key protease recognition sites of BSA. These combined effects limited protease accessibility and processing capacity, explaining a structural basis for the “high uptake with low activation” phenotype observed with high hapten density. Because antigen fragmentation and epitope release directly determine the efficiency of MHC-II loading and subsequent CD4⁺ T cell help, orchestrating hapten density of

conjugates is therefore important for antigen stability, antigen processing and presentation efficiency.

6. Hapten Density Dictates the Quality of B and T Cell Responses

We next examined whether the density-dependent differences in antigen processing translate into altered T and B cell responses *in vivo*. Mice were immunized with AMDH-BSA conjugates C1, C3, C5, C6, C8, and PBS following the immunization schedule as shown in Fig. 5A, and samples were collected at the indicated time points (Supplementary Table 3). Given that effective antibody maturation rely on the germinal center (GC) reaction, where antigen-activated B cells undergo somatic hypermutation, class switch recombination, and clonal expansion with CD4⁺ T cell help^{49,50}, We isolated splenocytes 7 days after the first booster immunizations, and analyzed GC B cell and T cell responses (Fig. 5B-E). As shown in Fig. 5C, conjugates C1, C3, and C5 with hapten densities up to 17.2 induced a higher overall proportion of B cells (27.5%, 27.9% and 28.3%) than conjugates C6 and C8 (24.8% and 24.9%). However, within the GC compartment, only C1 and C3 increased GC B cell frequency (1.0 and 1.2%), whereas C5, C6, and C8 all showed similarly lower levels (0.78%, 0.75% and 0.79%, respectively). This finding suggests that GC B cell generation is more sensitive to hapten density than the overall B cell response and begins to decline once the hapten density reaches approximately 17.2. T cell subtypes analysis demonstrated that mice immunized with C1, C3, and C5 maintained higher proportion of CD4⁺ T cells, whereas C6 and C8 shifted the balance toward CD8⁺ T

cell (Tc) as shown in Fig. 5D and 5E. Accordingly, C1, C3, and C5 exhibited higher CD4⁺/CD8⁺ T cell ratios of 2.8-3.0 than C6 and C8 of 2.4 and 2.5, indicating a bias toward humoral immunity for conjugates with hapten density below 17.2 (Supplementary Fig. 4A).

To elucidate how hapten density affects lymphocyte activation, we restimulated splenocytes from immunized mice *in vitro* with each conjugate alone or in combination with ConA (T cell stimulant) or LPS (B cell stimulant). As shown in Fig. 5F-G and Supplementary Fig. 4B-D, C5, C6, and C8 with hapten densities above 17.2 exhibited higher stimulation indexes for total lymphocyte proliferation and T cell proliferation. Among these, C5 with hapten density of 17.2 exhibited the strongest stimulation index for T cell proliferation, approximately 1.4-fold higher than C1, consistent with its the highest MHC-II expression and enhanced CD4⁺ T cell activation (Fig. 2E and 5E). In contrast, B cell proliferation was most pronounced by C1, C3, and C5, with C3 with hapten density of 6.4 achieved the highest stimulation index (Fig. 5H and Supplementary Fig. 4D). Cytokine secretion analysis revealed no significant differences in TNF- α , IL-6, or IFN- γ levels across groups, although an upward trend in TNF- α secretion was observed from C6 and C8 (Fig. 5I-K), suggesting a slightly more pro-inflammatory milieu associated with high hapten density.

7. Hapten Density Modulates Hapten-Specific Antibody Responses

Antibody titer and affinity are key readouts of how hapten density shapes the

immunogenicity of hapten-protein conjugates. To dissect antibody responses to each antigenic component, we longitudinally quantified antibodies specific for AMDH-BSA conjugate, the hapten AMDH, and the carrier protein BSA over the course of immunization time (Fig. 6, and Supplementary Fig. 5A-C). Following the first booster immunization on day 28, AMDH-BSA-specific antibody titers increased markedly in all immunized groups compared to the PBS control, with significant differences among conjugates. C3 and C5 with hapten density of 6.4 and 17.2 induced significantly higher AMDH-BSA-specific antibody titers by 2- to 4-folds compared to other conjugates (Fig. 6A). Similarly, C1, C3, and C5 with hapten density of 1.8-17.2 induced AMDH-specific titers that were 2- to 12-folds higher than those elicited by the C6 and C8 with higher hapten density of 26.7-36.3 (Fig. 6B). Titers plateaued after the second booster immunization on Day 49 while maintaining the original trends observed on Day 28. These findings reveal conjugates with high hapten density above 26.7 reduced immune responses to both AMDH-BSA and AMDH.

We next analyzed antibody subclasses. OD values of IgM, IgG1, IgG2a, and IgG2b were measured on Days 7 and 28. IgG3 and IgA levels remained near background (Supplementary Fig. 5D). On Day 7, AMDH-specific IgM and IgG subclasses from C1, C3, C5 and C6 showed an overall negative correlation with hapten density (Fig. 6C). Interestingly, C8 with the highest hapten density induced a strong initial IgM response, but this response showed limited effectively class-switch to IgG subclasses upon the first booster immunizations (Fig. 6D). Following the first booster immunization on Day 28, C1, C3 and C5 significantly increased IgG1 and IgG2b

secretion compared to C6 and C8 by 1.7- to 2.2-fold and 1.1- to 2.5-fold, respectively.

As expected, analysis of the Th1/Th2 index revealed a Th2-biased immune response to hapten AMDH across all immunized groups (Fig. 6E). Notably, the Th1/Th2 index was negatively correlated with hapten density and C8 with the highest hapten density increased the Th1 bias, consistent with the T cell subset analysis (Fig. 5E, 6F, and Supplementary Fig. 4).

Beyond titers, antibody affinity against target molecule is also involved in the hapten-specific antibody response¹⁰. Antibody affinity was evaluated by using the inhibition rate of AMD by ic-ELISA, where higher inhibition rate indicates higher affinity. Early after immunization (day 7), C8 with the highest hapten density, initially induced the highest antibody affinity to AMD likely due to repetitive epitope presentation on the carrier protein facilitating T-independent B cell activation.⁵¹ (Fig. 6F). In contrast, after subsequent booster immunizations, C1 and C3 with lower hapten densities of 1.8 and 6.4 induced significantly higher antibody affinities, up to 2- to 3-fold higher than C6 and C8. These results suggested that C1, C3, and C5 promoted antibody affinity maturation and long-lasting humoral immune response to the target molecule, whereas conjugates with high hapten density above 26.7 in the study can rapidly induce an early antibody production with low affinity but fail to promote effective antibody subclass switching and high-quality affinity maturation.

We also examined antibody responses to the carrier protein BSA, as carrier-specific antibodies can potentially suppress hapten-specific responses through carrier-induced epitope suppression⁵². The results in Fig. 6G revealed all AMDH-BSA conjugates

significantly reduced BSA-specific antibody titers compared to native BSA ($p < 0.0001$). As expected, increasing hapten density was inversely correlated with BSA-specific antibody production. C6 and C8 with high hapten density of 26.7-36.3 induced lower BSA-specific antibody titers at all time points, especially IgG1 after the first immunization and IgG2b after the second immunization (Fig. 6H and 6I). However, because C6 and C8 simultaneously exhibited low levels of AMDH-BSA-specific, AMDH-specific, and BSA-specific immune responses, high hapten density appears to exert an overall adverse effect on the antibody response rather than specific epitope suppression, at least in this model. We found hapten densities within 1.8-17.2 are optimal for inducing stronger specific humoral responses to hapten molecule, characterized by high titers, enhanced affinity maturation, and a balanced carrier response, whereas densities of 26.7-36.3 attenuate these features and overall weaken antibody quality. These data establish the optimal hapten density window for programming vaccine immunity

8. Hapten Density Elicited Distinct Transcriptional Programs

To gain mechanistic insights into how hapten density reshapes the global immune landscape, we performed bulk RNA-seq and scRNA-seq on spleen samples from mice immunized with BSA, C1, C3, and C8. Bulk RNA-seq revealed broad downregulation of immune-related genes across all AMDH-BSA conjugate groups compared to BSA alone, with the most pronounced suppression observed in the highest hapten density group C8 (Supplementary Fig. 6A). Immunoglobulin genes

were significantly reduced in all conjugate-immunized mice, such as Igkv12-46, Ighg1, and Ighv14-3. Compared to C1 and C3, C8 showed further downregulation of immunoglobulin genes such as Ighv6-6, Igkv3-2, Ighg1, suggesting attenuated humoral immune activity at high hapten density⁵³, consistent with the reduced antibody production observed for high hapten density of conjugates (Fig. 6).

Gene ontology (GO) analysis comparing C8 with C1 or C3 showed that the top 20 downregulated GO terms were predominantly associated with adaptive immune functions, such as adaptive immune response, immunoglobulin complex, antigen binding and BCR signaling, revealing a global attenuation of adaptive immune response against C8 (Supplementary Fig. 6B). Notably, these transcriptional suppressions were more pronounced in the C8 versus C3 comparison than in C8 versus C1, in line with the serological data showing stronger humoral responses induced by C3 than by C8 (Fig. 6). This hapten density-dependent transcriptional attenuation points to a threshold effect, whereby moderate hapten density (C3) optimizes immunogenicity, while excessive hapten density (C8) actively suppresses it.

To resolve the cellular basis of these transcriptional changes, we conducted scRNA-seq on splenic immune cells from the C3 and C8 groups, respectively. Uniform manifold approximation and projection (UMAP) identified all major immune cell populations, including B cells, macrophages, neutrophils, and T cells (Fig. 7A and 7B). Compared with C3, C8 provided a pronounced neutrophil-enriched composition, accompanied by reduced frequencies of B cells, macrophages and T cells. Subclustering of B and T cells revealed that C8 immunization resulted in

increased proportions of naive B cells and naive CD4⁺ T cells, together with a marked depletion of marginal zone B cells (MZB) and CD4⁺ memory T cells (CD4⁺ T_m) (Fig. 7C and 7D). The reduction of CD4⁺ T_m cells was accompanied by modest, albeit non-significant, increases in naive CD4⁺ T cells and regulatory T cells (Treg). These shifts indicate that high hapten density is associated with a neutrophil-biased splenic immune landscape and attenuated accumulation of antigen-experienced adaptive subsets, consistent with the impaired cellular and humoral responses observed for high hapten density.

We next focused on GC B cells and CD4⁺ T_m cells that were the key adaptive subsets driving affinity maturation and long-lived immunity, and performed differential expression gene (DEG) analysis between C8 with the highest hapten and C3 with optimal hapten density. In GC B cells, C8 induced downregulation of 44 and upregulation of 57 genes compared to C3; in CD4⁺ T_m cells, 38 genes were upregulated and 74 genes downregulated in response to C8. Overall, C8 elicited a transcriptional response characterized by predominant downregulation in both GC B cells and CD4⁺ T_m cells types (Fig. 7E and 7F). GO analysis of GC B cells in C8 exhibited downregulation of genes involved in cytoplasm and cytosol, RNA binding and mRNA processing, cytoplasmic translation, endoplasmic reticulum lumen, regulation of BCR signaling, and cell-cycle phase transition (Fig. 7G, left). These patterns suggest a coordinated suppression of RNA metabolic and translational programs, together with reduced BCR signaling and proliferative capacity in GC B cells under high hapten density. The KEGG analysis placed the significantly

downregulated genes in BCR signaling, Fcγ R-mediated phagocytosis, NF-κB signaling, and ribosomal protein synthesis (Fig. 7H, left). Reduced BCR signaling and downstream NF-κB activation are expected to impair clonal expansion and selection of GC B cells, ultimately limiting the generation of antibodies with high affinity⁵⁴, while ribosomal downregulation implies a diminished capacity for protein synthesis, proliferation, and antibody secretion in GC B cells.

Similarly, GO analysis of CD4⁺ Tm cells in C8 showed enrichment of downregulated genes in pathways related to cellular stress responses, inflammation, and cell survival (Fig. 7G, right). The KEGG enrichment analysis of CD4⁺ Tm cells further pointed downregulation in antigen processing and presentation, as well as C-type lectin receptor, TNF signaling pathway, and multiple infection-associated modules (Fig. 7H, right). These findings suggest that conjugates with high hapten density reduces antigen presentation efficiency and dampens proinflammatory and costimulatory signaling in CD4⁺ memory T cells, further contributing to the blunted adaptive immune activation. These transcriptional alterations align with the observed reduction in antigen processing efficiency and proteolytic cleavage in conjugate C8 with high hapten density (Fig. 3), and support a model in which excessive hapten modification leads to impaired antigen presentation, and suppressed adaptive immune activation.

9. Hapten Density Shapes the BCR and TCR Clonotype Repertoire

To assess the effect of hapten density on B and T cell clonal diversity and

expansion, we performed clonal analyses and VDJ repertoire mapping using bulk TCR/BCR-seq and scTCR/BCR-seq. Bulk TCR/BCR-seq was performed on splenic samples from mice immunized with BSA, C1, C3, and C8, while scTCR/BCR-seq focused on C3 and C8 to provide higher resolution insights.

Bulk TCR/BCR-seq revealed striking differences in repertoire diversity across immunization groups. The diversity of TRA, TRB and IGH was significantly decreased in BSA, C1, and C3 compared to C8, a signal of immunogen-driven clonal expansion⁵⁵ (Fig. 8A). This reduced diversity indicates that effective immunogens promote the selective outgrowth of specific clonotypes, whereas high hapten density fails to impose such selective pressure. Systematic clonality maps of TRA, TRB, IGH and IGK repertoires further showed a significant decreased proportion of expanded clonotypes (>0.001 clonotype proportions) in C8 group compared to other groups (Fig. 8B and 8C). These data suggest that while C8 induces a broad, polyclonal repertoire, it lacks the focused expansion necessary for robust adaptive immunity.

Furthermore, scTCR/BCR-seq confirmed these findings, showing a higher proportion of expanded clonotypes in C3 compared to C8 (Fig. 8D). When we analyzed the top 20 most frequent TCR and BCR clonotypes across all samples, C8 exhibited significantly fewer dominant clonotypes, with only 7 TCR clonotypes (clonotype10, 17, 20, 23, 28, 29 and 35) and 3 BCR clonotypes (clonotype12, 32 and 33). In contrast, C3-immunized mice displayed a robust set of expanded clonotypes, indicating successful clonal selection and amplification of antigen-specific lymphocytes.

Analysis of VJ gene usage further highlighted the profound impact of hapten density on TCR and BCR repertoire composition. As shown in Fig. 8E, the top 20 most abundant VJ combinations varied significantly between C3 and C8, indicating that hapten density not only affects the magnitude of clonal expansion but also shapes the genetic architecture of the responding repertoire. The distinct VJ usage patterns suggest that conjugates with different hapten densities engage fundamentally different sets of precursor B and T cell clones, potentially reflecting differences in antigen processing, epitope presentation, or the avidity thresholds required for lymphocyte activation.

These bulk and single-cell repertoire data demonstrate that lower hapten densities (e.g., C3) promote focused clonal expansion and the emergence of dominant antigen-specific BCR and TCR clonotypes, whereas higher hapten density (e.g., C8) leads to a more diverse but less clonally expanded repertoire, reflecting a failure to effectively select and expand high affinity antigen-specific clones and thereby contributing to impaired humoral responses to the hapten epitope.

Discussion

Despite the widespread use of hapten-protein conjugates in vaccine design, antidote development and antibody production, creating optimal conjugates that elicit robust immune responses against haptens with single epitopes, such as polysaccharides, hazardous compounds, addictive drugs and antibacterial agents, always remains a

significant challenge⁵⁶. Among the most critical yet underexplored factors governing conjugate immunogenicity is hapten density. While widely recognized as a key determinant, its mechanistic role lacks a clear scientific basis, with the literature offering limited and often contradictory findings¹⁶. In this study, we precisely synthesized a series of AMDH-BSA conjugates covering a near-theoretical maximum of hapten density from 1.8 to 36.3, as defined by MALDI-TOF MS. Complementary characterization by intrinsic fluorescence spectroscopy, CD, and TNBS assay corroborated the density measurements and revealed hapten-induced concomitant structural alterations. For the first time, we systematically elucidated the multidimensional relationships between hapten density and immune response to a hapten epitope, providing a unified mechanism for tuning hapten density to program vaccine immunity.

Our data reveal a fundamental paradox. Conjugates with higher hapten density (26.7-36.3) exhibited enhanced internalization by APCs, yet this increased uptake does not translate into enhanced APC activation. Instead, surface expression of key activation markers including CD80, CD86, CD40, and MHC-II, was markedly reduced, meaning defective APC activation and impaired antigen presentation⁵⁷. To investigate the molecular basis of the observation of this “high uptake with low activation” phenomenon, we integrated cathepsin proteolysis assays with MD simulations of AMDH-BSA conjugates with various hapten density. Through LC-MS/MS mapping of AMDH-modified peptides following cathepsin digestion, we first ranked probable conjugation sites and structurally mapped them onto the BSA to

generate accurate AMDH-BSA models for MD simulations. These analyses revealed that excessive hapten conjugation stabilizes the protein scaffold through the formation of additional exogenous hydrogen bonds, promotes a rigid, α -helix-rich conformation, and sterically hinders cathepsin cleavage sites. Consequently, high density conjugates were less efficiently cleaved by cathepsins and generated fewer modified peptides, providing a direct molecular explanation for impaired antigen processing. This interpretation agrees with previous work showing that hapten conjugation can alter lysosomal cathepsin-mediated degradation and thereby change the peptide repertoire generated from a carrier protein, with direct consequences for MHC class II peptide loading and T-cell recognition⁵⁸. In this context, reduced peptide release constrains MHC-II loading and the availability of carrier-derived T-cell epitopes, thereby limiting CD4⁺ T-cell priming and downstream adaptive immune responses.

Following initial immunization, high hapten density conjugates primarily induced IgM-based hapten-specific antibodies, consistent with studies showing that highly multivalent antigens can rapidly activate B cells and broaden the range of recruited precursor affinities^{59,60}. Kato et al. demonstrated that high-valency protein immunogens promoted early extrafollicular plasma cell and GC responses by recruiting B cells across a broad affinity range, whereas low-valency antigens imposed more stringent selection and preferentially favored high-affinity clones⁵⁹. Similarly, Ols et al. showed that multivalent nanoparticle display increased clonotype diversity and enabled the recruitment of low-affinity precursors, some of which subsequently matured into highly potent or cross-neutralizing antibodies through

633 avidity-assisted entry into the response⁶⁰. In our system, however, this early advantage
634 did not translate into superior long-term humoral immunity. Instead, conjugates with
635 high hapten density failed to sustain a strong antibody response to hapten epitope,
636 exhibiting lower antibody affinity and titers compared to low density conjugates. A
637 likely explanation lies in a key chemical difference. Unlike structurally ordered
638 multivalent nanoparticles, where antigen spacing is uniform and epitope accessibility
639 is preserved, our high hapten density in conjugates are characterized by random,
640 crowded surface modification that induces global conformational rigidity. This
641 excessive hapten density simultaneously compromises antigen processing and limits
642 the generation of carrier-derived peptides for MHC-II presentation, thereby reducing
643 the availability of CD4⁺ T-cell help required for productive GC reactions. Consistent
644 with this interpretation, high hapten density conjugates in our study were associated
645 with reduced GC B-cell and CD4⁺ T-cell responses after boosting. Transcriptomic
646 profiling further revealed downregulation of TNF and C-type lectin receptor signaling
647 in CD4⁺ memory T cells, together with reduced BCR and NF-κB signaling in GC B
648 cells, changes that would be expected to impair clonal expansion, selection and
649 affinity maturation. In line with this, V(D)J repertoire analysis underscored the
650 influence of hapten density on clonal selection. The observed differences in V-J gene
651 usage and serological results indicate that conjugates with low density may
652 preferentially recruit high-affinity B cell clones, facilitating more stringent GC
653 selection and affinity maturation. In contrast, conjugates with high hapten density fail

to generate sufficient selective pressure for affinity-based clonal expansion, potentially leading to a broader but lower affinity immune repertoire.

In addition to immunological effects, we observed significant structural differences among conjugates with varied hapten densities, which may also underlie their distinct immune response. Conjugates with lower hapten density exhibited a more disordered secondary structure, characterized by reduced α -helical content and increased random coil components. Conversely, conjugates with higher hapten density adopted more ordered conformations, with enhanced α -helical content and reduced structural flexibility. These hapten density-dependent structural changes not only affect secondary structure but likely influence tertiary conformation, potentially modulating the spatial arrangement and altering accessibility and presentation of conformational epitopes recognized by BCRs^{61,62}. More importantly, the increased structural order and rigidity of conjugates with high hapten density impaired proteolytic processing within APCs, leading to reduced diversity of T cell epitopes and compromised antigen processing, consistent with our experimental observations of diminished CD4⁺ T cell activation and attenuated humoral responses⁶³. The masking or altered presentation of B cell epitopes may affect BCR engagement and activation efficiency, while limited antigen processing further weakens T cell help, jointly restricting GC formation and antibody affinity maturation⁶⁴. Future high-resolution structural studies, such as cryo-electron microscopy or X-ray crystallography, combined with epitope mapping and proteolytic kinetic analyses, could elucidate how hapten density precisely influences epitope exposure, BCR recognition, and antigen processing, providing a structural

basis for the rational design of conjugate antigens with tunable immunomodulatory functions.

Our findings highlight hapten density as a critical design parameter for effective hapten-protein conjugates. Taking the BSA conjugate in this study as an example with hapten densities between 1.8 and 17.2 maximizing overall antibody response, while densities around 6.4 are most effective for inducing high-affinity hapten-specific antibodies. This aligns with earlier BSA-based studies showing that moderate, rather than maximal, hapten density generally yields better antibody responses^{25,65}. By contrast, high hapten densities (26.7-36.3 haptens per BSA) reduced carrier-specific responses, but only at the expense of weaker GC and CD4⁺ T-cell support, poorer affinity maturation, and overall reduced hapten-specific immunity. Hapten density should therefore be selected according to the intended outcome, maximizing antibody titer, improving antibody affinity, or limiting carrier immunogenicity, rather than simply increased to the highest achievable hapten density^{16,17}.

In conclusion, this study provides a mechanism for understanding how hapten density orchestrates immune responses to hapten molecule. We demonstrate that hapten density influences antigen processing, T cell activation, GC response, clonotype landscape and antibody affinity maturation through a chemically defined mechanism. These insights position hapten density as a critically tunable variable for the rational design of conjugate vaccines and antigens. Tuning hapten density on protein conjugates thus provides an effective strategy to program vaccine immunity.

Materials and method

1. Materials and Reagents

AMD standard was purchased from TCI (Shanghai, China). HAT medium, and horseradish peroxidase (HRP) labeled goat anti-mouse IgG were purchased from Sigma-Aldrich (MO, USA). Incomplete Freund's adjuvant (IFA), complete Freund's adjuvant (CFA), and fetal calf serum were bought from Gibco BRL (Carlsbad, CA, USA). Other chemical reagents were provided by Sinopharm Chemical Reagent, Inc. (Beijing, China). The T75 cell culture flasks, 96-well cell culture plates and polystyrene microtiter plates were purchased from Corning Incorporated (NY, USA). Cell Counting Kit-8 (CKK-8) and fluorescein isothiocyanate (FITC) were acquired from Solarbio Science and Technology (Beijing, China). Live/Dead Aqua and MHC-II were obtained from ThermoFisher Scientific (MA, USA). Staining antibodies against CD3, CD4, CD8, CD19, CD95, GL7, CD80, CD86, CD 40 and MHC-II were purchased from Biolegend (Kristiansand, Norway). Assay kits for TNF- α , IFN- γ , IL-6 were purchased from MultiSciences Technology (Zhejiang, China).

2. Hapten Design, Synthesis and Characterization

The structures of AMD and hapten AMDH were built and optimized based on density functional theory (DFT) at the TVZP/M06-2X level using Gaussian 16 A.03 (CT, USA). Three-dimensional conformations, molecular descriptors and electrostatic potential (ESP) analysis were then conducted. ESP maps ($p(r) = 0.001$ a.u) were

generated in Visual Molecular Dynamics software (VMD) based on files exported from Multiwfn 3.7(dev)^{66,67}.

For chemical synthesis of hapten AMDH as shown in Supplementary Fig. 7A, 5.04 g of AMD (33.38 mmol) was dissolved in 15 mL of DMF, followed by the addition of 9.1 g of ethyl bromoacetate (36.23 mmol) and 9.1 g of K₂CO₃ (65.84 mmol). The mixture was stirred at 60°C for 16 h. After dilution with 100 mL of water, the solution was extracted with ethyl acetate (3 × 50 mL), combined, dried, and vacuum-concentrated, yielding purified 8-(adamantyl) amino octanoate.

Next, 4 g of the intermediate was dissolved in a 1:1 ethanol-water solution, and 0.9 g of NaOH was added. After 1 h of stirring, ethanol was removed, and the pH was adjusted to 5 using 3 M of HCl. The solution was vacuum-concentrated, yielding 1.3 g of hapten AMDH as a white solid. The chemical structure of AMDH was confirmed via mass spectrometry (Thermo, Waltham, MA, USA) and nuclear magnetic resonance (NMR) spectroscopy (Bruker, Rheinstetten, Germany) as shown in Supplementary Fig. 7B-D. Mass spectrometry results (ESI): m/z calcd. for C₁₈H₃₀NO₂⁻ [M-H]⁻ 292, found 292. The ¹H NMR (400 MHz, DMSO-d₆) δ 1.29 (s, 6H), 1.48-1.53 (m, 2H), 1.58-1.67 (s, 8H), 1.87 (s, 6H), 2.11 (s, 3H), 2.21 (t, J = 8.0 Hz, 2H), 2.81 (t, J = 8.0 Hz, 2H), 8.71 (s, 1H), and 11.91 (s, 1H). The ¹³C NMR (101 MHz, DMSO-d₆) δ 24.95, 26.53, 26.67, 28.87, 28.98, 29.08, 29.18, 34.15, 35.73, 37.92, 50.35, 174.93.

3. AMDH-BSA Conjugates Preparation and Characterization

Hapten AMDH was activated using the active-ester method and conjugated to BSA and ovalbumin (OVA) as immunogens and coating antigens, respectively. To obtain AMDH-BSA conjugates with different hapten densities, 1.5-150 μmol of AMDH was dissolved in 1.5 mL of DMF, followed by the addition of N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in a 1:1.5 molar ratio. The reaction mixture was stirred at room temperature for 8 h, filtered to remove precipitates, and then added dropwise into 10 mL of phosphate-buffered saline (PBS) containing BSA at molar ratios of AMDH:BSA with 5:1 to 500:1. For AMDH-OVA conjugate, 9.0 μmol of AMDH was reacted with NHS and DCC (1:1.5 molar ratio), and the ratio of hapten and OVA was set at 30:1. The prepared AMDH-BSA conjugates with increasing hapten density were orderly named C1 to C8. All conjugates were stirred at 4°C for 12 h, dialyzed in PBS for 72 h, aliquoted, and stored at -20°C.

Hapten densities of conjugates were first determined via MALDI-TOF/MS (Bruker, Rheinstetten, Germany) and the hapten density were calculated as:

$$\text{hapten density} = [M(\text{conjugation}) - M(\text{protein})] / M(\text{hapten})$$

Then, hapten densities of conjugates were characterized by 2,4,6-trinitrobenzene sulfonic acid (TNBS) and SDS-PAGE methods. TNBS selectively reacts with free amines of BSA, resulting in a yellow-colored product detectable at 420 nm⁶⁸. In brief, the free carrier protein and conjugates were suspended in 0.1 M NaHCO₃ buffer at pH 8.0 and treated with TNBS (50 μL , 0.01% in 0.1 M NaHCO₃). The final volume and

protein concentration of the reaction mixture were adjusted to 150 μ L. The reaction was then allowed to proceed for 2 h at 37 $^{\circ}$ C, followed by absorbance measurement at 420 nm. The percentage of amino groups consumed in AMDH-BSA conjugates was determined by referencing a calibration curve generated by using BSA standards. SDS-PAGE was used to estimate the apparent molecular weight of BSA and C1, C3, C5, C6 and C8 conjugates. Samples were mixed with Laemmli loading buffer (with reducing agent), heated at 95 $^{\circ}$ C for 5 min, and separated on polyacrylamide gels under denaturing conditions alongside a protein molecular-weight marker. Gels were Coomassie-stained and imaged, and apparent molecular weights were determined by comparing band migration distance and a protein molecular-weight marker.

Fluorescence spectra of BSA and AMDH-BSA conjugates (100 μ L protein content of 1 mg/mL) and AMDH (100 μ L hapten content of 1 μ g/mL) were measured by an EnVision multimode plate reader (Perkin Elmer, USA). The emission spectrum was scanned from 0 to 600 nm after excitation at 290 nm (tryptophan excitation) and used with a scanning step of 2 nm. Each spectrum was an average of three scans.

The secondary structure of BSA and AMDH-BSA conjugates was assessed using circular dichroism (CD) spectroscopy in the far-UV region (195-250 nm). The CD spectra were recorded using a Chirascan Plus spectrometer (Applied Photophysics Ltd., UK) at room temperature. Samples (0.25 mg/mL protein content) were placed in a 0.1 cm pathlength quartz cell, with a scanning interval of 1.0 nm and a scanning speed of 1.0 nm/s. Each spectrum represented the average of three scans and was corrected for buffer signals. Data were expressed in milli-degrees (mdeg) and

analyzed using CDNN 2.1 software to determine the secondary structure composition, including α -helix, β -sheet, β -turn, and random coil.

4. Macrophage Uptake and Activation Assays

AMDH-BSA conjugates and native BSA were labeled with FITC. RAW264.7 cells were incubated with either FITC-BSA (50 μ g/mL) or FITC-labeled AMDH-BSA conjugates (50 μ g/mL) for 30 min and 2 h. The mean fluorescence intensity (MFI) of FITC within the cells was measured by flow cytometry (Sony Biotechnology, California, USA) to assess macrophage cellular uptake levels.

To visualize cellular uptake, RAW264.7 cells were incubated with FITC-labeled BSA or AMDH-BSA conjugates (C1, C5, and C8) at 50 μ g/mL for 2 h. Lysosomes were stained using LysoTracker dye (red fluorescence), and nuclei were counterstained with Hoechst dye (blue fluorescence). Images were captured using a confocal laser scanning microscope (CLSM).

RAW264.7 cells were treated with native BSA or AMDH-BSA conjugates (50 μ g/mL) for 24 h. The surface expression of CD40, CD80, CD86, and MHC-II was then quantified via flow cytometry (Sony Biotechnology, California, USA). Data were analyzed using FlowJo software, and statistical evaluations were conducted in Prism 10.4.0⁶⁹.

The proliferation activity of RAW264.7 cell were assessed using CCK-8 method. RAW264.7 cells were seeded into the 96-well flat bottom microtiter plates and then

treated with 50 µg/mL of BSA or AMDH-BSA conjugates with varying hapten densities. For controls, a positive control group was treated with 10 µL of 250 µg/mL lipopolysaccharide (LPS), while a blank control group received PBS. After 24 or 48 h of incubation, 10 µL of CCK-8 solution was added to each well. Plates were further incubated for 2 h under the same conditions, and optical density (OD) at 450 nm was measured using a microplate reader.

5. Enzymatic Degradation Assay *in vitro*

The proteolytic degradation of BSA and AMDH-BSA conjugates (C1, C3, C5, C6, and C8) was evaluated using recombinant mouse cathepsin S (MCE, Newjersey, USA) derived from *E. coli*. Reactions were performed in duplicate in a total volume of 300 µL, containing 100 mM sodium citrate buffer (pH 6.0), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 300 ng of cathepsin S, and 30 µg of protein. Samples were incubated at 37°C for 48 h.

Peptides were identified and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein quantification was performed using MaxQuant's Label-Free Quantification (LFQ) algorithm, which integrated isotope peak intensities of peptides across runs. Protein ratios were determined as the median of shared peptide ratios. Post-analysis filtering excluded proteins identified solely by modified sites, reverse database matches, or common contaminants.

6. Molecular Dynamics Simulation of AMDH-BSA Conjugates

The crystal structure of BSA was obtained from the Protein Data Bank (PDB ID: 4JK4). Four AMDH-BSA conjugates (C1, C3, C5, and C6) were generated by modifying the BSA structure with varying numbers of hapten AMDH using YASARA software. Molecular dynamics (MD) simulations were performed using GROMACS 2024.4 under periodic boundary conditions with the Amber14SB all-atom force field and the TIP3P water model. The system was solvated in a cubic simulation box with a minimum distance of 1 nm from the protein to the box edges and neutralized with counterions. Hydrogen bonds were constrained using the LINCS algorithm, with an integration time step of 2 fs. Electrostatic interactions were computed using the Particle Mesh Ewald (PME) method, and non-bonded interactions were truncated at 10 Å with neighbor lists updated every 10 steps. Temperature (300 K) and pressure (1 bar) were controlled using the V-rescale thermostat and Parrinello-Rahman barostat, respectively. The simulation protocol included energy minimization using the steepest descent algorithm, equilibration under an NVT ensemble at 300 K for 100 ps, and a 200 ns production MD simulation for each of the six independent systems, with trajectory snapshots recorded every 20 ps. Trajectory analysis, including root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), and secondary structure analysis, was performed using built-in GROMACS tools, and molecular visualization was conducted using ChimeraX 1.9.

7. Animals Immunization and Antisera Characterization

All animal experiments were approved by the Animal Welfare Committee of China Agricultural University and followed the “Guide for Care and Use of Laboratory Animals” (China Agricultural University). Selected AMDH-BSA conjugates with various hapten densities (1 mg/mL) were emulsified with an equal volume of adjuvant as immunogens. The initial immunization used CFA, while booster injections used IFA. Female BALB/c mice (6-8 weeks old) were randomly assigned to six groups and subcutaneously injected with 100 µg of immunogen. Mice received three immunizations at three-week intervals. Blood samples (50 µL) were collected from the retro-orbital sinus one week after each immunization for antisera characterization.

Antibody titers were determined via non-competitive (nc-ELISA) and expressed as the reciprocal of the highest dilution with an OD of antisera above the mean plus three standard deviations (SD) of control wells. Relative binding affinity was assessed using indirect competitive ELISA (ic-ELISA), with inhibition rates calculated as:

$$\text{Inhibition rates} = [(B_0 - B) / B_0]$$

where B_0 is the OD value without AMD and B is the OD value in the presence of 100 µg/L AMD under the same conditions. The higher inhibition rates mean the lower antibody affinity.

Supernatants from splenic lymphocyte cultures were analyzed for TNF- α , IFN- γ , and IL-6 levels using commercial ELISA kits per the manufacturer’s instructions.

8. Splenocyte Phenotyping and Proliferation Assay

One week after immunization, mice were anesthetized with isoflurane and euthanized via cervical dislocation. Splenocytes were isolated by mechanical disruption of the spleen and filtration through a 20 µm mesh filter. Single-cell suspensions were stained with antibody panels specific for T cell markers and B cell markers, incubated on ice for 30 min, washed, and filtered before flow cytometric analysis.

The spleen lymphocytes were also assessed using the CCK-8 method. The test was divided into the following experimental groups: RPMI-1640 complete medium with PBS; RPMI-1640 medium containing 50 µg/mL of conjugates (C1, C3, C5, C6, C8); RPMI-1640 medium with 50 µg/mL C1, C3, C5, C6, C8 plus 10 µg/mL concanavalin A (ConA, T cell stimulant) or 12.5 µg/mL lipopolysaccharide (LPS, B cell stimulant). Each group was tested in six replicate wells. ConA was used to stimulate T cell proliferation, while LPS was used to stimulate B cell proliferation. Cells were incubated at 37°C with 5% CO₂ for 72 h, followed by the addition of 10 µL of CCK-8 solution to each well. After an additional 4 h incubation, optical density (OD) at 450 nm was measured using a microplate reader. The proliferation rate was expressed as the stimulation index (SI):

$$SI = (\text{experimental group } OD_{450nm} - \text{blank group } OD_{450nm}) / (\text{negative control } OD_{450nm} - \text{blank group } OD_{450nm})$$

9. Bulk RNA Sequencing and Immune Repertoire Analysis

Bulk RNA sequencing (bulk RNA-seq) was performed to quantify gene expression levels using Htseq (v0.6.1), with normalization by FPKM (Fragments Per Kilobase of transcript per Million mapped reads). Differential gene expression analysis was conducted using edgeR (v3.28.1), applying a threshold of fold change (FC) ≥ 2 and a false discovery rate (FDR)-adjusted p-value (q-value) ≤ 0.05 . Gene Ontology (GO) enrichment analysis was carried out using GOrse, which corrects for gene length bias based on the Wallenius non-central hypergeometric distribution.

For immune repertoire analysis, MiXCR (v3.0.13) was used for two-step sequence alignment against the IMGT database to identify V, D, and J gene usage and annotate CDR1, CDR2, and CDR3 regions. Clonal clustering was performed based on sequence similarity. Immune repertoire diversity was assessed by analyzing CDR3 sequence richness and rank-abundance distribution, with diversity trends visualized using rank-abundance curves. Clonal expansion analysis was conducted by quantifying TRA, TRB, IGH, IGK, and IGL clonotypes, which were classified into three frequency intervals: high (>0.01), intermediate ($0.01 \geq x > 0.001$), and low (≤ 0.001). These analyses provided insights into immune repertoire diversity and antigen-specific clonal expansion.

10. Single-cell RNA Sequencing and Immune Repertoire Analysis

Single-cell RNA sequencing (scRNA-seq) and immune repertoire profiling were performed using the 10×Genomics Single Cell Immune Profiling Solution, following the manufacturer’s protocol. Single-cell suspensions were adjusted to a concentration of 700-1200 cells/μL before being encapsulated in barcoded gel beads within a microfluidic system to form Gel Beads-in-Emulsions (GEMs). Reverse transcription and cDNA amplification were performed within the GEMs, followed by PCR amplification to generate sequencing libraries. Transcriptomic libraries were prepared by enzymatically fragmenting cDNA (200-300 bp), ligating sequencing adapters, and performing PCR amplification. Immune repertoire libraries were generated by enriching V(D)J segments through nested PCR with TCR/BCR constant region-specific primers, followed by fragmentation and adapter ligation to capture full-length V(D)J sequences. Libraries were sequenced on the Illumina platform using paired-end sequencing.

Raw sequencing data were processed using Cell Ranger (10×Genomics) for demultiplexing, genome alignment, UMI counting, and quality control. The resulting gene-barcode matrix was analyzed using Seurat, with low-quality cells removed based on mitochondrial gene expression and feature counts. Cell clustering and subtype identification were performed through Principal Component Analysis (PCA), FindNeighbors, and FindClusters, with visualization using Uniform Manifold Approximation and Projection (UMAP). Differential expression gene (DEG)s were

identified across clusters ($|\log_2FC| > 0.26$, p value < 0.01), followed by GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

For immune repertoire analysis, cell barcode correction and UMI filtering were performed to remove low-quality sequences, and adapter sequences were trimmed using Cutadapt. Reads were de novo assembled into contigs and aligned to the V(D)J reference database for gene annotation. CDR3 sequences were identified to determine functional TCR/BCR sequences, and clonotypes were assigned based on identical CDR3 sequences. Clonal expansion was assessed by ranking the top 20 clonotypes and calculating their frequencies across samples.

11. Quantification and Statistical Analysis

All data are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was carried out using Prism statistical analysis software. For each experiment, the type of statistical testing, n values, summary statistics, and levels of significance can be found in the figures and corresponding figure legends.

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Contributions

R.L.: Investigation (lead), Formal analysis (lead), Visualization (lead), Writing – original draft (lead), Conceptualization (equal). Y.Z.: Visualization (equal), Methodology (equal), Writing – original draft (equal). L.N.: Project administration (lead). Y.L. and X.Y.: Methodology (equal), Data curation (equal). F.Z. and J.Z.: Conceptualization (equal), Writing – review and editing (equal). F.Z.: Methodology (supporting). J.Z.: Investigation (equal), Methodology (equal). K.W.: Methodology (equal), Project administration (equal). J.S., X.Yu. and Z.W.: Conceptualization (lead), Funding acquisition (lead), Supervision (lead), Writing – review and editing (lead).

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement

Animal care and all animal procedures were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals produced by the China Agricultural University. The study was approved by the Ethics Committee of the

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Tables Captions:

Table 1. Molecular mass and calculated hapten density of AMDH-BSA/OVA conjugates characterized by MOLDI-TOF MS.

Conjugates	AMDH to BSA/OVA	Observed molecular	Molecular mass	Hapten density
names	feeding mole ratios	mass (Da)	variation (ΔM)	($\Delta M/Mh^*$) [†]
BSA	0:1	66422.1	0	0
C1	5:1	66915.4	493.4	1.8
C2	10:1	67406.5	984.4	3.6
C3	20:1	68189.9	1767.8	6.4
C4	40:1	69989.9	3567.8	13.0
C5	60:1	71158.0	4735.9	17.2
C6	100:1	73770.3	7348.3	26.7
C7	200:1	75984.0	9562.0	34.7
C8	500:1	76432.7	10010.6	36.3
OVA	0:1	49267.1	0	0
AMDH- OVA	30:1	52461.2	3200.1	10.1

* Mh indicates molecular weight of AMDH minus H₂O, Mh = 275.6; [†] Values in parentheses indicate calculated numbers of AMDH on each BSA or OVA molecule.

Figure Captions:

Fig. 1 Validation, preparation, and characterization of AMD, hapten AMDH and AMDH-BSA conjugates with various hapten densities. (A) Similarity of AMD and AMDH was validated using area distribution of different ESP intervals (left), ESP mapping on the van der Waals surface (i.e., = 0.001 a.u. isosurface) and superposition of the optimized geometries of AMD and AMDH (right). (B) Schematic diagram of AMDH-BSA conjugates preparation, with feed molar ratios of AMDH to BSA increasing from 5:1 to 500:1. The carboxyl groups of AMDH are conjugated to ϵ -amino groups of lysine (LYS) of BSA, forming amide bonds. (C) Molecular weight (MW) characterization of AMDH-BSA conjugates by MALDI-TOF/MS. The calculated hapten densities are presented in Table 1. The conjugates with increasing hapten density were orderly named C1-C8 and the selected conjugates C1, C3, C5, C6, and C8 were used as immunogens and further characterized. (D) Characterization of tertiary structures of AMDH-BSA conjugates C1, C3, C5, C6, and C8 by fluorescence spectrum. (E) Characterization of secondary structures of AMDH-BSA conjugates C1, C3, C5, C6, and C8 by CD spectrum. (F) Secondary structure composition of AMDH-BSA conjugates C1, C3, C5, C6, and C8 in term of α -helix, β -sheet, β -turn and random coil and detailed data were provided in Fig. S2F.

Fig. 2 Effect of AMDH-BSA conjugates with varied hapten densities on the cellular uptake and activation of RAW264.7 macrophages *in vitro*. (A) Flow cytometric histograms of FITC labeled BSA and FITC labeled AMDH-BSA conjugates C1, C3, C5, C6, C8 uptake

by RAW264.7 cell at 50 µg/mL for 30 min and 2 h (n=3). (B) Cumulative mean fluorescence intensity (MFI) analysis of the same uptake data (n=3). (C) Confocal laser scanning microscope (CLSM) of RAW264.7 cells after incubation with FITC labeled BSA and FITC labeled AMDH-BSA conjugates C1, C5, C8 at 50 µg/mL for 2 h. The nuclei of RAW264.7 cells were visualized with Hoechst emitting blue light and lysosomes of RAW264.7 cells were stained with LysoTracker dye emitting red light. Scale bar, 10 µm (n=3). (D) CCK-8 assay of RAW264.7 cell viabilities after treatment with AMDH-BSA conjugates C1, C3, C5, C6, C8, BSA or LPS at concentrations of 3.25, 7.5, 15, 30, 60 µg/mL for 2 h; PBS and LPS served as negative and positive controls, respectively (n=4). (E) Flow cytometric quantitative analysis of MHC-II, CD40, CD80, and CD86 expression of RAW264.7 cells after incubation with AMDH-BSA conjugates C1, C3, C5, C6, C8 and BSA at 50 µg/mL for 24 h (n=3). (F) Representative flow cytometric contour plot showing CD80 and CD86 expression on RAW264.7 cells after incubation with AMDH-BSA conjugates C1, C3, C5, C6, C8 and BSA at 50 µg/mL for 24 h (n=3); * $p < 0.05$, * * $p < 0.005$, * * * $p < 0.001$, * * * * $p < 0.0001$. Data are shown as mean $\pm$ SD. Statistical significance was determined by ANOVA with multiple comparison testing among all conjugate groups and BSA.

Fig. 3 Effect of hapten density on peptide diversity and abundance of AMDH-BSA conjugates after *in vitro* digestion with cathepsin S analyzed by LC-MS/MS. (A) Proposed hapten conjugation sites on BSA based on lysine conjugation site mapping; Lysine (K) residues of BSA highlighted in red indicate potential hapten conjugation sites, with surface-exposed K shown in bold. These sites were derived from PSM analysis of hapten-

modified peptides generated by enzymatic cleavage of BSA and AMDH-BSA conjugates. The speculated hapten conjugation sites for C1, C3, C5, C6, and C8 are marked by yellow, green, red, and purple stars, respectively. (B) Locations of peptides generated from enzymatic degradation of AMDH-BSA conjugates C1, C3, C5, C6, and C8. Each conjugate was tested twice in duplicate; All enzymatically cleaved peptides of AMDH-BSA conjugates were represented as lines mapped onto the full-length sequence of BSA and arranged in a 5' to 3' order; The y-axis is plotted at a fixed height to illustrate the diversity of identified peptides, while the x-axis corresponds to the specific amino acid positions within the BSA sequence. (C) Differences in abundance of various peptide clusters across AMDH-BSA conjugates C1, C3, C5, C6, and C8. The total number of peptide-spectrum matches (PSM) within pie charts was obtained by summing all PSMs for the conjugates. Peptide clusters that were defined based on shared start or end residues, as well as the extent of overlap among conjugates. (D) Representative heat map of PSMs differences for the YST393-413 peptide cluster of BSA. PSMs were quantified with posterior error probability (PEP) values of <0.01 . Data in C, D, and E represent two independent experiments performed per conjugate tested in single determinations due to antigen availability and the quantity required per assay. Two independent experiments in D are labeled by appending “-1” or “-2” after each conjugate name, respectively.

Fig. 4 Effect of hapten density on conformational stability and protease cleavage site accessibility of BSA via 200 ns molecular dynamics (MD) simulations. (A) Root mean square deviation (RMSD) analysis of BSA and AMDH-BSA conjugates C1, C3, C5, C6. (B)

Root mean square fluctuation (RMSF) analysis of residue flexibility across BSA and conjugates C1, C3, C5, C6. Individual residue fluctuations compared among systems are shown as Δ RMSF. (C) Radius of gyration (Rg) analysis of BSA and conjugates C1, C3, C5, C6. (D) Solvent-accessible surface area (SASA) analysis of BSA and conjugates C1, C3, C5, C6. (E) Secondary structure composition of BSA and AMDH-BSA conjugates at 200 ns, showing the distribution of α -helix, β -sheet, β -turn, and random coil structures. (F) Visualization of the local binding regions of conjugates C3, C5, and C6 at 200 ns, showing additional hydrogen bonds (blue dashed lines) formed between the AMDH carbonyl group (C=O) and nearby amino acid residues. For clarity, only residues directly involved in hydrogen bonding with the hapten are shown. (G) Visualization of protease cleavage site accessibility in BSA and AMDH-BSA conjugates at 200 ns. Key cathepsin cleavage sites (Val, Ile, Leu, Met) are highlighted in different colors, with hapten conjugated regions shown in yellow.

Fig. 5 Effect of AMDH-BSA conjugates with varied hapten densities on T and B cell response. (A) Schematic timeline of the immunization regimen and sample collection. Six-week-old BALB/c mice were immunized with AMDH-BSA conjugates C1, C3, C5, C6, C8, or PBS every three weeks for a total of three immunizations. Antisera were collected on day 7, 28, and 49 and analyzed by nc-ELISA and ic-ELISA. Splenocytes were sorted on day 28 for flow cytometric analysis and transcriptional analysis, and on day 49 for lymphocyte proliferation and cytokines analysis. (B) Representative flow cytometry dot plots showing the gating strategy of B cells (CD19⁺) and GC B cells (CD19⁺CD95⁺GL7⁺). (C) Percentages of B

cells and GC B cells among viable splenocytes in respective immunized-group mice (n=3 mice/group). (D) Representative flow cytometry dot plots showing the gating strategy of CD4⁺ T cells (CD3⁺CD4⁺CD8⁻) and CD8⁺ T (CD3⁺CD4⁻CD8⁺). (E) Percentages of CD4⁺ and CD8⁺ T cells among viable splenocytes in respective immunized-group mice (n= 3 mice/group). (F) Stimulation index of splenocytes proliferation after treatment with 50 µg/mL AMDH-BSA conjugates C1, C3, C5, C6, C8, or PBS for 72 h *in vitro*. (G) Stimulation index of splenocytes proliferation after treatment with AMDH-BSA conjugates C1, C3, C5, C6, C8 in the presence of 10 µg/mL ConA (T cell stimulant). (H) Stimulation index of splenocyte proliferation after treatment with AMDH-BSA conjugates C1, C3, C5, C6, and C8 in the presence of 12.5 µg/mL LPS (B cell stimulant). (I, J and K) Levels of TNF-α, IL-6 and IFN-γ released by splenocytes after re-stimulation with AMDH-BSA conjugates or PBS *in vitro*. * 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.

Fig. 6 Effect of AMDH-BSA conjugates with varied hapten densities on specific antibody response. (A, B) Titers of AMDH-BSA-specific and AMDH-specific antibodies in antisera measured by nc-ELISA on day 7, 28 and 49. AMDH-BSA (C4) and AMDH-OVA were used as coating antigen at 2 µg/mL (n=6-8 mice/group). (C, D) OD₄₅₀ value of AMDH-specific IgM and IgG subclasses (IgG1, IgG2a, IgG2b) in 1:1600 diluted antisera measured by nc-ELISA on days 7 and 28. AMDH-BSA (C4) and AMDH-OVA as coating antigen at 2 µg/mL (n=6-8 mice/group). (E) Th1/Th2 index of the immune response based on IgG subclasses

distribution on day 28, calculated as $Th1/Th2 = (IgG2a + IgG3)/(2 \times IgG1)$. (F) Inhibition rates (%) of specific antibodies against AMD at 100 μ g/L, determined by ic-ELISA using AMDH-OVA as coating antigen on day 7, 28, and 49. Higher inhibition rates of antibodies indicate lower antibody affinity to AMD in the study. (G) Titers of BSA-specific antibodies in antisera measured by nc-ELISA using BSA as coating antigen at 2 μ g/mL on day 7, 28, and 49 (n=6-8 mice/group). (H, I) OD₄₅₀ value of BSA-specific IgM and three IgG subclasses (IgG1, IgG2a, IgG2b) in 1:1600 diluted antisera measured by nc-ELISA using BSA as coating antigen at 2 μ g/mL on day 7 and 28 (n=6-8 mice/group). * $p < 0.05$, * * $p < 0.005$, * * * $p < 0.001$, * * * * $p < 0.0001$. Means and standard deviations are displayed (n=6-8 mice/group). Statistical significance for A–F was determined by ANOVA with multiple comparison testing among all groups except the PBS group. For G–I, ANOVA with pairwise testing against the BSA group was used.

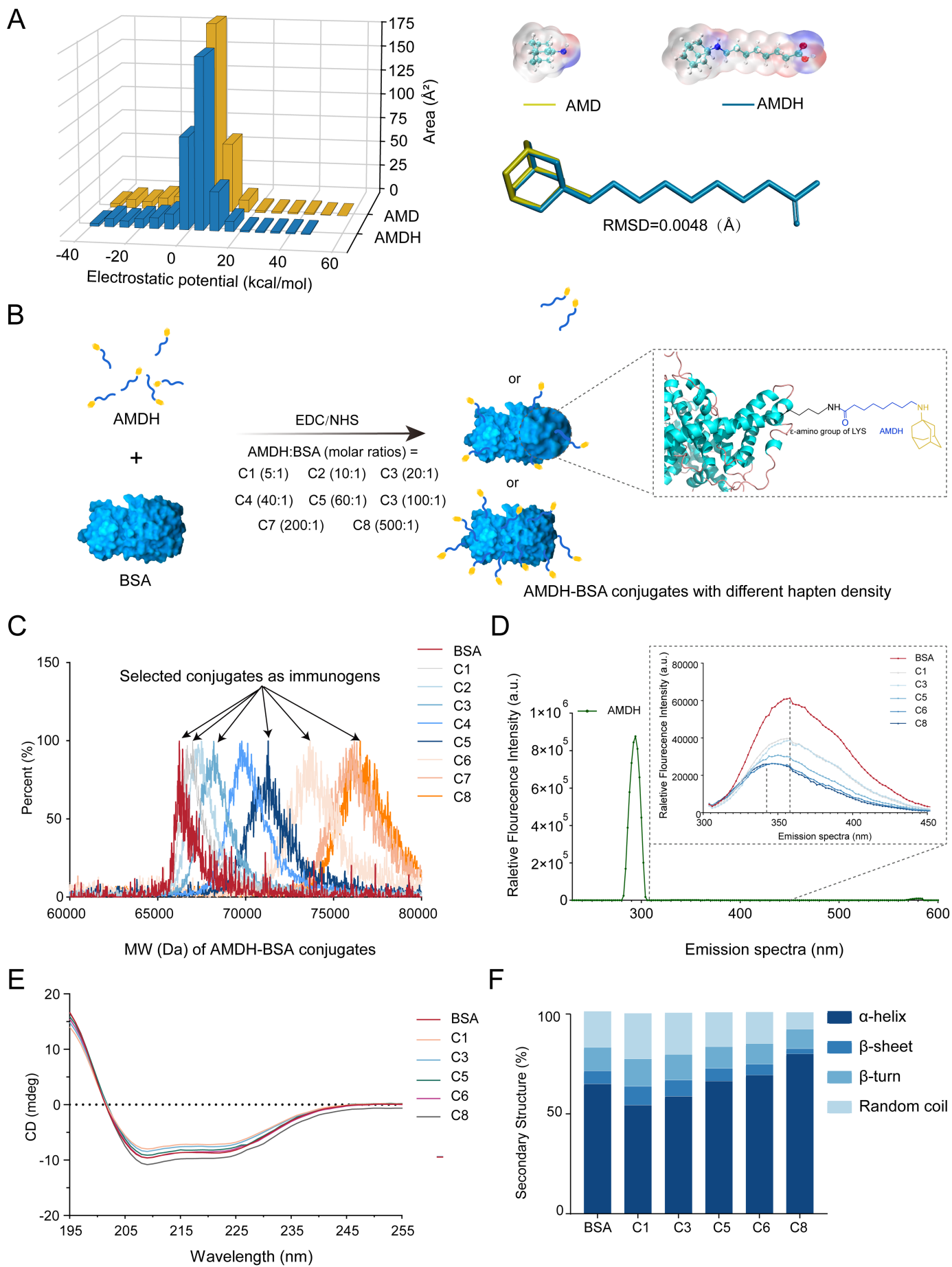
Fig. 7 Single-cell transcriptomic analysis of splenic immune cells from mice immunized with AMDH-BSA conjugates C3 and C8. (A) Uniform manifold approximation and projection (UMAP) visualizations of all 30,483 cells (left) and stacked bar chart showing cell type proportions in C3 and C8 immunization groups (right; n=2 mice/group). Clusters were labeled by cell types that were assigned based on the expression of cell type-specific markers; DC, dendritic cell; LPC, lymphoid progenitor cell; NK, natural killer cell. (B) Bubble plot of selected marker genes for each cell cluster. Bubble size indicates the frequency of cells with gene expression and color represents the level of RNA expression. (C, D) UMAP visualizations of T cell and B cell subpopulations (left) and stacked bar chart showing

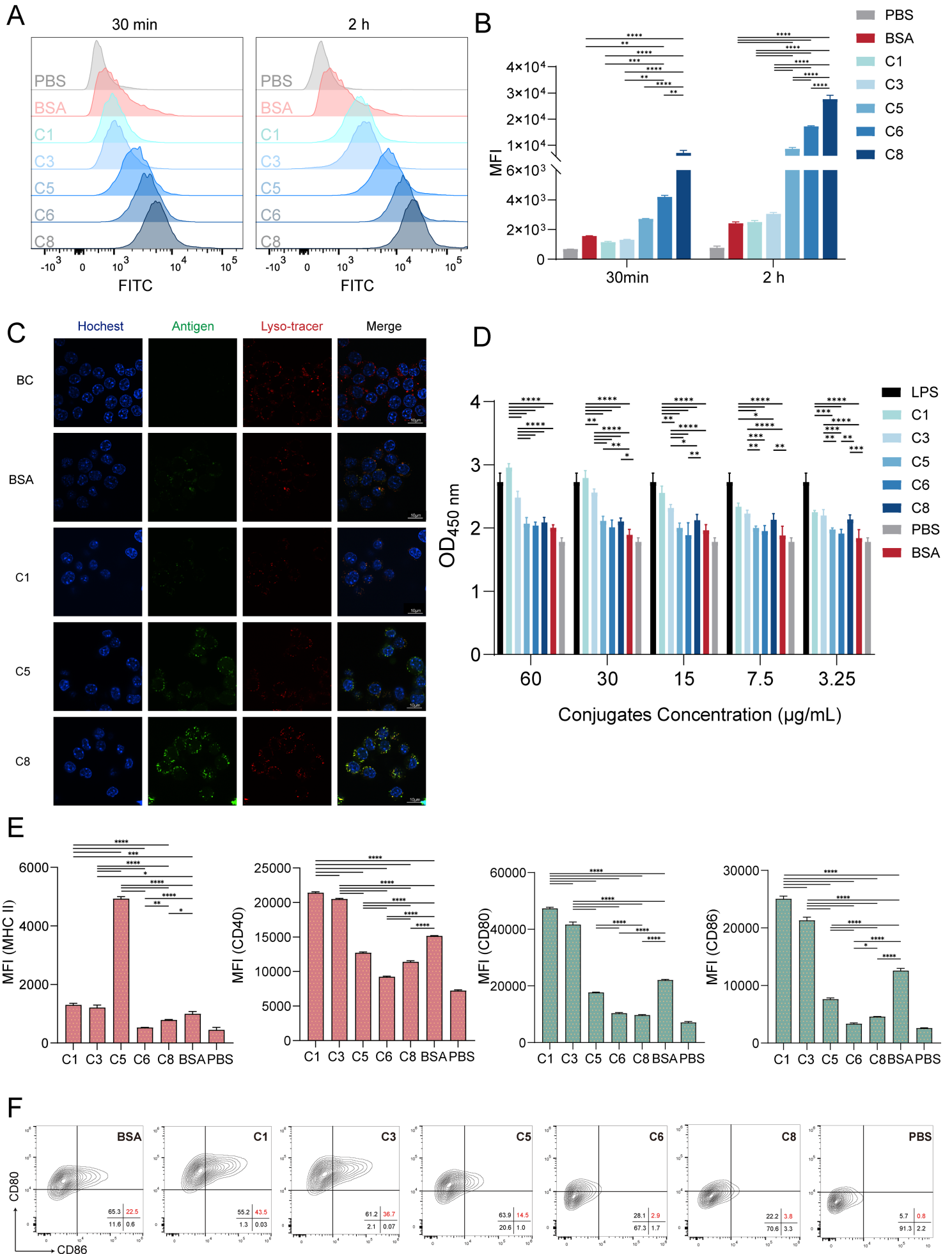
subpopulation proportions in C3 and C8 immunization groups (right, n=2 mice/group); Subclusters were labeled by cell types, assigned by the expression of cell type-specific markers; GC B, germinal center B cell; MBC, memory B cell; MZB, marginal zone B cell; pDC, plasmacytoid dendritic cell; CD4⁺ Tm, CD4⁺ memory T cell; Treg, regulatory T cell. (E, F) Volcano plots of differential expression analysis in GC B cells and CD4⁺ Tm cells comparing C8 versus C3. (G) Top 10 Gene Ontology (GO) terms for downregulated genes in GC B cells and CD4⁺ Tm cells comparing C8 versus C3; Receptor Tyrosine Kinase, RTK. (H) Top 10 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for downregulated genes in GC B cells and CD4⁺ Tm cells comparing C8 versus C3.

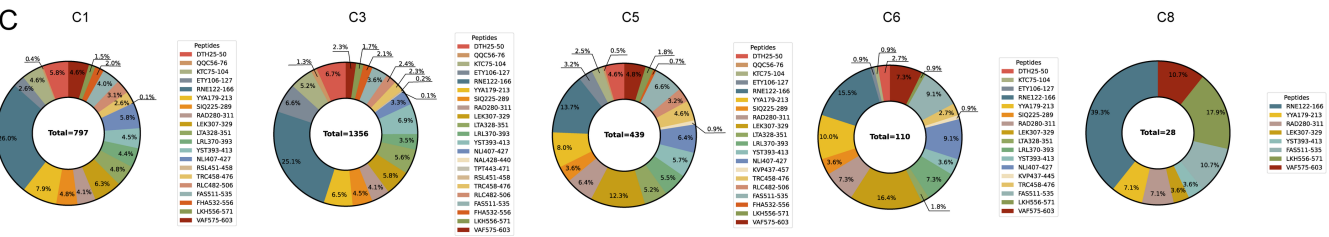
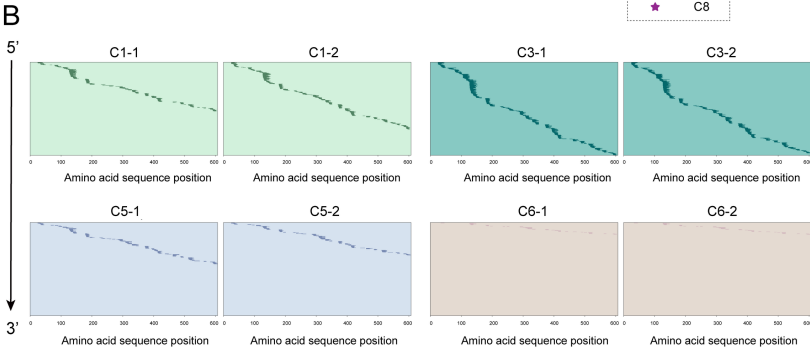
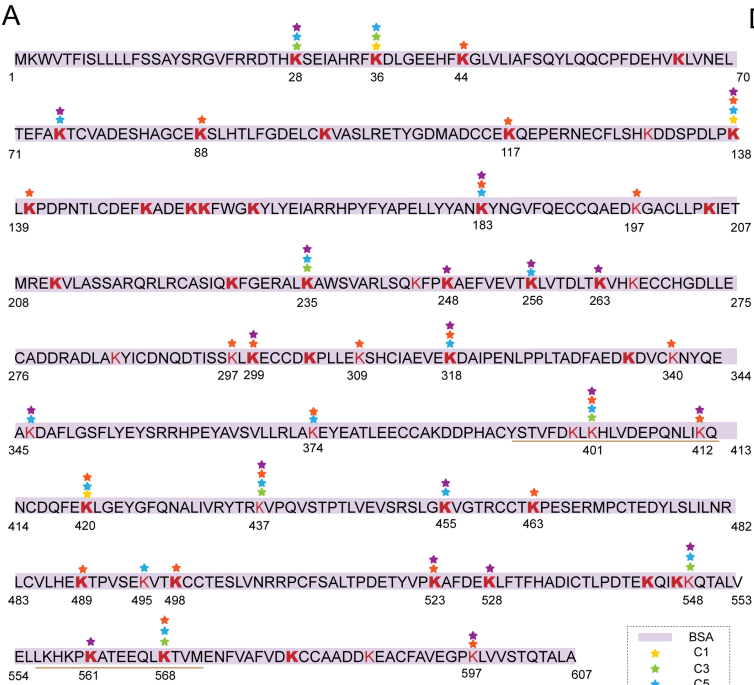
Fig. 8 Effect of AMDH-BSA conjugates with varied hapten densities on TCR and BCR diversity. (A) Number of unique TCR and BCR clonotypes in bulk BCR-seq dataset (n=3 mice/group). (B, C) Proportion of the BCR and TCR repertoire occupied by clones of a given size in the bulk BCR-seq dataset. (D) Top 20 most abundant clonotypes in the single-cell TCR-seq dataset (left) and single-cell BCR-seq dataset (right) (n=2 mice/group). (E) Circos plots showing V-J clonotype distribution for the single-cell TCR-seq dataset (left) and single-cell BCR-seq dataset (right); The 20 most abundant V-J combinations are shown for pooled vaccination group. For the bulk BCR-seq dataset, n = 3 mice per group for BSA, C1, C3, and C8 groups. Three independent experiments are labeled by appending “-1”, “-2” or “-3” after each immunization group name; For the single-cell dataset, n=2 mice per group for C1 and C8 groups; Two independent experiments are labeled by appending “-1” or “-2” after each immunization group name, respectively; * p < 0.05, * * p < 0.005, * * * p < 0.001,

1344 * * * * $p < 0.0001$. Means and standard deviations are displayed. Statistical significance

1345 was determined by ANOVA with multiple comparison testing among all groups.







D

C18H30NO - AMDH hapten

	C1	C3	C5	C6	C8
AKDDPHACYSTVFDK(C18H30NO)LKH	0	1	0	0	0
AKDDPHACYSTVFDK(LK)(C18H30NO)	0	1	0	0	0
AK(C18H30NO)DDPHACYSTVFDK	0	1	0	0	0
ACYSTVFDK(LK)(C18H30NO)H	0	2	0	0	0
ACYSTVFDK(LK)(C18H30NO)	0	5	0	0	0
YSTVFDK(C18H30NO)LKHLVDEPQNLKQ	0	1	0	0	0
YSTVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
YSTVFDK(LK)(C18H30NO)HLVDEPQ	2	4	0	0	0
YSTVFDK(LK)(C18H30NO)HL	0	1	0	0	0
YSTVFDK(LK)(C18H30NO)H	4	4	2	1	0
YSTVFDK(LK)(C18H30NO)H	3	2	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	1	0	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	6	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	2	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	4	5	1	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	1	1	0	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	2	1	2	0	0
STVFDK(LK)(C18H30NO)HLVDEPQNLKQ	2	3	2	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	4	0	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	3	4	1	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	2	0	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
TVFDK(LK)(C18H30NO)HLVDEPQNLKQ	3	3	2	0	0
VFDK(LK)(C18H30NO)HLVDEPQNLKQ	4	3	1	0	0
VFDK(LK)(C18H30NO)HLVDEPQNLKQ	1	0	0	0	0
DK(LK)(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
DK(C18H30NO)LKHLVDEPQNLKQ	0	1	0	0	0
KLK(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
KLKHLVDEPQNLK(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
K(C18H30NO)HLVDEPQNLKQ	0	1	0	0	0
K(C18H30NO)HLVDEPQNLKQ	0	3	0	0	0
K(C18H30NO)HLVDEPQNLKQ	1	3	0	0	0
K(C18H30NO)HLVDEPQNLKQ	2	2	1	0	0
HLVDEPQ(NQ)N(LK)(C18H30NO)Q	0	1	0	0	0
HLVDEPQ(NQ)N(LK)(C18H30NO)Q	0	3	2	0	0
HLVDEPQ(NQ)N(LK)(C18H30NO)Q	0	4	4	0	0
LVDEPQNLK(C18H30NO)Q	0	2	0	0	0
LVDEPQNLK(C18H30NO)Q	0	2	2	1	0
LVDEPQNLK(C18H30NO)Q	0	2	2	2	1
VDEPQNLK(C18H30NO)Q	0	2	0	0	0
VDEPQNLK(C18H30NO)Q	0	2	0	0	0
EPQNLK(C18H30NO)Q	0	1	2	0	0
EPQNLK(C18H30NO)Q	0	0	1	0	0
NLIKQNCQDFEK(C18H30NO)LGEYGFQ	1	0	0	0	0
NLIKQNCQDFEK(C18H30NO)LG	1	0	0	0	0
NLIK(C18H30NO)QNCDC	1	0	0	0	0

