Developing Acid-Responsive Glyco-Nanoplatform Based Vaccines for Enhanced Cytotoxic T-lymphocyte Responses Against Cancer and SARS-CoV-2

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Cytotoxic T-lymphocytes (CTLs) are central for eliciting protective immunity against malignancies and infectious diseases. Here, for the first time, partially oxidized acetalated dextran nanoparticles (Ox-AcDEX NPs) with an average diameter of 100 nm are fabricated as a general platform for vaccine delivery. To develop effective anticancer vaccines, Ox-AcDEX NPs are conjugated with a representative CTL peptide epitope (CTLp) from human mucin-1 (MUC1) with the sequence of TSAPDTRPAP (referred to as Mp1) and an immune-enhancing adjuvant R837 (referred to as R) via imine bond formation affording AcDEX-(imine)-Mp1-R NPs. Administration of AcDEX-(imine)-Mp1-R NPs results in robust and long-lasting anti-MUC1 CTL immune responses, which provides mice with superior protection from the tumor. To verify its universality, this nanoplatform is also exploited to deliver epitopes from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to prevent coronavirus disease 2019 (COVID-19). By conjugating Ox-AcDEX NPs with the potential CTL epitope of SARS-CoV-2 (referred to as Sp) and R837, AcDEX-(imine)-Sp-R NPs are fabricated for anti-SARS-CoV-2 vaccine candidates. Several epitopes potentially contributing to the induction of potent and protective anti-SARS-CoV-2 CTL responses are examined and discussed. Collectively, these findings shed light on the universal use of Ox-AcDEX NPs to deliver both tumor-associated and virus-associated epitopes.

1. Introduction
Fatal diseases, such as cancer and viral infections, seriously threaten human health. Globally, in 2020, there were 19.3 million new cancer cases, and by 2040, this number may increase to 28.4 million.[1] In addition, as of July 2, 2021, there are 182,392,61 confirmed cases of the new coronavirus pneumonia (COVID-19) worldwide caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), including 3,954,324 deaths, as reported by the World Health Organization.[2] Thus, the quest for effective and safe means against cancer and SARS-CoV-2 has been highly pursued.

Vaccines can elicit B-cell and/or T-cell responses, enabling them to be the most effective tools in fighting cancer and viral infections.[3–8] As an essential component of T cells, cytotoxic T lymphocytes can recognize short peptide epitopes (8–11 amino acids)[9] and play a critical role in killing tumor cells and virus-infected cells.[10–13] However, direct administration of CTL peptides (CTLp) usually fails to induce sufficient CTL responses,[14,15] possibly associated with the short retention time of CTLp kept in antigen-presenting cells. Thus, new strategies that can efficiently activate CTLs are in high demand.

The use of carriers for delivering epitopes is an appealing strategy to elicit robust CTL activities.[15–18] As a highly attractive carrier, acetalated dextran (AcDEX) is a pH-responsive polysaccharide with good biocompatibility.[15,18–21] Compared with the commonly used poly-(lactic-co-glycolic acid) (PLGA) particles, the particles formed by AcDEX polymer can elicit stronger immune responses.[15,18] AcDEX nanoparticles were developed to deliver antigens and/or adjuvants against various targets, including cancer, anthrax, bacterial infection, and influenza.[14,22–26] For instance, a hydrophobic ovalbumin peptide (OVAp, SIINFEKL) non-covalently encapsulated within the AcDEX particles by the double emulsion-evaporation technology led to effective antitumor efficacy.[14,27] Moreover, a drawback of the AcDEX system is that the AcDEX particles are less effective in encapsulating hydrophilic peptide epitopes, such as a peptide with the sequence of PDTRPAP from human mucin-1.
MUC1 is a high-ranked tumor-associated antigen, and its conjugates with a carrier moiety such as bacteriophage Qβ have been actively explored as potential anticancer vaccine candidates. In our study, by the emulsion–evaporation method, the AcDEX NPs were also not effective in encapsulating reported hydrophilic epitopes of MUC1, including TSAP-DTRPA, RPAPGSTAP, and SAPDNRPAI (data not shown). While the electrospray (ES) method may improve encapsulation efficiency, AcDEX particles made by ES were larger (1–5 µm), which can affect cell targeting and reduce CTL activity. To broaden the utility of the AcDEX system, it would be desirable that methods can be developed so that AcDEX particles can be utilized to deliver a wide variety of CTL epitopes.

We have begun to investigate an efficient strategy based on the AcDEX system to deliver both the hydrophobic epitope of OVA and the hydrophilic epitope of MUC1. Currently, the biodegradable carboxy–dimethylmaleic amide linker (CDM) linked polymer conjugate of OVA (P-CDM-OVAp) provides a promising platform for enhancing OVAp-specific CTL activation. While the addition of bovine serum albumin (BSA) significantly increased the release of OVAp, the P–CDM–OVAp released less than 10% OVAp after 24 h at acidic conditions, which may limit CTL activation. As an alternative to the CDM linker, the acid-responsive imine bond (Schiff-base) formed between aldehyde and amine is of great interest, which can be utilized for the delivery of all CTL peptides.

In this work, we prepared for the first time, partially oxidized acetalated dextran nanoparticles (Ox-AcDEX NPs 1) with a uniform nanospherical structure. Compared with AcDEX NPs non-covalently loaded with OVAp, OVAp-conjugated AcDEX NPs via imine bond formation elicited a higher level of CTL activation in vitro. Considering that the presence of the CTL epitope is not the sole requirement to generate powerful CTL activities, we envision that AcDEX-(imine)-CTLp-R NPs, formed through the conjugation of both CTL peptide (CTLp) and Toll-like receptor 7 agonist R837 (R) with Ox-AcDEX NPs, may further potentiate CTL responses (Scheme 1b). To test our hypothesis, we conjugated the MUC1 peptide (Mp) and R837 (R) to Ox-AcDEX NPs. The resulting AcDEX-(imine)-Mp-R NPs significantly enhanced antitumor activities by eliciting potent and long-lasting anti-MUC1 CTL responses. Furthermore, we also applied this strategy to develop anti-SARS-CoV-2 vaccine candidates. Combining the predicted CTL peptide (Sp) and R837 (R) with Ox-AcDEX NPs produced AcDEX-(imine)-Sp-R NPs, which induced robust and protective CTL responses against SARS-CoV-2. Importantly, this platform provides a facile and novel strategy to identify the protective CTL epitopes of SARS-CoV-2 experimentally. To the best of our knowledge, this is the first report of the AcDEX system for anti-MUC1 and anti-SARS-CoV-2 immunotherapy.

2. Results and Discussion

2.1. Synthesis and Characterization of Ox-AcDEX NPs 1

The oxidation of dextran with an average molecular weight of 9–11 KDa from Leuconostoc mesenteroides with sodium periodate...
yielded the partially oxidized dextran (Ox-DEX).[44] The aldehyde content on OxDex was measured by a microplate bicinchoninic acid assay according to the manufacturer’s instructions, and it was found that every 100 mol of anhydroglucose units contained 8 mol of aldehyde functions (Figure S2, Supporting Information). Next, in the presence of pyridinium p-toluenesulfonate, Ox-DEX polymer was treated with 2-methoxypropene to yield partially oxidized acetalated dextran (Ox-AcDEX) polymer (Scheme 1a), which can be fabricated into conjugates via the formation of Schiff base linkages.[27] We tried to conjugate Ox-AcDEX polymer with a MUC1 CTLp with the sequence of TSAPDTRPAP, and then prepared the nanoparticles (NPs) by the double emulsion method. Very low conjugation efficiency of peptides in the NPs was found, possibly due to the cleavage of the formed Schiff base in dichloromethane used to prepare the NPs. Thus, by the double emulsion–evaporation technology, we directly fabricated Ox-AcDEX NPs 1 for conjugating peptides and/or adjuvants. The obtained NPs were characterized by transmission electron microscopy (TEM), with an average size of 100 nm (Figure 1a). Fourier transform infrared (FTIR) spectroscopy analysis revealed the appearance of a signal at 1737 cm⁻¹ in Ox-AcDEX NPs 1 that was absent in the AcDEX NPs without oxidation (Figure S3, Supporting Information), indicating the presence of aldehyde groups on Ox-AcDEX NPs 1. To our knowledge, no studies have reported Ox-AcDEX NPs with uniform spherical nanostructure with an average diameter of ≤100 nm.

Due to the acetal bond’s lability to acid hydrolysis,[45,46] the AcDEX NPs can be degraded in acidic organelles such as late endosomes or lysosomes, bestowing their good biocompatibility. To confirm pH responsiveness, Ox-AcDEX NPs 1 were spiked into PBS (phosphate-buffered saline) at pH 7.4, 6.5, 5.5, or 4.5 under RT (room temperature) and 37 °C, respectively. The UV–visible diffraction at 600 nm of the NP solution was recorded. As shown in Figure 1b, the NPs degraded gradually in PBS with pH values of 5.5 and 4.5 over 20 h. In contrast, when added to PBS with pH 7.4 and 6.5 at RT or 37 °C, minor absorbance changes were observed, suggesting the particles were largely intact under these conditions (Figure 1b).

2.2. Synthesis and Immunological Evaluation of AcDEX-(imine)-OVAp NPs 2

To demonstrate the effectiveness of imine-linked glyco-nano-platform based on Ox-AcDEX NPs 1 in activating CTLs, we prepared AcDEX-(imine)-OVAp NPs 2 by coupling a well-known CTLp from OVA with the sequence of SIINFEKL (referred to as OVAp) with Ox-AcDEX NPs 1 to yield AcDEX-(imine)-OVAp NPs 2 (Figure 2a). To benchmark the Schiff-base chemistry’s performance in the AcDEX system for OVAp delivery, we also synthesized OVAp-loaded AcDEX NPs (OVAp-AcDEX NPs 3, Figure 2a) by the noncovalent encapsulation method as a control.

By high-performance liquid chromatography (HPLC) analysis, the amounts of OVAp by AcDEX-(imine)-OVAp NPs 2 and OVAp-AcDEX NPs 3 were measured reproducibly with 67 and 61 μg of peptide per mg NP, respectively (Table S1, Supporting Information).

We next evaluated the effect of AcDEX-(imine)-OVA NPs 2 on CTL activation. Due to the expression of MHC-I, EL4 lymphoma cells have been widely utilized as target cells for CTL studies.[14,22,38] To determine whether CTLs can recognize OVAp-presented EL4 cells, we conducted a B3Z assay,[47,48] a convenient method to test CTL activation of OVAp in vitro. The T cell line B3Z expresses OVAp-specific T cell receptors for recognizing the OVAp/MHC-I H-2Kb complexes. When the OVAp/MHC-I H-2Kb complexes bind to the T cell receptors, B3Z cells get activated to secrete β-galactosidase, which can cleave chlorophenol red-β-D-galactopyranoside to produce chlorophenol red conveniently quantified with its optical absorbance value measured at 595 nm.

When performing the B3Z assay to assess in vitro CTL activation, EL4 cells were incubated with free OVAp, AcDEX-(imine)-OVAp NPs 2, or OVAp-AcDEX NPs 3. The resulting OVAp-loaded EL4 cells were then co-cultured with B3Z cells. As shown in Figure 2b, compared with free OVAp, incubation of AcDEX-(imine)-OVAp NPs 2 and OVAp-AcDEX NPs 3 showed more robust activation of B3Z cells. Interestingly, a comparison between AcDEX-(imine)-OVAp NPs 2 and OVAp-AcDEX NPs 3 showed that AcDEX-(imine)-OVAp NPs 2 enhanced B3Z cell activation, indicating that AcDEX-(imine)-OVAp NPs 2 is superior to OVAp-AcDEX NPs 3 for activating of OVA-specific CTLs.

The AcDEX NPs can be taken up by cells and enter the acidic organelles, such as late endosomes and lysosomes, where OVAp can be sustainably released from the NPs. The released OVAp can easily escape from the lysosome and enter the major histocompatibility complex class I (MHC-I) presentation pathway to present OVAp on the cell surface, readily leading to CTL activation. To demonstrate this, we investigated the release of OVAp from the NPs within EL4 cells. For visualization of OVAp in cells,
SIINFEKL conjugated with fluorescein isothiocyanate (OVApFITC, Scheme S1, Supporting Information) was synthesized and conjugated with Ox-AcDEX NPs 1. As shown in Figure 2c, EL4 cells treated with AcDEX-(imine)-OVApFITC NPs 4 showed strong green (FITC) fluorescence on their cell surfaces, not limited to lysosomes, indicating that OVApFITC was successfully released from the NPs and entered the MHC-I pathway to present OVAp. Unlike nanomaterials, the released OVA peptide (OVAp) can easily escape from lysosomes. Compared with free OVApFITC and OVApFITC-AcDEX NPs 5, AcDEX-(imine)-OVApFITC NPs 4 produced the strongest FITC fluorescence on the cell surfaces, highlighting the most superior presentation of OVApFITC on MHC-I by EL4 cells, which is essential for CTL activation. The increase in OVAp cross-presentation is good evidence that AcDEX-(imine)-OVAp NPs 2 can enhance CTL activation.

Activation of antigen-presenting cells (APCs) is a critical step in generating CTLs. We next evaluated the presentation of OVAp by bone marrow dendritic cells (BMDCs), which are an important type of APCs. As shown in Figure S5, Supporting Information, incubation of AcDEX-(imine)-OVApFITC NPs 6 with BMDCs led to enhanced OVAp presentation and increased activation of B3Z cells, further confirming the advantage of AcDEX-(imine)-OVApFITC NPs 4 for CTL activation. Together, the conjugation of CTL epitopes on the AcDEX NPs via imine bond formation is an effective strategy to boost CTL responses.

Figure 2. a) Synthesis of AcDEX-(imine)-OVAp NPs 2 and OVAp-AcDEX NPs 3. b) Co-culture of EL4 cells presented by free OVAp, AcDEX-(imine)-OVAp NPs 2, or OVAp-AcDEX NPs 3 with B3Z cells for in vitro CTL activation study. The error bars represent the standard error of the mean (SEM) of three replicates. The p value was obtained by two-way ANOVA Bonferroni post-test. *** p < 0.001. c) Synthesis of AcDEX-(imine)-OVApFITC NPs 4 and OVApFITC-AcDEX NPs 5. d) Colocalization of AcDEX-(imine)-OVApFITC NPs 4, OVApFITC-AcDEX NPs 5, and OVApFITC within EL4 cells upon incubation with the NPs containing 1 µg of OVApFITC for 6 h. The intracellular FITC signals were merged with LysoTracker Red, and the colocalization was shown in yellow. Scale bars: 20 µm.

2.3. Synthesis and Immunological Evaluation of AcDEX-(imine)-Mp1-R NPs

With the advantages of Ox-AcDEX NPs 1 demonstrated in OVAp delivery, we next investigated Ox-AcDEX NPs 1 for delivering epitopes from mucin-1 (MUC1). First, a representative CTL epitope from the variable number of tandem repeats (VNTR) region of MUC1 with the sequence of TSAPDTRPAP (Mp1) was synthesized. By augmenting with a Toll-like receptor 7 (TLR-7) agonist R837, the immunogenicity of Mp1 could be enhanced.[49] Hence, we prepared AcDEX-(imine)-Mp1-R NPs 6 by conjugating Mp1 and R837 with Ox-AcDEX NPs 1 (Figure 3a).

By HPLC determination, it was found that AcDEX-(imine)-Mp1-R NPs 6 contained 62 µg of Mp1 and 69 µg of R837 per mg of NP (Table S2, Supporting Information). In parallel, AcDEX-(imine)-Mp1-R NPs 7 as R837-free control and AcDEX-(imine)-R NPs 8 as Mp1-free control were also prepared (Figure 3a). The amounts of Mp1 and R837 were determined to be 66 µg of Mp1 per mg of 7 and 81 µg of R837 per mg of 8, respectively (Table S2, Supporting Information).

Next, we tested the release rates of conjugated Mp1 and R837 in AcDEX-(imine)-Mp1-R NPs 6 by treatment of the NPs in PBS with a pH value of 7.4, 6.0, 5.5, or 4.5 at 37 °C. As shown in Figure 3b,c, when spiked into PBS with pH 6.0 or pH 7.4, the spontaneous release of Mp1 and R837 from the NP was slow.
On the contrary, their release rates increased significantly at pH 4.5 and 5.5 (Figure 3b,c). This suggests Mp1 and R837 can be released in the acidic environment of intracellular lysosomes or endosomes.

Afterward, CTL activation in vivo was evaluated. How antigen is formulated with the adjuvant can significantly affect immune responses.[50] To establish the best combination of Mp1 and R837, we proposed two methods of introducing R837. One is to use AcDEX-(imine)-Mp1-R NPs 6 bearing Mp1 and R837 within one particle for immunization. The other is to immunize mice by a mixture of AcDEX-(imine)-Mp1 NPs 7 and AcDEX-(imine)-R NPs 8 with Mp1 and R837 in separate particles. Mice were vaccinated with free Mp1, AcDEX-(imine)-Mp1-R NPs 6, AcDEX-(imine)-Mp1 NPs 7, and AcDEX-(imine)-R NPs 8, respectively, by weekly injections. The induced antigen-specific T lymphocyte activities were evaluated by the in vivo CTL study using carboxyfluorescein succinimidyl ester (CFSE) assay.[51]

Once CTLs bearing specific TCRs find the cognate peptides associated with the MHC-I on target cells, the CTLs get activated to kill the target cells. It is noted that all nucleated cells express MHC-I and splenocytes are relatively easy to obtain in great numbers. Thus, syngeneic splenocytes were used as targets for the in vivo CTL assay. As shown in Figure 3d,e, AcDEX-(imine)-Mp1-R NPs 6 vaccination by subcutaneous route produced more robust MUC1-specific CTLs than other groups,
resulting in a higher population of peptide Mp1-pulsed target cells to be lysed, highlighting the advantages of conjugating Mp1 and R837 in an AcDEX particle. Besides, Mp1-specific CTLs (TSAPDRPAP-MHC I*CD8+ cells) were detected by Mp1-MHC I tetramer staining (Figure S8, Supporting Information), which is direct evidence of the generation of Mp1-specific CTLs.

Subsequently, we tested the persistence of CTL responses induced by the NPs. Intriguingly, robust MUC1-specific CTL activities can be detected in spleens on days 60, 120 after three vaccinations with AcDEX-(imine)-Mp1-R NPs 6 on days 0, 7, 14 (Figure 3f,g), indicating that long-lasting anti-MUC1 CTL responses were elicited. In addition to the spleen, we also tested anti-MUC1 CTL activities in the lymph nodes. Activation of lymph nodes is a prerequisite for CTL activation. As shown in Figure S9, Supporting Information, AcDEX-(imine)-Mp1-R NPs 6 vaccination upregulated CD8 and MHC-I levels on lymph node cells, demonstrating that AcDEX-(imine)-Mp1-R NPs 6 vaccination activated lymph nodes. Importantly, AcDEX-(imine)-Mp1-R NPs 6 vaccination activated robust and long-lasting (over 120 days) MUC1 specific CTLs in lymph nodes (Figure 3h), leading to significant death of MUC1 containing target cells. Furthermore, AcDEX-(imine)-Mp1-R NPs 6 did not induce superior humoral responses (Figure S10, Supporting Information), highlighting that CTL immune response is the main mechanism of AcDEX-(imine)-CTLp-R NPs for antitumor and antiviral immunotherapy.

By judiciously choosing the amount of R837 for conjugation, the loading ratio of Mp/R837 can be precisely altered and manipulated. To confirm this, in addition to AcDEX-(imine)-Mp1-R NPs 6 containing Mp1 and R837 with a ratio of 10:11, we also synthesized AcDEX-(imine)-Mp1-R NPs 9–11, which contained Mp1 and R837 with ratios of 10:7, 5:2, and 5:1, respectively (Table S2, Supporting Information). As shown in Figure S11, Supporting Information, AcDEX-(imine)-Mp1-R NPs 6 showed the most superior CTL activation in vivo among the groups, indicating a higher R837 level in a particle contributes to enhanced CTL activation. Thus, in our further vaccine fabrication, CTLp and R837 with a ratio of 1:1 were added for Ox-AcDEX NPs conjugation.

2.4. Synthesis and Immunological Evaluation of AcDEX-(imine)-Mp2-R NPs 12

To enhance CTL activation, we also studied the other MUC1 CTLp with the sequence of SAPDNRPAL in different regions of MUC1, which binds both human and mouse MHC-I with much higher affinities than those from the tandem repeat region.[44] AcDEX-(imine)-Mp2-R NPs 12 were fabricated by the conjugation of Mp2 and R837 with Ox-AcDEX NPs 1 (Figure 4a) bearing 56 µg of Mp2 and 51 µg of R837 per mg of NP (Table S2, Supporting Information). For comparison, AcDEX-(imine)-Mp2 NPs 13 with 68 µg of Mp2 per mg of NP was also prepared (Figure 4a). The release rates of conjugated Mp2 and R837 from AcDEX-(imine)-Mp2-R NPs 12 increased significantly at pH 5.5 and pH 4.5 (Figure 4b,c), which is consistent with AcDEX-(imine)-Mp1-R NPs 6. Furthermore, in vivo CTL assay showed AcDEX-(imine)-Mp2-R NPs 12 vaccination activated the highest Mp2-specific CTLs amongst the groups (Figure 4d,e). This further demonstrates the importance of combining MUC1 peptide and R837 in an AcDEX particle.

2.5. Tumor Challenge Study

With the superior CTL activation by AcDEX-(imine)-Mp-R NPs, we evaluated their abilities to provide tumor protection. Mice were subcutaneously implanted with B16-MUC1 tumor cells, which express MUC1 on the cell surface. One day after B16-MUC1 tumor cell injection, mice were immunized with various vaccine formulations every three days for a total of five injections, then the growth of the tumor was monitored. As shown in Figure 5, free Mp2 alone or AcDEX-(imine)-Mp2-R NPs 12 were not very effective in slowing down tumor growth than the mock group receiving AcDEX-(imine)-Mp1-R NPs 6 injection. In comparison, immunization with AcDEX-(imine)-Mp1-R NPs 6 drastically reduced tumor growth, highlighting the importance of Mp1 from the VNTR region of MUC1 in the NPs for eliciting potent CTL responses against tumors. Furthermore, the combination of AcDEX-(imine)-Mp1-R NPs 6 + AcDEX-(imine)-Mp2-R NPs 12 provided the strongest tumor protection (Figure 5), indicating the superiority of multiple epitope delivery. Next, we tried to reduce the dose of immunizations, so performed immunizations of various vaccine formulations every seven days for a total of three injections. Intriguingly, superior protection of B16-MUC1 tumors by the NPs 6 or 6 + 12 vaccination was further confirmed (Figure S12a–c, Supporting Information). Besides, no significant weight loss was observed in the mice, implying that the NPs were nontoxic despite the pronounced tumoricidal effects (Figure S12d, Supporting Information).

2.6. Design, Synthesis, and Immunological Evaluation of AcDEX-(imine)-Sp-R NPs 14–16

Since the end of 2019, coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has pushed the global public health system to the limit.[52] Comfortingly, with the report of the genome sequence of SARS-CoV-2,[53,54] much valuable experience has been achieved in combating COVID-19. Significantly, three vaccines (Pfizer-BioNTech, Moderna, and Janssen) have been authorized for emergency use by the U.S. Food and Drug Administration (FDA).[55] However, there is no clarity about the duration of immunity elicited by these vaccines. And these vaccines have noticeable side effects, including pain at the injection site, headache, fatigue, and muscle pain.[55] Furthermore, new SARS-CoV-2 variants continue to emerge,[56] some with the potential to escape vaccine-induced or therapeutic antibodies. COVID-19 appears likely to develop into a seasonal disease, underscoring the need to improve vaccines against SARS-CoV-2 continuously.[57]

Although the current COVID-19 vaccines are primarily based on generating neutralizing antibodies,[58,59] growing evidence suggests that T cell responses correlated with favorable disease outcomes can play a critical role in combating
Thus, it is an urgent matter to gain a deeper insight into T cell responses to SARS-CoV-2.

Given that AcDEX-(imine)-Mp-R NPs induced robust anti-cancer CTL activities, we are interested in exploiting the AcDEX NPs to deliver CTL epitopes of SARS-CoV-2 for vaccine candidates. For such vaccine design, a first and essential criterion is that immunogenic epitopes of SARS-CoV-2 should be identified. Many strategies, such as in silico epitope predictions and genome-wide screening technology,\(^{62,63}\) have provided crucial knowledge of SARS-CoV-2 T cell epitopes. However,
experimentally identifying the critical SARS-CoV-2 CTL epitopes that can trigger robust and protective CTL responses is difficult.

Interestingly, in our recent study, a protective B cell epitope from the S protein receptor-binding domain (RBD) of SARS-CoV-2’s spike (S) protein with the sequence of SYGFQPTNGVGYQPY (residues 494–508, referred to as Sp1) has been discovered,[64] which contains a top potential epitope with high MHC-I coverage predicted by Fast et al. [65] In addition, Fast et al. predicted two other top potential epitopes with the sequence of SIIAYTMSL (691–699, referred to as Sp2) and VVFLHVTVY (1060–1068, referred to as Sp3), [65] which showed very high binding affinity to mouse H2-Kβ MHC-I with the immune epitope database (IEDB) score of 0.818 and 0.793 (the higher the score, the higher the affinity), respectively (Table S3, Supporting Information). Therefore, peptides Sp1–Sp3 were first chosen for vaccine design.

Peptides Sp1–Sp3 were synthesized from the Wang resin using Fmoc chemistry and characterized by HPLC and HRMS. With the peptides in hand, Sp (Sp1–Sp3, respectively) and R837 were conjugated with Ox-AcDEX NPs [48] to afford AcDEX-(imine)-Sp-R NPs 14–16 (Figure 6a). The amounts of peptides and R837 on the NPs were determined by HPLC, shown in Table S4, Supporting Information.

Subsequently, the effect of AcDEX-(imine)-Sp-R NPs 14–16 on CTL activation was evaluated using the in vivo CTL assay. The mice were immunized weekly with free Sp1–3 and AcDEX-(imine)-Sp-R NPs 14–16, respectively. After two weeks, a mixture of CFSEhigh labeled SARS-CoV-2 peptide-pulsed target splenocytes and CFSELow labeled control cells (1:1) was injected into the immunized and non-treated mice, respectively. One day later, their splenocytes were analyzed by FACS. The p values were analyzed by a two-tailed unpaired Student’s t-test with GraphPad Prism. *p < 0.05, **p < 0.01.

![Figure 6](image-url)

**Figure 6.** a) Synthetic AcDEX-(imine)-Sp-R NPs 14–16 by the conjugation of CTL epitopes Sp1–3, (respectively) derived from SARS-CoV-2 and R837 (R) with Ox-AcDEX NPs 1 for enhanced CTL responses against SARS-CoV-2. b–d) In vivo CTL activities against SARS-CoV-2 peptides. Mice were respectively immunized weekly with free Sp1–3 (n = 2 mice for each group) or AcDEX-(imine)-Sp-R NPs 14–16 (n = 3 mice for each group). On day 14 after two vaccinations, a mixture of CFSEhigh labeled the corresponding peptide (Sp1, Sp2, or Sp3)-pulsed target cells and CFSELow labeled control cells (1:1) was injected into the immunized and non-treated mice, respectively. One day later, their splenocytes were analyzed by FACS. The p values were analyzed by a two-tailed unpaired Student’s t-test with GraphPad Prism. *p < 0.05, **p < 0.01.
To further confirm the protection of the NPs-induced CTLs in the fight against SARS-CoV-2 infected cells, we also conducted in vivo CTL activities against the SARS-CoV-2 spike pseudovirus, which contains the complete S protein. Intriguingly, AcDEX-(imine)-Sp-R NPs 14 and 15 elicited potent in vivo CTL activation against the spike pseudovirus resulting in a significant killing of target cells pulsing the spike pseudovirus (Figure 7c). This indicates that the SARS-CoV-2 spike pseudovirus can be processed to produce identical/similar Sp1 and Sp2 after infecting the target cells. Collectively, AcDEX-(imine)-Sp-R NPs 14 and 15 can effectively eliminate SARS-CoV-2 infected cells.

Furthermore, vaccination of AcDEX-(imine)-Sp-R NPs 14 and 15 upregulated MHC-I, CD86, CD8, and CD4 levels on splenic immune cells (Figures S14 and S15, Supporting Information), highlighting effective CTL immune memory was induced by these NPs. Finally, no detectable serum TNF-α and IFN-γ were observed in the sera from vaccinated mice (Figure S16, Supporting Information), suggesting that AcDEX-(imine)-Sp-R NPs 14 and 15 can induce strong anti-SARS-CoV-2 CTL immune responses without generating a harmful cytokine storm.

2.7. Design, Synthesis, and Immunological Evaluation of AcDEX-(imine)-Sp-R NPs 17–24

Given the exciting results obtained, we hope to develop a facile method to prove SARS-CoV-2 CTL epitopes’ effectiveness. As shown in Figures 3d,e, 4d, 6b,c immunization of free MUC1 peptides (Mp1, Mp2) or SARS-CoV-2 peptides (Sp1, Sp2) failed to generate enough CTL responses. Indeed, in our studies, these peptides are critically protective CTL epitopes against cancer or SARS-CoV-2. Therefore, it may not be an effective method to verify whether the predicted peptide is a protective epitope by directly administering the free peptide. Excitingly, the combination of CTLp and B37 on AcDEX NPs leads to AcDEX-(imine)-CTLP-R NPs, which offers a facile platform for effectively testing the protection of predicted CTLp.

To further demonstrate our strategy’s utility, we explored the delivery of other predicted CTL epitopes from SARS-CoV-2. Ferretti et al. revealed 29 shared epitopes in COVID-19 patients with only three located in the S protein, whereas most were located in the ORF1ab or nucleocapsid (N) protein. We are interested in some epitopes shared by most COVID-19 convalescent patients, including two from the S protein (residues 378–386 with the sequence of KCYGVSPHTK and residues 269–277 with the sequence of YLQPRTFLL), four from ORF1ab (residues 3886–3894 with the sequence of KLWAQCVQ, residues 4094–4102 with the sequence of ALWEIQQV, residues 1637–1646 with the sequence of TTDPFSLGRY, and residues 4163–4172 with the sequence of CTDDNALAYY), and one from N protein at residues 361–369 with the sequence of YGYLQPRTF. It is noted that these reported peptides from humans are leukocyte antigen (HLA)-restricted epitopes. SARS-CoV-2 nucleoprotein (Mp1) identified by Joag et al. [66] which is consistent with a reported epitope from Martelli et al. [67]
Figure 8. 

a) Synthetic AcDEX-(imine)-Sp-R NPs 17–24 by the conjugation of R837 (R) and CTL epitopes Sp4–11, respectively, derived from SARS-CoV-2 with Ox-AcDEX NPs 1 for enhanced CTL responses against SARS-CoV-2. 

b–i) CTL activation in vivo study. Mice were respectively vaccinated weekly with free Sp4–11 (n = 2 mice for each group) and AcDEX-(imine)-Sp-R NPs 17–24 (n = 3 mice for each group). On day 14 after two vaccinations, a mixture of CFSEhigh labeled peptide-pulsed target cells and CFSElow labeled control cells (1:1) was injected into the immunized and non-treated mice, respectively. One day after injection, their splenocytes were analyzed by FACS. 

j) For testing in vivo CTL activities against the N protein of SARS-CoV-2, 14 days later, a mixture of CFSEhigh labeled N protein (SARS-CoV-2 nucleocapsid recombinant protein)-pulsed target cells and CFSElow labeled control cells with a ratio of 1:1 was injected into AcDEX-(imine)-Sp11-R NPs 24 immunized and non-treated mice (n = 2 mice for each group), respectively. One day later, their splenocytes were analyzed by FACS. 

The p values were analyzed by a two-tailed unpaired Student’s t-test with GraphPad Prism. * p < 0.05, ** p < 0.01, *** p < 0.001. ns: no significant difference.
Peptides Sp4–Sp11 were synthesized and respectively conjugated with Ox-AcDEX NPs to afford AcDEX-(imine)-Sp-R NPs 17–24 (Figure 8a). When screened by the in vivo CTL assay, we found that free predicted peptides other than Sp9 and Sp11 were not effective in inducing potent CTL responses. Compared with the non-treated groups, AcDEX-(imine)-Sp-R NPs 17–19 did not induce significant CTL activation in vivo (Figure 8b–d), suggesting that Sp4–6 were non-protective epitopes. AcDEX-(imine)-Sp-R NPs 20, 21, and 23 led to a certain degree of CTL activation resulting in 20% of target cells to be killed (Figure 8e,f, h), indicating that Sp7, Sp8, and Sp10 are weakly protective epitopes. Usually, peptides may induce insufficient CTL responses. Interestingly, both Sp9 and Sp11 led to almost 30% of target cells to be lysed (Figure 8g, i). Compared with Sp9 and Sp11, AcDEX-(imine)-Sp9-R and AcDEX-(imine)-Sp11-R NPs 22 and 24 led to a higher population of target cells averaging 57% for 22 and 39% for 24 to be lysed (Figure 8g, i), suggesting the high protection of Sp9 and Sp11, and indicating the great potentials of 22 and 24 as anti-SARS-CoV-2 vaccine candidates. Collectively, the AcDEX-(imine)-Sp-R NPs provide a facile platform to identify the protective CTL epitopes of SARS-CoV-2 experimentally, highlighting the advantages of the NPs for effectively excluding non-protective epitopes from lots of predicted ones.

Due to the availability of the N protein of SARS-CoV-2 from the company, we finally evaluated in vivo CTL activities of AcDEX-(imine)-Sp9-R NPs 24 against the N protein of SARS-CoV-2 using a mixture of CFSEhigh labeled N protein-pulsed target cells and CFSElow labeled control cells for mouse injection. Excitingly, AcDEX-(imine)-Sp9-R 24 induced effective CTL activation in vivo against the N protein, leading to a higher number of N protein loaded target cells being killed (Figure 8j), indicating that N protein can be processed to present the identical/similar sequence of Sp11 on the MHC-I by target cells. Thus, AcDEX-(imine)-Sp-R NPs 24 has a great potential to eliminate SARS-CoV-2 infected cells.

3. Conclusion

In summary, we have reported a versatile acid-responsive nano-platform AcDEX-(imine)-CTLp-R NPs by conjugating CTL peptide and TLR-7 agonist R837 with partially oxidized acetalated dextran nanoparticles (Ox-AcDEX NPs), which effectively enhanced CTL responses. When applied to anticancer vaccines, the MUC1 CTLp (Mp1) with the sequence of TSAPDTRPAP and R837 were conjugated with Ox-AcDEX NPs to obtain AcDEX-(imine)-Mpl-R NPs, which produced potent MUC1-specific CTLs and allowed for superior antitumor CTL immunotherapy. Furthermore, based on AcDEX-(imine)-Sp-R NPs, through coupling Ox-AcDEX NPs with R837 and four critical CTLp of SARS-CoV-2 (Sp), respectively, in vivo CTL activation studies discovered several protective CTL epitopes, including two from the spike (S) protein of SARS-CoV-2 with the sequences of SYG-FQPTNGVGYQPY (Sp1) and SIIAYTMSL (Sp2), one from the ORF1ab polyprotein with the sequence of NALAYYNTT (Sp9), and one from the nucleocapsid (N) protein with the sequence of LALLLDLRL (Sp11). The resulting AcDEX-(imine)-Sp-R NPs produced robust CTL activities to SARS-CoV-2, highlighting their great translation potential against SARS-CoV-2. It is worth mentioning that an important future direction can be to combine these four epitopes and R837 with Ox-AcDEX NPs in a particle to design a multivalent vaccine against SARS-CoV-2.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

cancer, cytotoxic T lymphocytes, immunotherapy, nanoparticles, SARS-CoV-2

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