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Synthesis and immunological evaluation of TLR1/2 ligand-conjugated RBDs as self-adjuvanting vaccine candidates against SARS-CoV-2†

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We synthesized and evaluated Pam_3CSK_4-conjugated receptor binding domain (RBD)/deglycosylated RBD as potential anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine candidates. Our investigation revealed the critical importance of limiting the number of introduced Pam_3CSK_4 molecules to the RBD in order to preserve its antigenicity. We also confirmed the harmonious integration of the adjuvant-conjugation strategy with the glycan-shield removal strategy.

Vaccination is of significant importance in suppressing infectious diseases and has played a pivotal role in addressing the coronavirus (COVID)-19 pandemic. Notably, RNA vaccines have been practically used as SARS-CoV-2 vaccines, demonstrating profound efficacy.[1–3] Nonetheless, adverse effects, such as fever and soreness at the injection site, have been noted. In this context, a strategic blueprint is required to develop milder yet potent vaccines to improve readiness against impending outbreaks of infectious diseases.

Vaccines comprise antigens and adjuvants (immunoenhancers). Innate immune ligands, particularly toll-like receptor (TLR) agonists, are promising adjuvants,[4–13] although they can potentially cause adverse inflammatory reactions. The conjugation of antigens with adjuvants, resulting in self-adjuvanting vaccines, offers a potential option for eliciting antigen-specific immune responses without inducing undue inflammation, thereby presenting a promising strategy for vaccine development (Fig. 1a).[14–16] Since the pioneering work by Boons *et al.*,[17] Pam_3CSK_4,[18] a TLR1/2 agonist, has been widely used in self-adjuvanting vaccines.[17,19–22] We have also reported Pam_3CSK_4-conjugated vaccines in our studies.[23–25] Other innate immune ligands, including α-GalCer[25–29] and MPL,[30,31] have also been harnessed. Trumenba, a recombinant lipoprotein serving as a TLR1/2 agonist ligand, has been developed as a self-adjuvanting vaccine for preventing meningococcal B (MenB) infections. This practical application underscores the effectiveness and safety of the self-adjuvanting approach in vaccination.[32,33]

The structure of antigens plays a pivotal role in vaccine development. Glycosylations, the most common post-translational modifications, bear the inherent capability to shield the antigen epitope, thus attenuating antigenicity.[34–36] Guided by this insight, Ma and Wong *et al.* engineered an anti-influenza vaccine using hemagglutinin featuring trimmed glycans, which yielded heightened effectiveness (Fig. 1b).[37] Furthermore, they substantiated the potency of this

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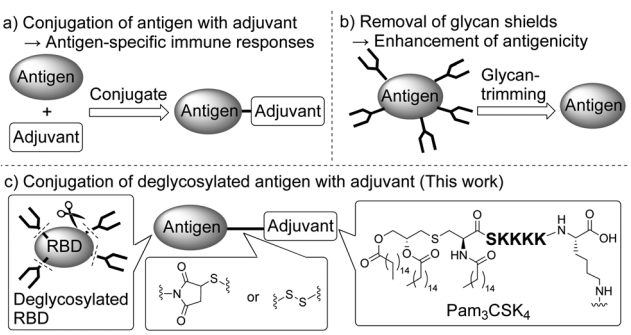

Fig. 1 Strategies for developing efficient vaccines. (a) Conjugating an antigen with an adjuvant (self-adjuvanting strategy). (b) Glycan-shield removal strategy by glycan-trimming. (c) Combinational use of a self-adjuvanting strategy and glycan-shield removal strategy (this work).

glycan-trimming strategy in the context of a SARS-CoV-2 vaccine using their spike protein (S-protein).[38]

In the present study, we investigated the development of a COVID-19 vaccine that integrates a conjugation-based self-adjuvanting strategy with a glycan-shield removal strategy. Herein, receptor binding domains (RBDs) of the S-protein,[39–42] which are commonly used in COVID-19 vaccine development and possess two asparagine-linked glycans (*N*-glycans),[36,43] were used as antigens. Specifically, we harnessed the RBD and its deglycosylated counterpart (deglyRBD) for conjugation with Pam_3CSK_4 through either maleimide–thiol ligation or disulfide bond formation directed toward the cysteine (Cys) residues of the RBDs. Maleimide confers a relatively stable bond, preventing the dissociation of Pam_3CSK_4 from the RBDs within the biological milieu. Conversely, the relatively labile disulfide linkage is expected to undergo smooth digestion after incorporation into antigen presenting cells (APCs), thereby minimizing the antigenic impairment caused by the modification. Conjugation-based self-adjuvanting strategies have predominantly been applied to small-molecule antigens, including peptides and glycans. Consequently, guidelines for the design of protein-based self-adjuvanting vaccines remain unclear. Guo *et al.* reported promising self-adjuvating vaccine candidates using RBD, wherein Pam_3CSK_4 was selectively conjugated to the *N*-terminal amino acid of RBD.[44] In contrast, the present study applied the aforementioned conjugation scheme targeting Cys residues in RBDs, enabling us to analyze the effect of the Pam_3CSK_4-introduction quantity and linker stability. These results emphasized the importance of the Pam_3CSK_4-introduction ratio; specifically, an excessive amount of Pam_3CSK_4 led to decreased RBD antigenicity. Furthermore, linker stability influenced the potency of vaccine candidates. Importantly, we substantiated the ability of deglyRBD to induce immune responses against RBD, confirming the harmonious interplay between the self-adjuvanting and glycan-shield removal strategies. Therefore, this study provides a blueprint for the development of protein-based self-adjuvanting vaccines.

Fig. 2 summarizes the preparation of the Pam_3CSK_4-conjugated RBD vaccine candidates. The initial step involved the synthesis of maleimide-/pyridyl disulfide-functionalized Pam_3CSK_4 **1** and **2** through Fmoc solid-phase peptide synthesis (Fig. 2a and Fig. S1 and S2, ESI†). After the construction of Pam_3CSK_4 on the resin carrying the ivDde-protected lysine (Lys) residue, selective cleavage of ivDde by hydrazine and conjugation with a maleimide-/pyridyl disulfide-functionalized linker, followed by global deprotection under acidic conditions, afforded the desired **1** and **2**.

Compounds **1** and **2** were further conjugated to both the RBD and deglyRBD (Fig. 2b). It has been reported that the RBD possesses nine Cys, where eight partake in the formation of intramolecular disulfide bonds, and the remaining SH forges a disulfide bond with glutathione, a pivotal component in the RBD expression process.[39–42,45] Therefore, RBD and deglyRBD were treated with tris(2-carboxyethyl)phosphine (TCEP) to reduce disulfide bonds. The resulting SH groups were then reacted with **1** or **2** to produce RBD–mal-Pam_3CSK_4, RBD–SS-Pam_3CSK_4, deglyRBD–mal-Pam_3CSK_4, and deglyRBD–SS-Pam_3CSK_4. The approximate introduction ratios of Pam_3CSK_4 to RBD–mal-Pam_3CSK_4, RBD–SS-Pam_3CSK_4, deglyRBD–mal-Pam_3CSK_4, and deglyRBD–SS-Pam_3CSK_4

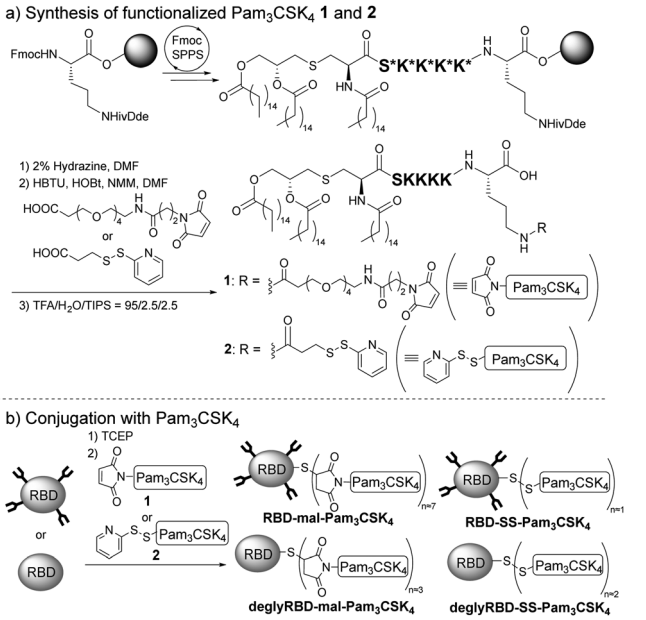

Fig. 2 Preparation of Pam_3CSK_4-conjugated RBD/deglyRBD. (a) Synthesis of maleimide-/pyridyl disulfide-functionalized Pam_3CSK_4 1 and 2. (b) Conjugation with Pam_3CSK_4.

were estimated to be 7, 1, 3, and 2, respectively, using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDA–PAGE) analysis (Fig. S3–S6, ESI†). The approach employed in this study involved a random conjugation reaction against nine free SH groups in the RBD, thereby hindering precise control of the Pam_3CSK_4 introduction ratio.

The vaccine entries are listed in Table 1. V1/V2 corresponds to RBD/deglyRBD, whereas V3/V4 denotes a mixture of RBD/deglyRBD and Pam_3CSK_4. V5/V6 encompassed RBD–mal-Pam_3CSK_4/deglyRBD–mal-Pam_3CSK_4 used maleimide–thiol ligation, whereas V7/V8 represented RBD–SS-Pam_3CSK_4/deglyRBD–SS-Pam_3CSK_4, which was implemented through disulfide bond formation. These entries were administered intraperitoneally to 8-week-old wild-type (WT) BALB/c mice on day 1. The immunization schedule included three additional administrations on days 14 and 28. To assess their self-adjuvanting properties, no additional adjuvants, such as Freund's adjuvant, were co-administered. Blood was collected from each mouse before immunization on day 0 (pre-immunization) and 1 week after each immunization (days 8, 21, and 35).

Table 1 Vaccine entries for *in vivo* mouse immunization

Vaccine entries	Immunized compounds
V1	RBD
V2	deglyRBD
V3	RBD + Pam_3CSK_4
V4	deglyRBD + Pam_3CSK_4
V5	RBD–(mal-Pam_3CSK_4)$_{n\approx7}$
V6	deglyRBD–(mal-Pam_3CSK_4)$_{n\approx3}$
V7	RBD–(SS-Pam_3CSK_4)$_{n\approx1}$
V8	deglyRBD–(SS-Pam_3CSK_4)$_{n\approx2}$

Anti-RBD antibody titers after the third vaccination (day 35) were quantified using enzyme-linked immunosorbent assay (ELISA). V1 and V2 produced minimal anti-RBD antibodies, and the addition of Pam_3CSK_4 (V3 and V4) resulted in a modest increase in antibody production, although the increase remained insignificant. With regard to self-adjuvanting vaccines conjugated with Pam_3CSK_4 through maleimide–thiol ligation, V5 using the RBD displayed minimal antibody titers, in contrast to V6, which, involving degly RBD, induced substantial antibody production. Interestingly, distinct outcomes were noted for vaccines conjugated with disulfide bonds; V7, using RBDs, showed significant induction of antibody production, whereas V8, prepared from deglyRBD, demonstrated negligible antibody production. Both V6 and V7 evoked high IgG titers, but low IgM titers, suggesting efficient class-switching from IgM to IgG (Fig. S8, ESI†). The enhancement of IgG antibody production through repetitive vaccinations was also confirmed in V6 and V7 (Fig. S9, ESI†). V6 produced both IgG1 and IgG2a, whereas V7 predominantly produced IgG1, indicating a balanced Th1/Th2 immune response in V6 and a Th2-biased immune response in V7 (Fig. S10, ESI†). Previously, Wong *et al.* reported that immunization with the S protein possessing *N*-glycans trimmed to the mono-GlcNAc elicited stronger immune responses, characterized by a more balanced Th1/Th2 responses, than the fully glycosylated S protein.[38] In this study, a similar effect by the glycan de-shielding on the RBD was observed, suggesting that the *N*-glycans have the ability to regulate Th1/Th2 immune responses. It is noteworthy that the antibody titers against deglyRBD mirrored those against the RBD for V2, V4, V6, and V8, where deglyRBD was employed as an antigen (Fig. S7, ESI†), confirming the versatility and efficacy of the glycan shield removal strategy.

Interestingly, only V6 and V7 prominently induced anti-RBD antibody production. Despite both V5 and V6 utilizing maleimide-based conjugation, V5 introduced a considerable amount of Pam_3CSK_4 (approximately 7 out of 9 Cys), whereas the incorporation in V6 was low (approximately 3). The antigenicity of V5 may have been impaired by the excessive introduction of Pam_3CSK_4. In contrast, in conjugation mediated by disulfide bond formation, while the content of Pam_3CSK_4 was controlled in both V7 (approximately 1) and V8 (approximately 2), interestingly, significant antibody production was observed only in V7. Presumably, in the conjugation mediated by disulfide bond formation, glycan removal might have altered the accessibility of the conjugation reagent **2**, leading to the introduction of Pam_3CSK_4 at sites significantly altering antigenicity in V8. We also assumed that the removal of glycans from the RBD may reduce bulkiness, weaken disulfide bonds, and subsequently diminish metabolic stability, thereby impairing self-adjuvanting properties (Fig. 3).

In the previous report by Guo *et al.*, a conjugate of Pam_3CSK_4 and RBD with the molecular ratio of 1 to 1 induced a remarkable antibody production.[44] Their results indicated that conjugation of only a single Pam_3CSK_4 unit is adequate to exhibit adjuvant effects. Trumenba®, an FDA-approved self-adjuvanting vaccine used against *Neisseria meningitidis* group B, is composed of two recombinant lipoprotein antigens, each incorporating an N-terminal lipid moiety with TLR2 agonist activity.[33] In addition, introducing a single TLR2 agonist through ligation reaction using sortase A against a recombinant protein, a model group A Streptococcus (GAS) recombinant polytope antigen, significantly enhanced its antigenicity.[46] Our results also indicated that conjugation of a single Pam_3CSK_4 unit is sufficient to exhibit adjuvant effects.

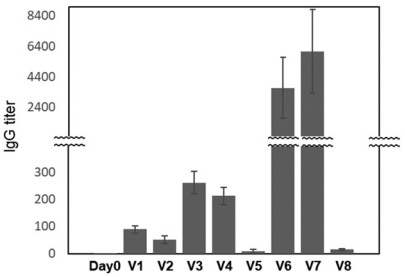

Fig. 3 IgG antibody titters against RBD after third immunizations. Data represent the results from five experiments (n = 5). The error bars represent the standard error of the mean value.

Based on these results, the following design principles for protein-based self-adjuvanting vaccines were proposed: limiting the number of conjugated adjuvants to preserve antigenicity, and ensuring stable linkages between antigens and adjuvants to prevent dissociation within the biological milieu.

In summary, we synthesized Pam_3CSK_4-conjugated RBD/degly RBD as anti-SARS-CoV-2 vaccine candidates and evaluated their functions. Importantly, the deglyRBD-based vaccine material V6 elicited the production of antibodies against RBD, thereby demonstrating the applicability of glycan-shield removal strategy in realm of vaccine development. In this study, we adopted a concise and versatile approach to fabricate a Pam_3CSK_4-conjugate by leveraging the Cys residue of an antigenic protein. Some of the resultant Pam_3CSK_4-conjugated vaccine candidates exhibited marked antibody production, thus presenting a viable avenue for efficacious vaccine construction. The present conjugation method based on stochastic Pam_3CSK_4 introduction into the Cys or antigenic protein remains an inherent concern, hindering the preparation of homogeneous and structurally well-defined vaccine materials. However, this aspect can be addressed through the application of selective-biocompatible reactions and their combined use with genetic and protein engineering.[47] Actually, Guo *et al.* achieved the production of homogeneous Pam_3CSK_4-RBD conjugate using selective ligation reaction.[44] While further investigation is necessary, we believe that this study can offer an invaluable guiding principle for the advancement of protein-based self-adjuvanting vaccines: limiting the number of conjugated adjuvants to preserve antigenicity. Notably, the present vaccine candidates did not require the co-administration of additional adjuvants to elicit substantial antibody production. Such self-adjuvanting vaccines are envisaged to induce antigen-specific immune responses with minimal inflammatory repercussions, and are expected to contribute to the emergence of safer and more effective next-generation vaccine formulations.

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Foundation – Osaka University Project for Infectious Disease Prevention". Y. M., K. K., and K. F. conceived the study and designed the experiments. B. G. R. and K. I. performed synthesis and biological assays. Y. M. wrote the manuscript. R. H.-G. provided the RBD and deglyRBD. All the authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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