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Precise immunological evaluation rationalizes the design of a self-adjuvanting vaccine composed of glycan antigen, TLR1/2 ligand, and T-helper cell epitope[†]

 Tsung-Che Chang,^{‡,a} Yoshiyuki Manabe,^{‡,a} Keita Ito,^a Ryuku Yamamoto,^a Kazuya Kabayama,^{ab} Shino Ohshima,^c Yoshie Kametani,^{*c} Yukari Fujimoto,^{‡,d} Chun-Cheng Lin^{‡,e} and Koichi Fukase^{*ab}

Sialyl-Tn (STn), overexpressed on various tumors, has been investigated for its application in anti-cancer vaccine therapy. However, Theratope, an STn-based vaccine, failed in the phase III clinical trial due to poor immunogenicity and epitope suppression by the foreign carrier protein. We therefore developed a self-adjuvanting STn based-vaccine, a conjugate of clustered STn (triSTn) antigen, TLR1/2 ligand (Pam₃CSK₄), and T-helper (Th) cell epitope, and found that this three-component self-adjuvanting vaccine effectively resulted in the production of anti-triSTn IgG antibodies. We herein analyzed immune responses induced by this self-adjuvanting vaccine in detail. We newly synthesized two-component vaccines, *i.e.*, Pam₃CSK₄- or Th epitope-conjugated triSTn, as references to evaluate the immune-stimulating functions of Pam₃CSK₄ and Th epitope. Immunological evaluation of the synthesized vaccine candidates revealed that Pam₃CSK₄ was essential for antibody production, indicating that the uptake of triSTn antigen by antigen-presenting cells (APCs) was promoted by the recognition of Pam₃CSK₄ by TLR1/2. The function of the Th epitope was also confirmed. Th cell activation was important for boosting antibody production and IgG subclass switching. Furthermore, flow cytometric analyses of immune cells, including T cells, B cells, dendritic cells, and other monocytes, were first employed in the evaluation of self-adjuvanting vaccines and revealed that the three-component vaccine was able to induce antigen-specific immune responses for efficient antibody production without excessive inflammatory responses. Importantly, the co-administration of Freund's adjuvants was suggested to cause excessive myeloid cell accumulation and decreased plasma cell differentiation. These results demonstrate that vaccines can be designed to achieve the desired immune responses *via* the bottom-up construction of each immune element.

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Introduction

Tumor-associated carbohydrate antigens (TACAs),¹ which are overexpressed on tumor cells and rarely expressed on normal

cells, can be used for cancer vaccine therapy.² In fact, many vaccines have entered clinical trials, including phase III trials. However, no TACA-based cancer vaccine has been approved for clinical use yet. Since TACAs employed in clinical trials are small T cell-independent antigens, they exhibit poor immunogenicity and cannot induce immunoglobulin (Ig) class switching. Therefore, they have been conjugated with immunogenic carrier proteins, such as keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), or diphtheria toxin (CRM₁₉₇).³ Although these carrier proteins possess T cell epitopes and the conjugates can elicit a strong immune response, they can sometimes induce suppression of the target epitope-specific reaction, reducing their efficacy.^{4,5}

It has been necessary to control adverse reactions while ensuring efficacy for current vaccine development. Self-adjuvanting vaccines, the conjugates of antigens and immunoenhancers, are expected to induce antigen-specific immune

^aDepartment of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka, 560-0043, Japan. E-mail: Koichi@chem.sci.osaka-u.ac.jp

^bForefront Research Center, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

^cFaculty of Medicine, School of Medicine, Tokai University, 143 Shimokasuya, Isehara-shi, Kanagawa 259-1193, Japan

^dDepartment of Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan

^eDepartment of Chemistry, National Tsing Hua University, 101 Sec. 2, Kuang Fu Rd., Hsinchu 30013, Taiwan

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[‡] Equal contribution.



responses without excessive inflammation.^{6–8} In addition, they have well-defined structures and hence facilitate quality control of vaccine products.

In the self-adjuvanting vaccine strategy, T-helper (Th) cell epitopes and innate immune ligands are usually conjugated as immunoenhancers. Conjugation with Th epitopes can induce T cell-dependent immune responses, such as antigen-specific B cell activation and IgG class switching. Kunz *et al.* introduced various Th epitopes or multiple Th epitopes in MUC1 glycopeptide-based vaccines and evaluated their effects.^{9,10}

Innate immune ligands, especially Toll-like receptor (TLR) agonists, are promising adjuvants and have been applied to practical use.^{11–20} Since TLRs are expressed on antigen presenting cells (APCs) such as macrophages, B cells, and dendritic cells, the conjugation of antigens with TLR ligands is expected to promote antigen uptake by APCs and also to upregulate adoptive immune responses through the induction of costimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) responsible for T cell activation. The TLR family is divided into largely two groups, which contain extracellular and intracellular receptors.^{13,21–23} Especially, TLR-1, TLR-2 and TLR-6, which are of the former TLRs, tend to induce humoral immune responses, whereas intracellular TLRs enhance Th1 and cellular immune responses. Although cancer immunity has focused on cellular immunity, humoral immunity might exert strong effects to overcome the cancer if functional anti-cancer IgGs are extensively produced.

Extracellular TLR ligands, such as TLR2 ligands and TLR4 ligands have been mainly utilized to develop self-adjuvanting vaccines. Trumenba, a recombinant lipoprotein with a TLR2-stimulating unit, is an FDA-approved self-adjuvanting vaccine that is used against *Neisseria meningitidis* group B disease.²⁴ Actually, TLR2 agonists, including Pam₃CSK₄, have often been used in many self-adjuvanting vaccines considering their immunostimulation efficacy and ease of synthesis.^{25–33} Boons *et al.* reported that a tumor-associated MUC1 glycopeptide conjugated with TLR1/2 ligand (Pam₃CSK₄) and a Th epitope induced high IgG titers against the MUC1 antigen.^{25,26} We have also previously reported Pam₃CSK₄-conjugated self-adjuvanting vaccines.^{30–32} TLR4 ligands have also been used for self-adjuvanting vaccines. Guo *et al.* reported that self-adjuvanting vaccines containing the mild TLR4 agonist monophosphoryl lipid A elicit TACA-specific IgG antibodies without Th epitope conjugation.^{34–37} Codée *et al.* also developed a self-adjuvanting vaccine conjugated with CRX-527, a lipid A analogue.³⁸ The lipidic property of TLR2 and TLR4 ligands might also contribute to the adjuvant effects *via* the formation of aggregates, which enables multivalent interactions between the conjugated antigens and B cell receptors (BCRs) to promote BCR clustering. In fact, Kiessling *et al.* have revealed that polymeric multivalent ligands induce BCR clustering to activate B cell responses.³⁹ The importance of the lipid moiety for the self-adjuvanting property was also suggested based on the adjuvant effects in several TACA-based vaccines.^{33,40–42} Intracellular TLR ligands and other innate immune ligands have been also employed in self-adjuvanting vaccines. A TLR9 agonist (CpG-ODNs) was less effective for antibody production compared to Pam₃CSK₄.²⁶ A

self-adjuvanting vaccine using a CD1d ligand (α -galactosylceramide),^{43–47} a zwitterionic polysaccharide,^{48–50} and a TLR7 ligand⁵¹ have also been investigated.

Despite many reports of excellent self-adjuvanting vaccines, most previous studies have focused on the antibody titer and level of secreted cytokines to evaluate the efficacy of self-adjuvanting vaccines, especially TACA-based vaccines. Exceptionally, BenMohamed *et al.* reported the efficacy of synthetic conjugated vaccines composed of the clustered tetravalent Tn carbohydrate antigen with Th (CD4+) and Tc (CD8+) epitopes as an antitumor vaccine in a mouse tumor model, though they did not analyze the innate immune responses.⁵² To understand the high efficacy of self-adjuvanting vaccines with low inflammatory effects, the cellular responses should be investigated. However, the role and effect of each component in self-adjuvanting vaccines have not been investigated well at the cellular level. Hence, in this study, we evaluated the immunological responses of the three-component self-adjuvanting vaccine 1,³⁰ composed of trimeric Sialyl-Tn cluster (TACA), Pam₃CSK₄, and a Th cell epitope, in comparison to those with Pam₃CSK₄- or Th epitope-conjugated triSTn (vaccine 2 and 3, respectively; Fig. 1). We herein analyzed the production of antibodies, as well as the activation of various immune cells, including T cells, B cells, and dendritic cells, by flow cytometry with staining for each cell marker. These analyses provided clear evidence that the covalently linked three-component vaccine can efficiently induce specific immune responses without causing unnecessary inflammation. A combination of the TLR2 ligand and Th epitope was found to be essential for the activation of B cells and ACPs, as well as T cells. Namely, we herein demonstrated the immunological rationality of the design of three-component vaccines, offering essential information for their clinical development.

In general, glycoconjugate vaccines require the co-administration of adjuvants such as alum for clinical use and Freund's adjuvants for experimental use.^{31,53} Freund's adjuvants are often used in mouse experiments, but are not approved for use in humans and are not recommended for preclinical studies. Until now, conflicting results have been reported on the effects of co-administering additional adjuvants in the self-adjuvanting system, and no detailed immunological analyses have been performed. This study clearly revealed that the co-administration of Freund's adjuvants induced nonspecific immune responses to interfere with the antigen-selective immune responses, proposing guidelines that additional adjuvants should not be added to the self-adjuvanting vaccines.

Sialyl-Tn antigen [STn, NeuAc α (2,6)GalNAc α -O-Ser/Thr] is abundantly expressed on many epithelium-derived tumors (*e.g.*, breast, pancreas, prostate, lung, colorectal, gastric, and ovarian)^{54,55} and correlates with invasion and aggressive potential. Therefore, cancer vaccines targeting STn-related mucins have been developed and investigated over the past several decades.^{56–60} Theratope (STn-KLH conjugate) was developed as a therapeutic vaccine for the treatment of metastatic breast cancer.⁶¹ However, it failed in the phase III clinical trial because statistical significance was not observed in the extension of overall survival and disease progression time.^{62,63} *Post hoc*



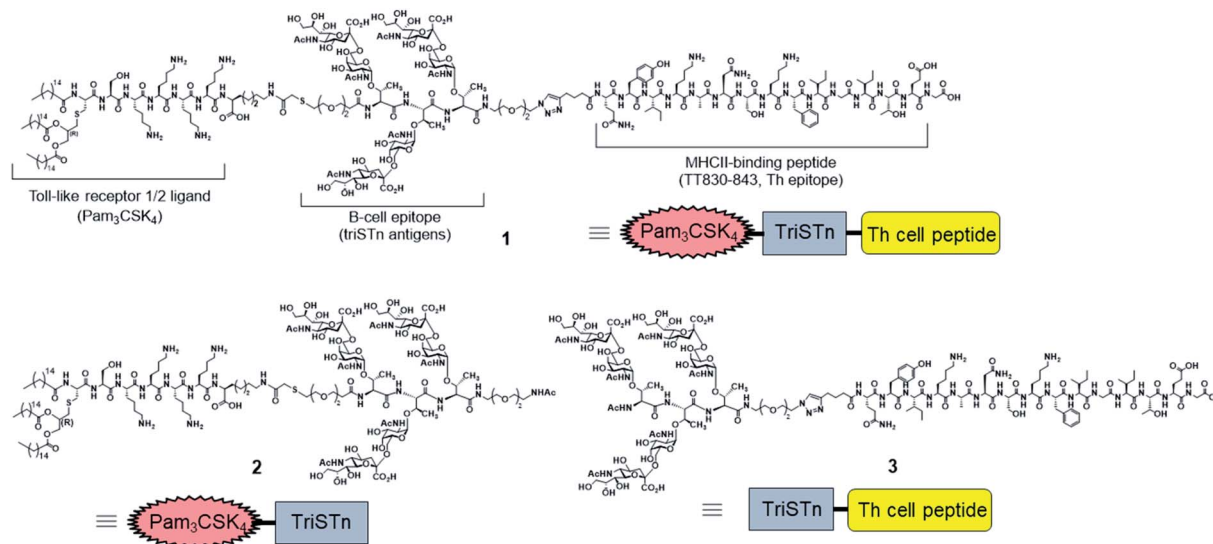


Fig. 1 The chemical structures of vaccine candidates 1–3. Vaccine candidate 1: three-component (triSTn, Pam₃CSK₄, and Th cell epitope); vaccine candidate 2: Pam₃CSK₄ conjugated triSTn; vaccine candidate 3: Th cell epitope conjugated triSTn.

analysis revealed that overall survival was significantly improved in patients exhibiting a high antibody titer against ovine submaxillary mucin (OSM), whereas the antibody titer against STn did not correlate with the overall survival. Since OSM multivalently expresses STn, the clustered STn seemed to be an appropriate antigen for vaccines against breast cancer. Moreover, the clustered STn structures are expressed on the mucins of most adenocarcinomas with high specificity.^{64–66} Therefore, anti-STn vaccines that can effectively induce immune responses against clustered STn are expected to effectively target cancer cells. The three-component self-adjuvanting vaccine 1, developed by our group, effectively produced IgG antibodies, which selectively recognized clustered STn (triSTn) over STn.³⁰ Thus, we herein analyzed immunological responses induced by 1 in detail to obtain the guidelines for efficient TACA-based cancer vaccine development.

Results and discussion

The vaccine candidates in this study and their chemical structures are presented in Table 1 and Fig. 1, respectively. Two-component conjugated vaccines, Pam₃CSK₄-triSTn 2 and triSTn-Th epitope 3, were newly synthesized to investigate the function of the TLR2 ligand and Th immunomodulator (Schemes S1–S3 and Fig. S1–S6†). The preparation of three-

component vaccine 1, composed of triSTn antigen, Pam₃CSK₄, and Th epitope, has been previously reported.³⁰ Compound 1 was used for immunization with or without Freund's adjuvants (V1' and V1, respectively), whereas compounds 2 and 3 were used for vaccination without Freund's adjuvants (V2 and V3).

Immunization was performed according to the standard protocol. Compounds 1–3 in phosphate-buffered saline (PBS) were intraperitoneally administered to 8 week-old wild-type BALB/c mice on day 1. The immunization schedule included the administration of three booster doses for each mouse on days 14, 28, and 42 *via* injection of the same vaccine materials. Regarding V1', the first immunization was performed with complete Freund's adjuvant, whereas the booster doses were with incomplete Freund's adjuvant. Blood was collected from each mouse before immunization on day 0 (blank controls) and 1 week after each immunization (days 8, 21, 35, and 49). The blood samples were used to prepare plasma *via* the standard method. TriSTn-, STn-, and linker (composed of PEG linker and tri Thr without STn glycan)-immobilized bovine serum albumin (BSA-TriSTn, BSA-STn, and BSA-linker, respectively)³⁰ were used to evaluate the antibody titers against each epitope by enzyme-linked immunosorbent assay (ELISA).

An estimation of antibody production after four doses clearly revealed the usefulness of the three-component vaccine 1 (Fig. 2a and b). In our previous report,³⁰ 1 induced robust immune responses, whereas the mixture of the respective components (STn antigen, Pam₃CSK₄, and Th epitope) did not induce significant antibody production (Fig. S7†), indicating the importance of covalent linkage for antigen-specific immune responses. Furthermore, the titer with V1 was slightly higher than that for V1' (Fig. S7†), suggesting that additional adjuvant administration did not improve antibody production. Moreover, the present study clearly demonstrates that robust immune responses to the three-component vaccine 1 require the conjugation of both Pam₃CSK₄ and Th epitope; the antibody

Table 1 Vaccine candidates for *in vivo* mouse immunization

Vaccine candidate	Immunization compounds
V1	Pam ₃ CSK ₄ -triSTn-Th epitope 1
V1'	Pam ₃ CSK ₄ -triSTn-Th epitope 1 + Freund's adjuvant
V2	Pam ₃ CSK ₄ -triSTn 2
V3	triSTn-Th epitope 3



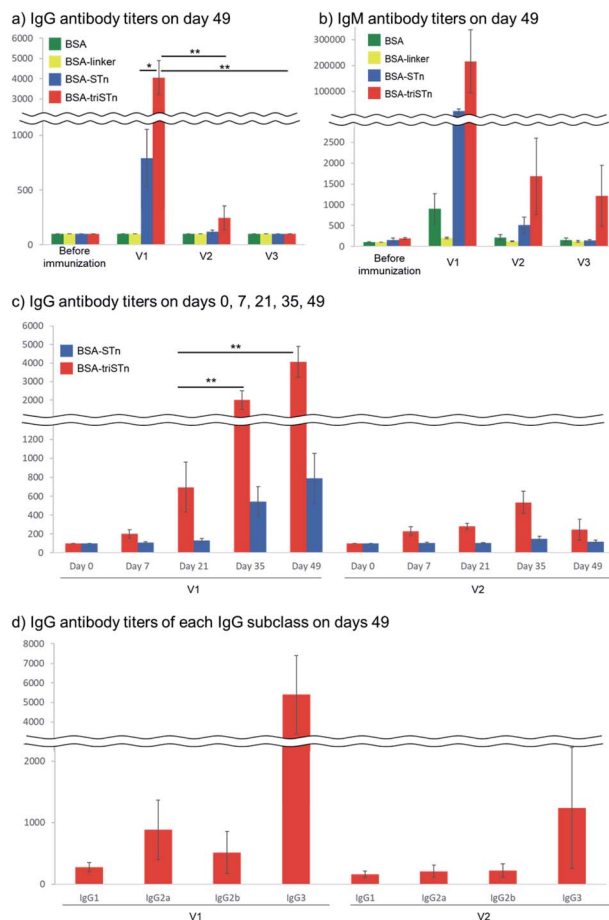


Fig. 2 Antibody titers induced by V1–V3. a and b) IgG and IgM antibody titers on day 49 induced by V1, V2, or V3. c) IgG antibody titers on days 0, 7, 21, 35, and 49 induced by V1 or V2. d) IgG antibody titers against tri-STn for each IgG subclass on day 49 induced by V1 or V2. ELISA plates were coated with BSA-triSTn to measure the anti-triSTn antibody titers and BSA-STn to measure the anti-STn antibody titers. *: $p < 0.05$, **: $p < 0.01$.

titers for the two-component vaccine 2, Pam₃CSK₄-triSTn conjugate, were much lower than those induced by the three-component vaccine 1, whereas vaccine 3, Th epitope-triSTn conjugate, did not induce IgG production. V2 was able to induce a small amount of antibodies, whereas V3 was not, indicating that Pam₃CSK₄ was essential for antibody production. Probably, promotion of antigen uptake through the recognition by TLR is required for the initiation of immune responses.⁶⁷ It should also be mentioned that V1 produced antibodies that selectively recognize tri-STn over STn, as expected.

Next, we investigated the booster effect of vaccine candidates V1 and V2 (Fig. 2c). V1 resulted in a steady increase in IgG titers from day 0 to day 49, showing a significant booster effect as observed in the previous report of V1' (Fig. S8†). Conversely, V2 did not show any booster effect; IgG titers induced by V2 did not change from day 7 to 49. These results indicated that the Th epitope is necessary for Th cell activation to induce robust immune responses. In addition, memory B cells with highly

specific IgG expression might not be induced without Th epitope stimulation, and repetitive naïve B cell activation might result in the poor production of highly specific antibodies. This tendency was also observed for IgM antibody production (Fig. S9†), demonstrating that the Th epitope plays an important role in boosting IgM antibody production, as well as in IgM to IgG class switching.^{9,10}

The Th-epitope also influenced the produced IgG subclasses (Fig. 2d and S10†). The IgG3 isotype of anti-triSTn antibodies was observed as a major subclass with V1, V1', and V2 vaccinations. IgG2a and IgG2b antibodies were significantly induced by V1 and V1'. Mouse IgG3 can be produced against thymus-independent antigens such as bacterial carbohydrates without T cell assistance.^{68,69} Thus, IgG2a and IgG2b antibody production with V1 and V1' suggested that the three-component vaccine 1 induced adaptive T cell-mediated immunity and IgG subclass switching.

To evaluate the detailed immune responses induced by each vaccine candidate, immune cells from the spleen of each mouse collected on day 49 were analyzed by flow cytometry (Fig. 3). In addition, immune responses of non-vaccine treated mice (NT) were also analyzed as a control. Size-fractionation and staining with antibodies against the corresponding immune cell markers provided information on the activation and proportion of respective cells.

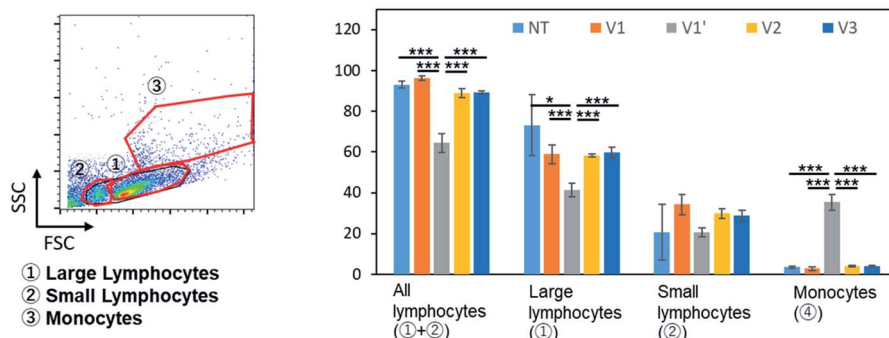
The number of spleen cells was first counted for each mouse. The spleen cells were divided into large lymphocytes, small lymphocytes, and monocytes based on forward scatter (FSC) and side scatter (SSC) (Fig. 3a). The co-administration of Freund's adjuvants increased the total number of immune cells (Fig. S28†), especially the number of monocytes, resulting in a highly skewed monocyte/lymphocyte ratio (average monocyte ratio, NT: 3.5%, V1: 2.8%, V1': 35.2%, V2: 4.1%, V3: 4.0%, *cf.* usual: less than 10%).

The increased expression of CD80, an activation marker of APCs, including dendritic cells and macrophages, with the three-component vaccine 1 was confirmed (Fig. 3b and c). V1 resulted in a higher mean fluorescence intensity (MFI) for CD80 than the other candidates. It is well-known that TLR2 ligands upregulate the expression of CD80. The present result indicates that the co-activation of TLR2 and MHC class II efficiently promotes CD80 expression, which is responsible for the subsequent T cell activation.

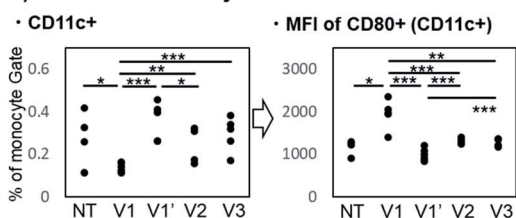
Although the number of dendritic cells and other monocytes (involving macrophages), stained with antibodies against CD11c and CD11b, respectively, were increased with the co-administration of Freund's adjuvants (V1'), CD80 expression decreased on these cells. Namely, the co-administration of Freund's adjuvants increased the number of myeloid-gated cells, but the co-receptor (CD80) expression on these cells was suppressed in the spleen. These results indicated that Freund's adjuvants might induce monocyte accumulation in the spleen and consequently suppress their activation. The development of suppressive cells, such as myeloid-derived suppressor cells (MDSCs), might be enhanced by the excess stimulation by Freund's adjuvants.⁷⁰



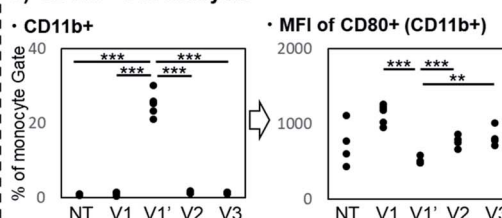
a) Gate for each cell and the ratio of each cell



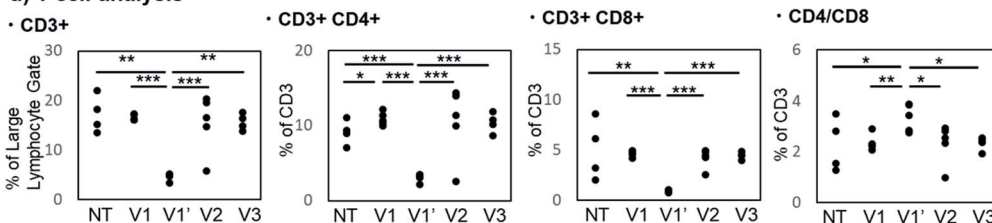
b) Dendritic cell analysis



c) CD11b+ cell analysis



d) T cell analysis



e) B cell analysis

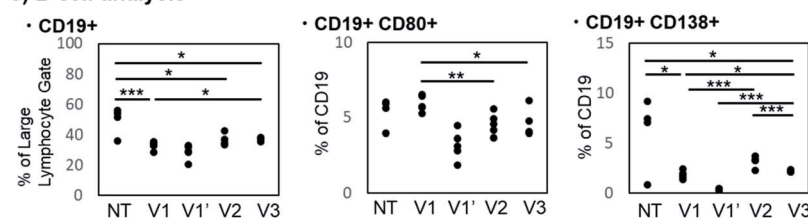
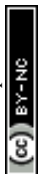


Fig. 3 Flow cytometric analyses of immune cells of the immunized mice. a) Cell classification based on forward scatter (FSC) and side scatter (SSC) and the ratio of each cell type (lymphocyte, large lymphocyte, small lymphocyte, and monocyte). Small lymphocytes are observed after freezing of the cells. Most of live cells are involved in the large gate. b) Dendritic cell analysis. Monocytes stained with an antibody against a dendritic cell marker (CD11c) were analyzed. CD11c: dendritic cell marker, CD80: activation marker of APCs. c) CD11b+ cell analysis. Monocytes stained with an antibody against a macrophage marker (CD11b) were analyzed. CD11b: macrophage marker, CD80: activation marker of APCs. d) T cell analysis. Large lymphocytes stained with an antibody against a T cell marker (CD3) were analyzed. CD3: T cell marker, CD4: Th cell marker, CD8: Tc cell marker. e) B cell analysis. Large lymphocytes stained with an antibody against a B cell marker (CD19) were analyzed. CD19: B cell marker, CD80: activated B cell marker, CD138: plasma/plasmablast marker. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

T cell analysis revealed that V1 activated T cells without affecting the Th/Tc balance. Spleen cells of immunized mice were stained with antibodies against a T cell marker (CD3) and differentiated cell markers [CD4: helper T (Th) cell marker, CD8: cytotoxic T (Tc) cell marker] (Fig. 3d). For all candidates, the Th(CD4+)/Tc(CD8+) ratio was almost the same as that for non-immunized BALB/c mice except with V1'; the CD4+/CD8+ ratios for NT, V1, V1', V2, and V3 were 1.8, 2.3, 3.3, 2.5, and 2.3, respectively, whereas that of nonimmunized BALB/c mice was

reported to be approximately 2.3.^{71,72} The IgG titer was significantly higher with V1 than with V2 (Fig. 2a), clearly indicating that the conjugated Th epitope was functional. However, the total Th cell number and ratio were not remarkably increased by the conjugation with Th epitope (V1 and V3), suggesting only the selective activation of specific Th cells by V1 and V3. Considering that Tc responses are also important for anti-tumor immunity, the induction of Th responses without affecting the Th/Tc balance is desirable. In contrast, the co-



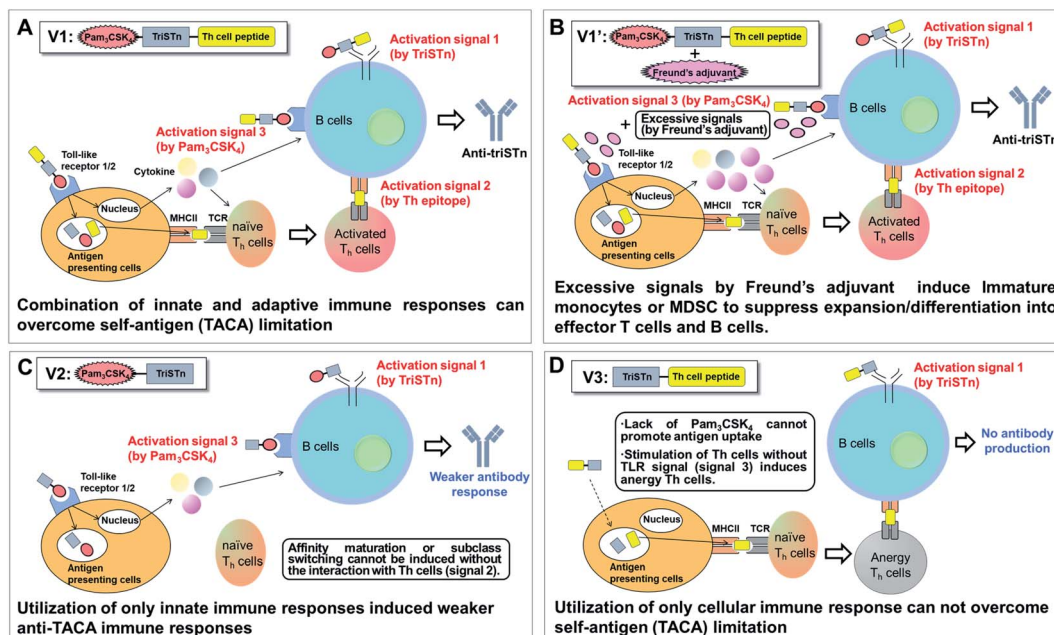


Fig. 4 Function of each component in each vaccine candidate (A: V1, B: V1', C: V2, D: V3).

administration of Freund's adjuvants (V1') decreased the proportion of activated T cells (both Th and Tc), indicating that the co-administration of additional adjuvants like Freund's adjuvant should be avoided for self-adjuncting vaccines.

B cell analysis also suggested that V1 can effectively stimulate the immune system to induce antibody production (Fig. 3e). The three conjugated vaccines 1, 2, and 3 did not significantly affect the number of B cells, which were stained with the antibody against a B cell marker (CD19). Meanwhile, V1, the three-component vaccine 1 administered without Freund's adjuvants, resulted in the highest ratio of B cells expressing an activated B cell marker (CD80). V1 exhibited higher differentiating activity than V2, whereas both V1 and V2 may directly stimulate B cells *via* TLR2,⁷³ suggesting that B cells need to be activated by an interaction between B cells and T cells *via* presentation of the Th epitope by MHC class II to the T cell receptor, as well as the recognition of co-stimulatory molecules such as CD80 on B cells by CD28 on T cells.

In contrast, V1' showed a significantly lower ratio of CD80+ B cells, suggesting that the excessive immune responses induced by Freund's adjuvants might suppress B cell activation. In addition, the proportion and number of plasma/plasmablasts, which are involved in antibody production, stained with an antibody against CD138, were decreased with the co-administration of Freund's adjuvants (average plasma/plasmablast ratio, NT: 6.1, V1: 1.8, V1': 0.3, V2: 3.2, and V3: 2.2). These results also suggest that the co-administration of additional Freund's adjuvants with self-adjuncting vaccines is not desirable.

Li *et al.* also reported that the co-administration of Freund's adjuvant with self-adjuncting vaccines reduced antibody production, though they did not analyze the cellular responses.^{28,74} In contrast, Toth *et al.* reported that

coadministration of Freund's adjuvant enhanced the antibody production by self-adjuncting vaccines.⁴¹ However, since they used a weak TLR2 ligand, it is likely that additional stimulation with Freund's adjuvant resulted in antibody production. Our results indicate that the co-administration of additional adjuvants to self-adjuncting vaccines should be avoided because it elicits non-specific inflammatory responses.

Conclusions

In conclusion, our analyses revealed that the three-component vaccine 1 induced robust immunity toward triSTn antigen as designed. Since vaccine 1 induced innate and adaptive immunity to effectively activate T cells, B cells, dendritic cells, and macrophages, V1 overcame the self-antigen (TACA) limitation (Fig. 4A). B cells are synergistically stimulated *via* direct interactions with V1 (activation signal 1), the interaction with T cells activated by dendritic cells (activation signal 2), and cytokines induced by a TLR agonist (activation signal 3) to efficiently achieve affinity maturation and class switching of anti-triSTn antibodies.⁷⁵ Conjugation with not only TLR ligand but also Th epitope has already been reported to be required to overcome the poor immunogenicity of T cell-independent TACA.^{33,41,42} Our analysis revealed that Th epitope and TLR ligand conjugation are essential for the activation of B cells and APCs, as well as T cells, demonstrating the immunological rationality of the design of three-component vaccines. Of course, the importance of covalent linkage between the triSTn antigen, Pam₃CSK₄, and Th epitope should be emphasized to obtain antigen-specific immune responses.

Importantly, the co-administration of Freund's adjuvants with vaccine 1 was suggested to cause splenomegaly, containing increased number of myeloid cells, which might include



immature monocytes or MDSCs, to suppress expansion/differentiation into effector T cells and B cells (Fig. 4B).^{76,77} This study demonstrated that the co-administration of an additional adjuvant induces nonspecific immune responses to interfere with the antigen-selective immune responses.

In contrast, the Pam₃CSK₄-triSTn conjugate V2 produced a weaker anti-triSTn humoral immune response than V1 (Fig. 4C). Although V2 can stimulate immune cells through TLR1/2 to produce anti-triSTn antibodies (activation signal 1 and 3), it cannot induce T cell–B cell interactions *via* the TCR, MHC, and antigen peptides due to the absence of a Th epitope and cannot promote the affinity maturation and class switching.

In spite of the trimeric presentation of STn antigen and conjugation with Th epitope, V3 did not trigger the production of any anti-triSTn IgG antibodies partly because of the small molecular weight of the glycopeptide (Fig. 4D). Lipophilic Pam3CSK4-conjugated vaccines tend to form supramolecular aggregates, which function as carriers.^{31,32} Kunz and Li *et al.* previously reported that self-adjuvanting vaccines composed of MUC1 glycopeptides and a T-cell epitope with Freund's adjuvants effectively induced antibodies against MUC1 glycopeptides, probably the molecular sizes of the vaccines were large enough for the antibody production.⁹ However, addition of Freund's adjuvant to V3 may not improve its immunogenicity because of its low molecular weight. Promotion of antigen uptake to APCs through the recognition by TLR might be essential step to initiate immune responses. The interaction between T cells and APCs without TLR stimulation might also induce the anergy of T cells due to the insufficient expression of MHC II and B7.⁷⁸ Hence, the intrinsic limitation of TACAs, self-antigens, probably cannot be overcome only by the involvement of a Th epitope.

Overall, our results indicate that the activation of both innate and adaptive immune responses is crucial in the design of fully synthetic self-adjuvanting TACA-based anti-cancer vaccines. Our results demonstrate that vaccines can be designed to achieve the desired immune responses through bottom-up construction of the necessary immune elements.

Data availability

We have provided all the necessary data in the ESI.†

Author contributions

T-C. C. synthesized all compounds employed in this study. T-C. C., K. I. and R. Y. performed ELISA assay. S. O. and Y. K. performed *in vivo* vaccination and flowcytometry analysis. Y. M., K. K., Y. K., Y. F., C-C. L., and K. F. designed the experiments. T-C. C., Y. M., Y. K., and K. F. mainly wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

There are no conflicts to declare.

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