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Synthesis and immunological evaluation of Mincle ligands-based antitumor vaccines

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ABSTRACT

The development of novel adjuvants constitutes a new strategy for the research of tumor vaccines. Immunomodulatory molecule adjuvants are one of the novel adjuvants that can effectively stimulate the pattern recognition receptors to activate the downstream pathways of immune cells. However, there are few studies on immunomodulatory molecular adjuvants associated with C-type lectin. It has been reported that $GlcC_{14}C_{18}$ is a Mincle ligand with a relatively simple structure and strong adjuvant activity *in vivo*. Herein, we coupled $GlcC_{14}C_{18}$ with MUC1 glycopeptide and evaluated its immune effect. In addition, we also synthesized α-$GlcC_{14}C_{18}$-MUC1 and β-$GlcC_{14}C_{18}$-MUC1 based on the two configurations of $GlcC_1 4C_{18}$ and compared their immune effects. The results show that both of the two configurations of the vaccine have a good immune effect, but to a certain extent, the immune effect of β-$GlcC_{14}C_{18}$-MUC1 is better than that of α-$GlcC_{14}C_{18}$-MUC1.

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Tumor immunotherapy has become a new method of tumor treatment. Its main principle is to enhance the body's adaptive immune response by promoting the ability of immune cells to recognize specific antigens on the surface of tumor cells [1,2]. Tumor antigen vaccines, mainly targets tumor-specific antigens, are important to tumor immunotherapy [3–5]. However, tumor antigens have the disadvantage of weak immunogenicity, leading to huge difficulties to the design of tumor antigen vaccines [6]. Immune adjuvants play an important role in improving the immunogenicity of tumor antigens and activating the body's adaptive immune response. Therefore, it provides a new strategy to solve this problem [7].

Adjuvant is a non-specific immune enhancer that can enhance the body's immune response to the antigen or change the type of response when used before or in combination with an antigen [8]. The continuous findings of new vaccines in recent years has also led to the development of new adjuvants, which can be divided into three categories: immunomodulatory molecule adjuvants, delivery adjuvants and combination adjuvants [9–11]. Immunomodulatory molecular adjuvants can stimulate pattern recognition receptors (PRR) on the surface of immune cells, and secrete cytokines by directly activating downstream pathways [12,13]. The C-type lectin receptor (CLR), a member of the PRR family, can recognize certain glycosylated or non-glycosylated targets and cause immune response [14–16].

Lectin is a protein that can bind carbohydrates, while C-type lectins are a special subfamily of lectins that are mainly decoupled from sugar groups by Ca^{2+} [17]. Mincle is a macrophage-inducible C-type lectin, which cannot transduce activation signals by itself like many other activation receptors. The presence of arginine with positive charges in the transmembrane region allows it to interact with the adaptor protein containing the Fc receptor γ (FcRγ) chain based on the activation motif of immunoreceptor tyrosine (ITAM) [18–20]. The mycobacterial cell wall is the main source of Mincle ligands. Ishikawa *et al.* demonstrated that Mincle could recognize mycobacteria by using the nuclear factor of green fluorescent protein (GFP) receptor cells driven by activated T cells. Then it was found that Trehalose-6,6′-dimycolate (TDM) was the direct ligand of Mincle in Mycobacterium tuberculosis by high-performance thin-layer chromatography (HPTLC) for lipid extraction and isolation [21]. After that, extensive research on TDM was carried out, and the trehalose dibehenate (TDB) was synthesized organically through structure analysis and restructure. TDM

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and TDB are considered to be two of the most biologically active components in *Mycobacterium tuberculosis* and have adjuvant activity [22–24]. Alexiane *et al.* took a further investigation on Mincle and its various ligands and found that macrophages showed a bias towards TLR2 and Mincle signals. They also proved that the simplest structure of Mincle ligands, $GlcC_{14}C_{18}$, showed a stronger immune boosting effect and lower toxicity than TDB [25].

There are many kinds of tumor cell surface antigens, among which MUC1 glycoprotein has attracted much attention in recent years and is considered to be one of the most promising targets in tumor immunotherapy [26]. MUC1 is a transmembrane macromolecular glycoprotein, which has a wide range of tissue distribution and is expressed on the surface of epithelial cells in most secretory organs such as breast, respiratory tract, gastrointestinal tract, urinary tract and reproductive tract [27–29]. MUC1 glycoprotein contains two subunits: the first is the N-terminal domain outside the cells, and its core protein contains a large number of variable number of tandem repeats (VNTR). Each VNTR consists of 20 amino acids, of which five amino acids is a potential glycosylation site (two threonines and three serines). In normal cells, the immunogenic epitopes in the VNTR region are widely covered by carbohydrate chain, while in cancer cells, they are exposed due to low levels of glycosylation. The second subunit is the C-terminal domain within the cell, which plays an important role in signal transduction. There are several phosphorylated sites in the cytoplasmic tail region that can interact with signal transduction proteins [27,30–32]. MUC1 expressed on healthy epithelial cells has a certain lubricating effect, and at the same time it is also a physical barrier that can resist external pathogens and protect epithelial cells from harm [33]. In addition to these basic functions, MUC1 can mediate the secretion of cytokines to activate cellular pathways, thereby allowing tumor cells to proliferate. It can also induce the expression of pro-angiogenic factors to promote tumor angiogenesis and promote tumor proliferation anti-inflammatory effect [34,35]. Because of its close relationship with tumors, MUC1 is a promising target for tumor immunotherapy [36,37]. However, the immunogenicity of natural glycopeptides associated with tumors is very low, which makes it difficult to achieve efficient immune effect [38]. A successful tumor vaccine can activate multiple branches of the immune system at the same time [39]. In order to overcome the shortcoming of natural glycopeptides, synthetic glycopeptide vaccines remain to be investigated for the development of MUC1 vaccines [40,41]. To improve the immune effect of MUC1-based synthetic vaccines, immune stimulatory components need to be induced. MUC1, as a B cell epitope, can activate B cells to produce a small amount of antibodies. Mincle ligands can effectively stimulate dendritic cells (DCs) and macrophages to targeted transport MUC1 to APCs, thereby promoting MUC1 antigen uptake and presentation, which result in an immune response and immune memory against MUC1 *in vivo*.

In this work, we used $GlcC_{14}C_{18}$, which has a simpler structure and lower toxicity among Mincle ligands, as an adjuvant. We coupled it with the tumor antigen MUC1 to obtain a glycopeptide antitumor vaccine against MUC1. Because $GlcC_{14}C_{18}$ has two stereo configurations, α and β, we obtained single-configuration α-$GlcC_{14}C_{18}$ and β-$GlcC_{14}C_{18}$ through two different synthetic routes, and then constructed α-$GlcC_{14}C_{18}$-MUC1 and β-$GlcC_{14}C_{18}$-MUC1 polypeptide as two vaccines through peptide solid-phase synthesis (Fig. 1). We compared the ability of the two stereoisomeric vaccines to induce tumor antigen-specific antibodies and cellular immune responses and found that β-$GlcC_{14}C_{18}$-MUC1 induced stronger immune responses *in vivo*. Our study provides a theoretical support that the immunostimulatory activity induced by adjuvant-receptor binding depends not only on the specific chemical structure of the adjuvant molecule, but also on the spatial complementarity of the structure and its receptor.

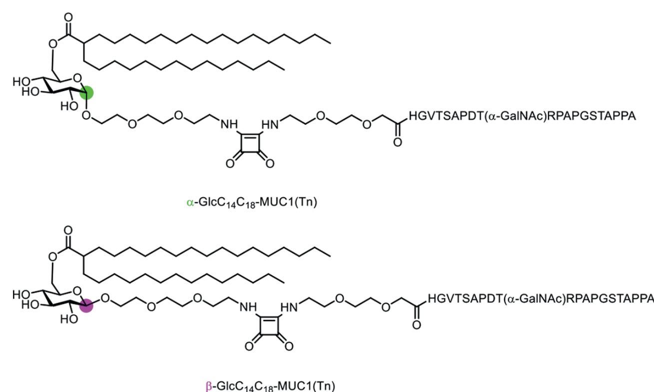

Fig. 1. Structure of α-$GlcC_{14}C_{18}$-MUC1 and β-$GlcC_{14}C_{18}$-MUC1.

Proinflammatory cytokines are linked to disease behaviors, which is related to immune response and tissue damage caused by malignant tumors [42]. In order to compare the immune-enhancing effects of Mincle ligands, two stereo structures were prepared, then we examined the ability of α-$GlcC_{14}C_{18}$ and β-$GlcC_{14}C_{18}$ to induce pro-inflammatory cytokine production in macrophages *in vitro*. We obtained peritoneal macrophages from C57BL/6 mice. After incubation with two compounds for 2 h respectively, the cytokines of IL-6, IL-12 and TNF-α were detected by ELISA. As shown in Fig. 2, the Mincle ligands can effectively stimulate macrophages to secrete IL-6, IL-12 and TNF-α. Moreover, β-$GlcC_{14}C_{18}$ induces macrophages to produce higher pro-inflammatory cytokines than that of α-$GlcC_{14}C_{18}$ *in vitro*, which means β-$GlcC_{14}C_{18}$ has a stronger immune-enhancing effects than α-$GlcC_{14}C_{18}$. The effect of immune stimulation varied with the configuration of $GlcC_{14}C_{18}$, which laid a foundation for the subsequent comparison of the immune-enhancement effect of vaccines formed by coupling with MUC1.

After understanding the immune-enhancing effect of two Mincle ligands, we constructed α-$GlcC_{14}C_{18}$-MUC1 and β-$GlcC_{14}C_{18}$-MUC1 vaccines using MUC1 as antigen through peptide solid-phase synthesis (Schemes S1 and S2 in Supporting information). We found that the immune effect of covalently linked $GlcC_{14}C_{18}$ and MUC1 was stronger than that of physical mixture. Therefore, in the subsequent experiments, we only compared the immune effect of the covalently linked $GlcC_{14}C_{18}$ and MUC1 (Figs. S1 and S2 in Supporting information). In order to further verify the *in vivo* immune response of the two vaccines α-$GlcC_{14}C_{18}$-MUC1 and β-$GlcC_{14}C_{18}$-MUC1, we conducted *in vivo* immunization experiments with C57BL/6 mice. Three groups of female C57BL/6 mice were injected with α-$GlcC_{14}C_{18}$-MUC1, β-$GlcC_{14}C_{18}$-MUC1 and PBS by intraperitoneal injection, once every two weeks for a total of 4 times.

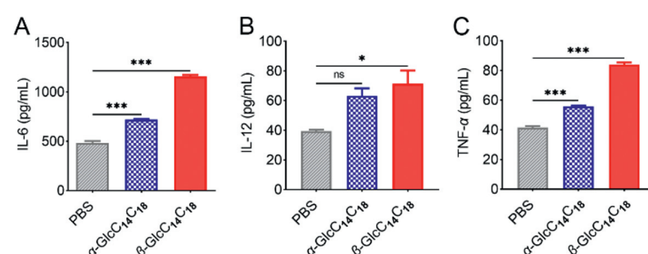

Fig. 2. The production of pro-inflammatory cytokines (IL-6 (A), IL-12 (B) and TNF-α (C)) from mouse peritoneal macrophages stimulated by α-$GlcC_{14}C_{18}$ and β-$GlcC_{14}C_{18}$. Three pro-inflammatory cytokines of the cell medium supernatant of mouse peritoneal macrophages incubated with α-$GlcC_{14}C_{18}$ and β-$GlcC_{14}C_{18}$ were analyzed by ELISA. Data are shown as mean ± SD. Differences are determined by one-way ANOVA analysis, ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

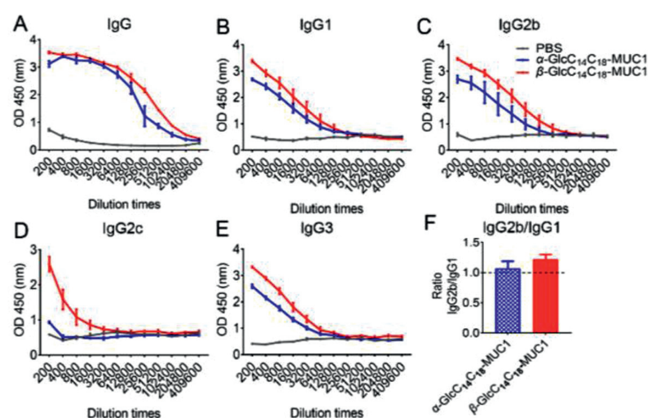

Fig. 3. Analysis of antibody titers and antibody subtypes induced by the vaccine candidates. (A) The antibody titers of the mouse serum induced by α-Glc$C_{14}C_{18}$-MUC1 and β-Glc$C_{14}C_{18}$-MUC1 were detected by ELISA. Data are shown as mean ± SD. The antibody subtypes IgG1 (B), IgG2b (C), IgG2c (D) and IgG3 (E) of the mouse serum induced by α-Glc$C_{14}C_{18}$-MUC1 and β-Glc$C_{14}C_{18}$-MUC1 were detected by ELISA. The plates were coated by MUC1 (Tn). (F) The ratio of IgG2b/IgG1. Data are shown as mean ± SD.

One week after the last injection, the antiserum and spleens of the mice were collected.

The IgG antibody titers were detected by ELISA. As shown in Fig. 3A, both α-Glc$C_{14}C_{18}$-MUC1 and β-Glc$C_{14}C_{18}$-MUC1 can effectively respond to the MUC1 antigen, which indicated Glc$C_{14}C_{18}$ serve as a vaccine adjuvant to enhance immunogenicity of MUC1. Compared with α-Glc$C_{14}C_{18}$-MUC1, β-Glc$C_{14}C_{18}$-MUC1 generated a higher IgG titer.

To investigate the immune characteristics of this vaccine further, we conducted the antibody subtypes experiment by ELISA. As shown in Figs. 3B-E, β-Glc$C_{14}C_{18}$-MUC1 increased the production of IgG1, IgG2b, IgG2c and IgG3 than α-Glc$C_{14}C_{18}$-MUC1, which was similar as the result of IgG titer. IgG1 subtype is related to Th2-biased immune response, while IgG2b is related to Th1-biased immune response. The ratio of IgG2b to IgG1 can well show the type of immune response induced by the vaccine. The results showed that the ratio of IgG2b to IgG1 is greater than 1 (Fig. 3F), indicating that the type of immune response induced by the vaccine may be more prone to Th1-biased immune response.

To further evaluate the type of immune response induced by the vaccine, we tested the cytokines secreted by splenocytes. The spleens collected from the mice after 4 times immunizations were prepared into single cell suspentions, and then incubated with MUC1 peptide. After a period of time, the cell supernatant was collected. The cytokines of IL-4, IL-6, IL-12 and IFN-γ were detected by ELISA kit. As we all know, the naïve $CD4^+$ T cells will differentiate into effector T-helper (Th) cells under the action of different cytokines secreted by DCs. The cytokines secreted by different types of helper T cells also play an important role in immune regulation. Th1 cells can secrete IL-12 and IFN-γ cytokines, while Th2 cells can secrete IL-4 and IL-6 cytokines. As shown in Figs. 4B-E, the amount of cytokines secreted by the splenocytes of the mice injected with the β-Glc$C_{14}C_{18}$-MUC1 vaccine was higher than that of α-Glc$C_{14}C_{18}$-MUC1 vaccine. Among the secreted cytokines, the highest amount of IFN-γ was detected, followed by IL-12, and the amount of cytokines secreted by Th1 type cells is higher than that of Th2 type cells. Therefore, it can be speculated that the main type of immune response induced by the vaccine may be Th1 type immune response, which is consistent with the results of antibody subtypes.

Cytotoxic T cells can eliminate tumor cells by releasing killing factors. In order to evaluate the killing effect of cytotoxic T cells on tumor cells induced by vaccines, CTL experiments were conducted.

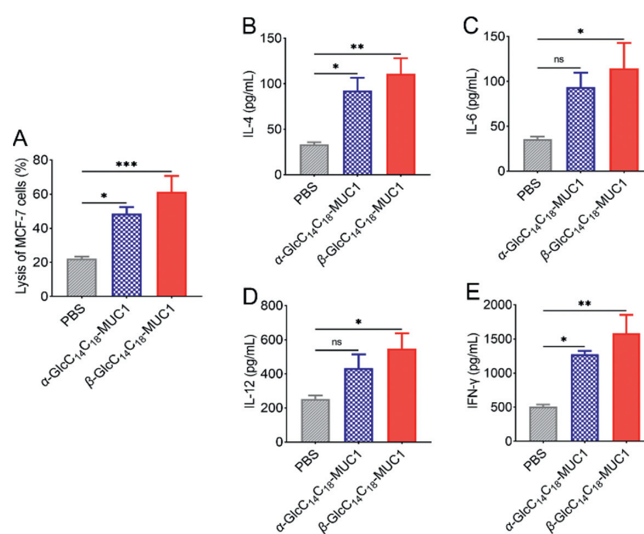

Fig. 4. Analysis of the CTL cytotoxicity to MCF-7 cells (A). The splenocytes collected from the mice immunized with vaccine candidates were used as effector cells and MCF-7 cells as target cells, the cells were co-incubated at the ratio of 50:1. The secretion of cytokines (IL-4 (B), IL-6 (C), IL-12 (D) and IFN-γ (E)) by mouse splenocytes stimulated by MUC1. The cytokines from the cell medium supernatant of mouse splenocytes incubated with MUC1 were analyzed by ELISA. Data are shown as mean ± SD. Differences among groups are determined by one-way ANOVA analysis, ns = not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The collected mouse splenocytes used as effector cells and MCF-7 as target cells were co-incubated at a cell number ratio of 50:1, and then detected by a microplate reader. As shown in Fig. 4A, the splenocytes of mice immunized with α-Glc$C_{14}C_{18}$-MUC1 and β-Glc$C_{14}C_{18}$-MUC1 have a killing rate of 47% and 60%, respectively. So, the splenocytes of the mice immunized with the two vaccines have a certain killing rate on the tumor cells. To a certain extent, the ability of the β-Glc$C_{14}C_{18}$-MUC1 vaccine may be slightly stronger than α-Glc$C_{14}C_{18}$-MUC1.

Then, for measure the binding properties of the antiserum with tumor cells, we used flow cytometry and laser scanning confocal microscope. The MCF-7 cells with high expression of MUC1 were incubated with the collected antisera. After a period of time, goat anti-mouse IgG conjugated with the FITC fluorescence channel was added for co-incubation, and the cell surface fluorescence was detected by flow cytometry and laser scanning confocal microscope. As shown in Figs. 5A and B, the antisera induced by the two vaccines can effectively bind to tumor cell surface antigens, and the antisera induced by β-Glc$C_{14}C_{18}$-MUC1 has better binding affinity than the antisera induced by α-Glc$C_{14}C_{18}$-MUC1. Therefore, the antiserum induced by β-Glc$C_{14}C_{18}$-MUC1 has stronger binding capacity than that of α-Glc$C_{14}C_{18}$-MUC1.

Complement can be combined with antibodies which bind to antigens to activate the complement system, thereby destroying target cells. We further completed the complement-dependent cytotoxicity (CDC). The antisera induced by the two vaccines were incubated with MCF-7 cells, and rabbit complement was added. Then the cells were incubated overnight at 37 °C, and CCK-8 reagent was added for detection with a microplate reader. As shown in Fig. 5C, the antisera induced by the two vaccines of α-Glc$C_{14}C1_{8}$-MUC1 and β-Glc$C_{14}C_{18}$-MUC1 had 21% and 31% lethality against tumor cells, respectively. β-Glc$C_{14}C_{18}$-MUC1 showed the stronger tumor cell killing rate than α-Glc$C_{14}C_{18}$-MUC1, so β-Glc$C_{14}C_{18}$-MUC1 could produce stronger complement dependent cytotoxicity.

To test the anti-tumor therapeutic effect of the vaccine *in vivo*, B16-MUC1 cells were embedded into the subcutaneous area of the right hind leg of female C57BL/6 mice. After a period of time, when the tumor diameter was about 5 mm, the mice were divided into

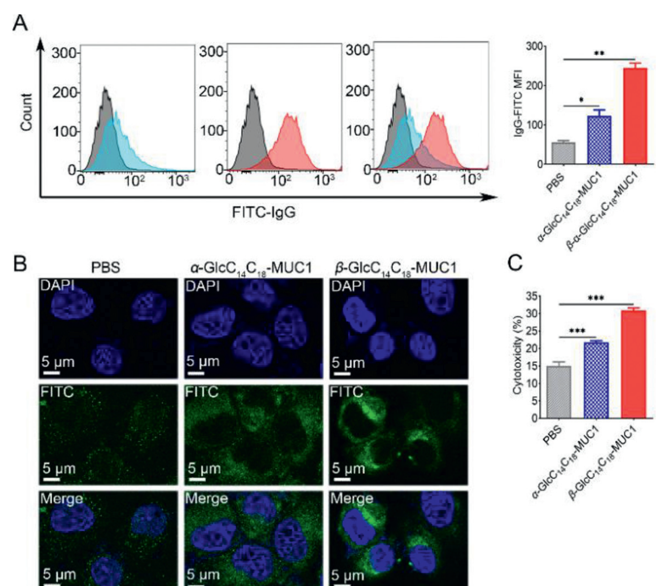

Fig. 5. Analysis of the effect of antibodies induced by α-GlcC$_{14}$C$_{18}$-MUC1 and β-GlcC$_{14}$C$_{18}$-MUC1 vaccine candidates. (A) FACS detects the binding ability of the MCF-7 cells with antiserum induced from α-GlcC$_{14}$C$_{18}$-MUC1 (blue), β-GlcC$_{14}$C$_{18}$-MUC1 (red) and PBS (black). The antiserum derived from mice immunized with PBS (black) was used as a negative control. (B) Laser confocal microscopy detects the ability of binding between the MCF-7 cells and antiserum. FITC-labeled anti-mouse IgG was used to label antiserum (green), the nucleus was stained with DAPI (blue). The scale bar was 5 µm. (C) Complement dependent cytotoxicity analyzed by the CCK-8 method. MCF-7 cells (5×10^3) incubated with 1:10 dilution of antiserum collected from vaccine candidates, then CCK-8 method was used to measure. Data are shown as mean ± SD. Differences among groups are determined by one-way ANOVA analysis, ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

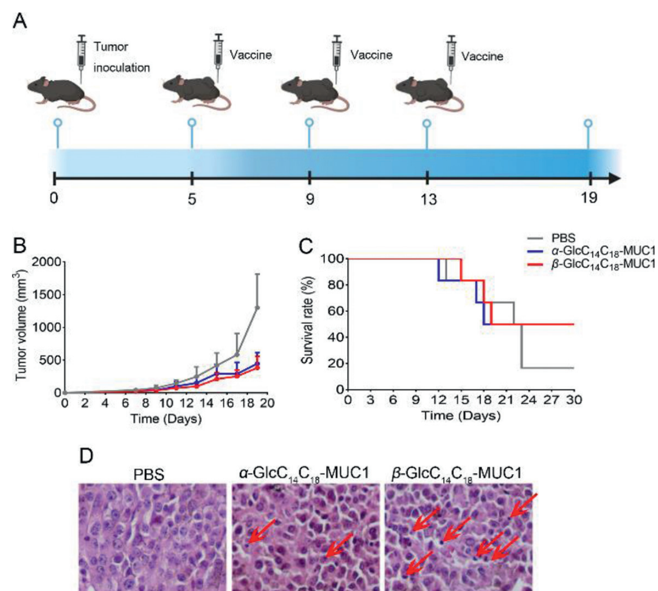

Fig. 6. The therapeutic effect of α-GlcC$_{14}$C$_{18}$-MUC1 and β-GlcC$_{14}$C$_{18}$-MUC1 on tumors. (A) Immune flowchart of the treatment process. After establishing the tumor-bearing mouse model, the vaccine candidates were injected for 3 times. (B) Growth curve of tumor volume in mice after injection of vaccine candidates ($n = 6$). When the tumor diameter reached 5 mm, they were measured every 2 days for a total of 7 times. (C) Survival curve of tumor-bearing mice after vaccination ($n = 6$). (D) Pathological sections of tumor tissue stained by hematoxylin and eosin. Inflammatory cells are indicated by the red arrows.

three groups. The mice were injected with α-GlcC$_{14}$C$_{18}$-MUC1, β-GlcC$_{14}$C$_{18}$-MUC1 and PBS by subcutaneous injection three times, and the tumor volume was measured every 2 days (Fig. 6A). At the same time, the survival rate of the mice was calculated. As shown in Figs. 6B and C, the tumor volume of mice injected with β-GlcC$_{14}$C$_{18}$-MUC1 was small, and the survival rate of the mice treated with β-GlcC$_{14}$C$_{18}$-MUC1 is high, which demonstrated that β-GlcC$_{14}$C$_{18}$-MUC1 not only has excellent tumor growth inhibition effect, but also can prolong the survival time of mice.

To study the inhibitory effect of the vaccine on tumor tissue intuitively, we made the tumor tissue into pathological sections. Observing the pathological characteristics of tumor tissue by hematoxylin and eosin (H&E) staining under the microscope, we can find that the tumor cells of the vaccinated mice showed the nuclear pyknosis and the infiltration of inflammatory cells. In the tumors of mouse that have been injected with β-GlcC$_{14}$C$_{18}$-MUC1 are more obvious than that in mice injected with α-GlcC$_{14}$C$_{18}$-MUC1 (Fig. 6D). These results prove that β-GlcC$_{14}$C$_{18}$-MUC1 has a better inhibitory and therapeutic effect on tumors than α isomer.

The specific cellular immune response mediated by T cells plays a significant role in the suppression of tumors. During the whole process, CD8$^+$ T cells, effector activated cytotoxic T cells , will infiltrate the tumor tissues to kill tumors by releasing killer factors, so the ratio of tumor infiltrating lymphocytes in tumor tissues can well reflect the immune effect of the vaccine. In order to measure the ratio of CD8$^+$ T cells in tumor tissue and spleen, a single cell suspension was made from the collected tumor tissue and spleen, then labeled with CD3 and CD8 antibodies, and finally analyzed by flow cytometry. As shown in Fig. 7, the proportion of CD8$^+$ T cells in splenocytes, of which induced by β-GlcC$_{14}$C$_{18}$-MUC1 vaccine was higher than that of α-GlcC$_{14}$C$_{18}$-MUC1, and the same results were obtained in tumor tissues. So it can be seen that β-GlcC$_{14}$C$_{18}$-MUC1 can induce CD8$^+$ T cells into tumor tissues better than α-GlcC$_{14}$C$_{18}$-MUC1, thereby causing antigen-specific cytotoxic T lymphocyte immune response to inhibit tumor growth.

Overall, based on the above experiments, GlcC$_{14}$C$_{18}$ has been shown to be useful as an immunopotentiator for MUC1-based antitumor vaccines, but its α and β configurations have different immunological enhancement effect. As an immunopotentiator derived from Mincle ligand, GlcC$_{14}$C$_{18}$ has a simple structure and low toxicity, which not only reduces the difficulty of obtaining it, but also enhances the body's tolerance. In addition, the vaccine obtained by coupling GlcC$_{14}$C$_{18}$ and MUC1 can simultaneously bind to two PRRs of immune cells (TLRs and CLRs), so that multiple branches of the immune system can be activated at the same time, adding an opportunity to improve the immune capacity of the body [43,44]. However, the immunostimulatory activity induced by adjuvant binding to receptors depends on not only the specific chemical structure of the adjuvant molecule, but also the steric complementarity of the structure with the receptor [45–48]. Hadar Feinberg *et al.* introduced the mechanism by which three ligand-binding sites in Mincle recognize pathogen-specific glycolipids in the binding model of Mincle and trehalose bismuth lipids. The ligand-binding site in Mincle consists of a typical C-type primary binding site centered on Ca^{2+}, supplemented on one side by a secondary binding site for the second glucose residue in the trehalose head group, and on the other side by a Hydrophobic channel complement for binding acyl groups [49]. In addition, Christian A. Söldner *et al.* also showed that as the acyl chain length increases, the interaction energy of the monoester becomes more favorable [50]. Therefore, the stronger immune ability of β-GlcC$_{14}$C$_{18}$-MUC1 may be related to its better binding to the three ligand-binding sites in Mincle. Moreover, the length of its acyl chain is slightly longer than that of α-GlcC$_{14}$C$_{18}$-MUC1, this may make it more favorable for binding to the ligand-binding site of Mincle.

In this study, two configurations of GlcC$_{14}$C$_{18}$ namely α-GlcC$_{14}$C$_{18}$ and β-GlcC$_{14}$C$_{18}$ were synthesized through different synthetic routes, the vaccine candidates of α-GlcC$_{14}$C$_{18}$-MUC1 and

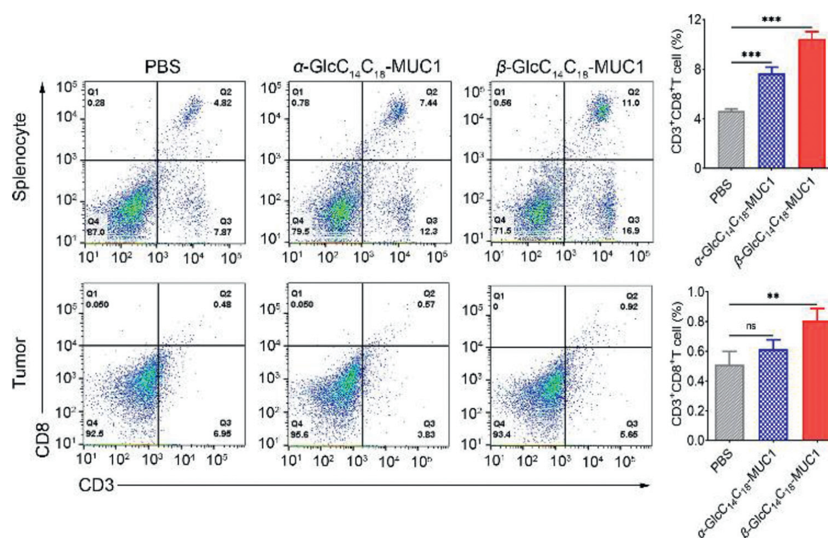

Fig. 7. FACS detects the ratio of $CD3^+/CD8^+$ T cells in splenocytes and tumor tissues collected from the immunized mice. Data are presented as the mean ± SD ($n = 3$). Differences among groups are determined by one-way ANOVA analysis, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

β-GlcC$_{14}$C$_{18}$-MUC1 were then constructed by solid-phase peptide synthesis. Immunological evaluation of these vaccines was then performed and found that β-GlcC$_{14}$C$_{18}$-MUC1 induced higher levels of antibodies and cytokines than α-GlcC$_{14}$C$_{18}$-MUC1. The binding ability of the antiserum induced by the vaccine to tumor cells and the induced CDC effect were both stronger for the vaccine with β configuration. Meanwhile, the β-configuration vaccine was stronger in enhancing the cytotoxicity of CTL, killing tumor cells and inhibiting tumor growth. In conclusion, this study provides novel adjuvants to construct vaccines and provides insights into the impact of adjuvant configuration on vaccine design.

Declaration of 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.108065.

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