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N-Glycosylated type II collagen peptides as therapeutic saccharide vaccines for rheumatoid arthritis

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The interaction among type II collagen (CII), human DR4 major histocompatibility complex type II molecule (MHC II) and T-cell receptor (TCR) is associated with the development of rheumatoid arthritis (RA). The activation of T cells can be reduced through exposure to modified CII(263–272) glycopeptide fragment *via* competitive inhibition with self-antigen. In this work, 30 peptides based on the sequence of CII(263–272) were prepared and evaluated for their binding to DR4 protein by surface plasmon resonance (SPR) assay. The effect on the secretion of pro-inflammatory factors by the spleen cells in collagen induced rheumatoid arthritis (CIA) mouse was also investigated. Two *N*-glycosylated CII peptides were identified to have strong binding to the human recombinant DR4 protein and weak proinflammatory effect. These glycopeptides could be developed as therapeutic saccharide vaccines for the treatment of rheumatoid arthritis (RA).

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Rheumatoid arthritis (RA) is a systemic autoimmune disease with an incidence of 0.5%–1.0% in the world, affecting peripheral joints by causing chronic inflammation that destroys cartilage and bone [1,2]. The etiology of RA is not fully understood, which greatly limits the development of clinical therapy. Type II collagen which is the major component in the cartilage matrix has been suggested to be an autoantigen associated with collagen-induced arthritis (CIA) [3]. The occurrence of CIA is related to the specific recognition of CII bound major histocompatibility complex class II (MHC II) molecules that are present on an antigen-presenting cell by T-cell receptors (TCR) [4]. The TCR/antigen/MHC II complex is essential for the immune system to distinguish the body's own constituents and foreign antigens [5].

The CII(263–272) fragment (Fig. 1A) is the immunodominant epitope of CII and the main antigen that binds to human leukocyte antigen DR (HLA-DR) molecule *via* hydrogen bonding [6]. Ile^{260} and Phe^{263} are anchored to the HLA-DR binding groove, constituting the main sites for HLA-DR molecular binding [6]. Glu^{266}, Lys^{270} and glycosylated Lys^{264} are the crucial sites for the recognition by TCR [7]. The carbohydrate moiety at Lys^{264} in natural CII greatly affects the incidence and severity of CIA [8]. There are two types of glycosylation modification in natural CII (Fig. 1B): galactose Gal(β1-*O*) or disaccharide Glc(α1–2)-Gal(β1-*O*) [9]. CII has important biological functions since 5–hydroxy-lysine can undergo such glycosylation modification.

Previous studies have shown that the structurally modified CII glycopeptide can significantly affect the activation of T cells by competitive inhibition with self-antigen, thereby inhibiting CIA. Those structural modifications have mainly focused on the substitution of amino acids on the CII immune dominant epitope [10–13] and the change of the hydroxyl of carbohydrate moiety sites [14,15]. The difficulty to synthesize 5-hydroxylysine glycosylated derivatives has greatly limited the study of structure-activity relationship of modified peptides. Glutamine (Gln) has a similar long side chain as that of 5-hydroxylysine. The more soluble and stable *N*-glycosylation derivatives at Gln-can be used to replace the *O*-glycosylated 5-hydroxylysine for specific recognition of CII peptide and TCR [16]. The synthesis of *N*-glycopeptides can be easily proceeded by using *N*-glycosylated Gln since *N*-glycosidic bond construction is more efficient. Moreover, the challenge to prepare chiral 5-hydroxylysine could be avoided [17–19]. Furthermore, large and complex carbohydrate moiety may eliminate the specific recognition of the CII glycopeptide with TCR [20]. In addition, it was shown that replacing Ile^{260} and Phe^{263} with Ala or Gly in CII results in markedly reduced affinities for the DR4 protein [21]. Similarly, recognition of the CII glycopeptide by TCR could be reduced with the substitutions of Ala or Gly for Gln^{267} and Lys^{270}.

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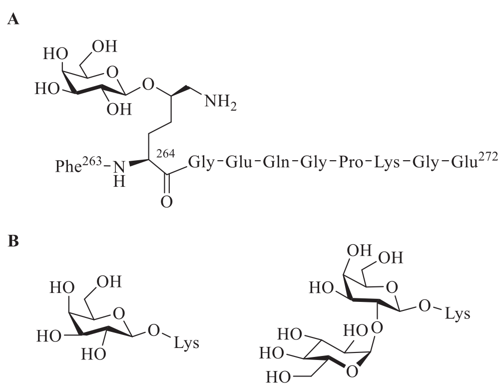


Fig. 1. (A) Chemical structure of glycopeptide CII(263−272). (B) Glycosylated lysine in native CII.

In this study, we designed and synthesized a new type of *N*-glycosylated Gln-modified CII peptides based on the sequence from CII(263–272), and explored their binding to DR4 protein as well as the recognition by TCR. *N*-Linked CII glycopeptides were synthesized from *N*-glycosylglutamic acid and non-glycosylated natural amino acids. Phe^{263} was retained in the designed peptides since it is crucial for binding to the MHC II molecule. Some amino acids in CII(263–272) were replaced with Ala or Gly with short side chains for the purpose of reducing T-cell recognition. All peptides were evaluated for their binding to DR4 protein and stimulation of spleen cells from CIA mice *in vitro* to investigate their potential to be used for the treatment of RA.

Lysine derivative **1-13** was synthesized from protected aspartic acid **1-1** in 4% yield as shown in Scheme S1 (Supporting information) [22], which was used for the synthesis of *O*-galactose lysine **3a**. To characterize the chiral purity of compound **1-13**, we tested the specific rotation of compound **1-9**, $[\alpha]_D^{20} = -20.7$ ($c = 1.000$, $CHCl_3$), $[\alpha]_D^{20} = -19.8$ as a reference value [22]. The synthesis of galactopyranosyl trichloroacetimidate **2a** [23] and aminoglycosides **2b**–**2d** are shown in Supporting information (Experimental Section) [24], which were used for the synthesis of glycosylated amino acids **3a**–**3d**.

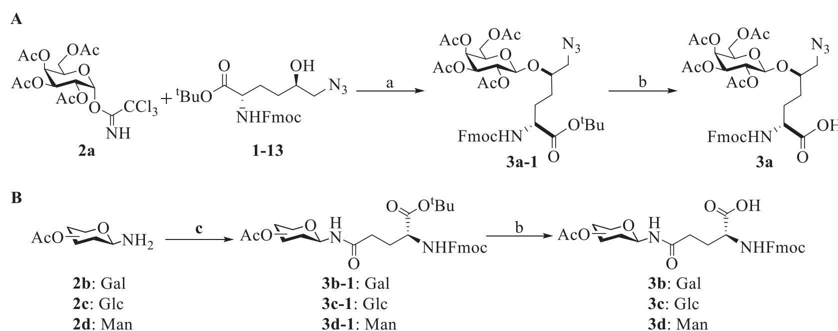
Galactosyl hydroxylysine **3a** was synthesized by the glycosylation of **2a** and **1-13** catalyzed by trimethylsilyl trifluoromethanesulfonate (TMSOTf), followed by the removal of *tert*–butyl with trifluoroacetic acid (TFA) (Scheme 1A) [25]. Glycosyl glutamates **3b**–**3d** were synthesized from aminoglycosides **2b**–**2d** with Fmoc-protected glutamic acid in the presence of 2-(1*H*-benzotriazol-1-yl)−1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*,*N*-diisopropylethylamine (DIEA)/*N*,*N*-dimethylformamide (DMF), followed by the removal of *tert*–butyl with TFA (Scheme 1B) [26]. They were used to synthesize a series of *N*-linked CII peptides.

Table 1
Designed peptides derived from CII(263–272).

Peptide	Amino acid sequence [a]	Yield (%)	K_D (μmol/mL) [b]
CII(263–272)	FKGEQGPKGE	28	6.52
AP1	FKGEQAGAGE	35	–
AP2	FEGEQGPKGE	34	7.57
AP3	FKGEQGPEGE	20	–
AP4	FEGEQGPEGE	20	–
AP5	FEGEQAGAGE	29	–
AP6	FEGAQGPKGE	34	–
AP7	FEGEAGPKGE	31	1.20
AP8	FEGEQGPAGE	34	–
4a	F(Gal-*O*-K)GEQGPKGE	7	4.84
4b-1	F(Gal-*N*-E)GEQGPKGE	8	2.82
4b-2	FKGEQGP(Gal-*N*-E)GE	13	6.52
4b-3	F(Gal-*N*-E)GEQGPEGE	11	2.38
4b-4	F(Gal-*N*-E)GEQGP(Gal-*N*-E)GE	10	4.11
4b-5	F(Gal-*N*-E)GEQAGAGE	12	6.20
4b-6	F(Gal-*N*-E)GAQGPKGE	11	–
4b-7	F(Gal-*N*-E)GEAGPKGE	7	–
4b-8	F(Gal-*N*-E)GEQGPAGE	10	–
4b-9	F(Gal-*N*-E)GEAGPAGE	5	–
4b-10	F(Gal-*N*-E)GEGAGAGE	7	1.44
4b-11	F(Gal-*N*-E)GAGAGAGE	4	–
4b-12	F(Gal-*N*-E)AEQGPKAE	4	4.30
4c-1	F(Glc-*N*-E)GEQGPKGE	7	–
4c-2	F(Glc-*N*-E)GEQAGAGE	10	–
4c-3	FKGEQGP(Glc-*N*-E)GE	4	–
4c-4	F(Glc-*N*-E)GEGAGAGE	3	–
4d-1	F(Man-*N*-E)GEQGPKGE	6	–
4d-2	F(Man-*N*-E))GEQAGAGE	6	–
4d-3	FKGEQGP(Man-*N*-E)GE	6	–
4d-4	F(Man-*N*-E)GEGAGAGE	4	–

[a] Gal-*O*-K: *β*-*O*-galactopyranosyl-lysine; Gal-*N*-E: *β*-*N*-galactopyranosyl-glutamine; Glc-*N*-E: *β*-*N*-gluctopyranosyl-glutamine; Man-*N*-E: *α*-*N*-mannopyranosyl-glutamine.
[b] K_D value of peptides binding to DR4 protein determined by SPR assay.

Thirty peptides based on sequence CII(263–272) were synthesized manually by Fmoc-based solid-phase synthesis (SPSS) using commercial Fmoc-protected amino acids and glycosylated amino acids **3a**–**3d** (Table 1). Cleavage from the solid support with TFA/H_2O/triisopropylsilane (TIS), followed by deacylation of the carbohydrate moieties by treatment with 5% hydrazine hydrate in DMF and purification by reversed-phase high performance liquid chromatography (HPLC) gave the designed peptides in 3%–35% yields. Native peptide fragment CII(263–272) and non-glycopeptides **AP1**–**AP8** were obtained in 20%–35% yields. *O*-Galactopeptide **4a** derived from galactosyl hydroxy–lysine **3a** were obtained in 7% yield. *N*-Linked glycopeptides were obtained in 3%–13% yields. The purities of all peptides were more than 95% by reversed-phase HPLC analysis and their structures were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.



Scheme 1. (A) Synthesis of compound **3a**. (B) Synthesis of glycosyl glutamates **3b**–**3d**. Reagents and conditions: (a) TMSOTf, DCM, 4 Å molecular sieve, 0 °C, Ar, 1 h, 51%. (b) TFA/DCM = 1:1, r.t., 3 h, 95%. (c) HBTU, DIEA, DMF, 0 °C to r.t., 15 h, 85% for **3b-1** from **2b**, 80% for **3c-1** and **3d-1** from **2c** and **2d** respectively.

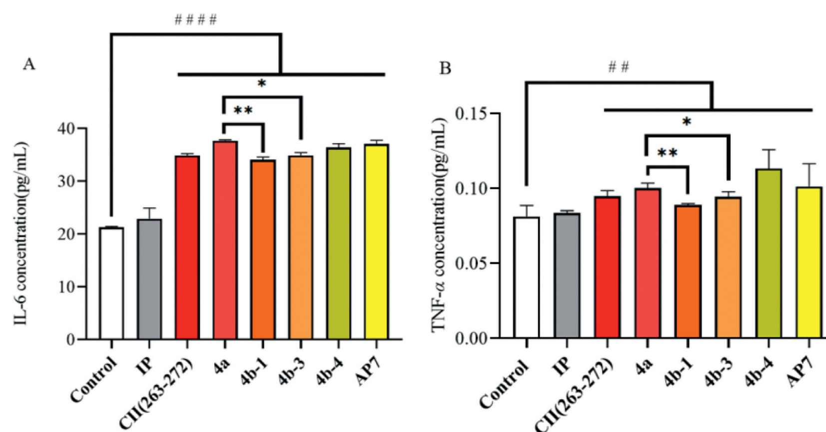


Fig. 2. The secretion of cytokines produced by spleen cells of CIA mice. (A) IL-6. (B) TNF-α. $^{##}P < 0.01$, $^{####}P < 0.0001$, compared with control group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with **4a**. $n = 4$. Values are mean ± standard deviation (SD) of three independent experiments.

The ability of the peptides to bind to human recombinant DR4 was determined by SPR assay. We found that 11 out of the 30 peptides were capable of binding to DR4 protein (Table 1). The binding ability of **4b-1**, **4b-3** and **4b-4** to DR4 was stronger than **4a**, while the binding ability of **4b-2**, **4b-5**, **4b-10** and **4b-12** to DR4 was weaker than **4a**. These results suggested that the designed *N*-glycosylated CII peptides could bind to DR4 protein more strongly than the *O*-glycosylated CII peptide, and our subsequent research work should focus on the modification of galactosylated CII peptide rather than other glycoforms.

A model of the CII(263–272) in complex with a DR4 crystal structure was constructed from a reported structure (PDB ID: 1J8H). The designed glycopeptides **4a** and **4b-1** were selected to be docked into the model to explore the comparative effects of the introduced glycosylated Lys^{264} moieties on the protein-ligand interactions (Fig. S1 in Supporting information). Assuming a similar binding mode as for the CII(263–272), the glycosylated Lys^{264} moieties in **4a** and **4b-1**, respectively, would be positioned on the border of the DR4 binding site. The backbones of the glycopeptides were similarly found fitting into the peptide-binding groove. The same conformations were also observed for the Gal *O*-glycosylated Lys^{264} (Gal-*O*-K, **4a**) and Gal *N*-glycosylated Gln^{264} (Gal-*N*-E, **4b-1**), which was consistent with the same binding affinities ($K_D = 4.84$ µmol/mL for **4a** and $K_D = 2.82$ µmol/mL for **4b-1**). But the replacement by Gal-*N*-E might affect the binding to TCR. Next, immunological assays were performed to evaluate the stimulation to TCR.

The effect of **4b-1** on the viability of spleen cells from CIA mice was detected by cell counting kit-8 (CCK-8) (Fig. S2 in Supporting information). Animal welfare and experiment process were reviewed and approved by Animal Ethics Committee of Peking University People's Hospital. The relative cell viability of each concentration group was slightly enhanced after 1.5 h treatment with compound **4b-1**, but with no significant difference compared to the control group. As the time prolonged, the relative cell viability of each concentration group showed a decreasing trend, which showed a statistical difference compared to the control group ($P < 0.05$) when it was treated for 3 h compared to the 1.5 h group ($P < 0.01$). When treated for 4 h with the peptide **4b-1**, there was a statistically significant difference in cell viability compared to the Control group ($P < 0.01$) as well as a statistically significant difference compared to the 1.5 h group ($P < 0.01$). However, there was no statistically significant difference in the cell viability between the 3 h group and the 4 h group. Peptide **4b-1** exhibited the highest cell viability at the concentration of 10 µg/mL. Therefore, the peptide concentration of 10 µg/mL and treatment time of 1.5 h on CIA mouse spleen cells were selected as the activity measurement conditions for subsequent experiments.

CII(263–272), **AP7**, **4a**, **4b-1**, **4b-3** and **4b-4** were selected for stimulation of the CIA mouse spleen cells *in vitro*. The secretion of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured by using the Elisa method (Fig. 2). We found that the secretion of IL-6 stimulated by *N*-glycopeptides **4b-1** and **4b-3** was significantly reduced compared to *O*-glycopeptide **4a** ($P < 0.01$ and $P < 0.05$). Similarly, the secretion of TNF-α stimulated by *N*-glycopeptides **4b-1** and **4b-3** was significantly reduced compared to *O*-glycopeptide **4a** ($P < 0.01$ and $P < 0.05$). These results indicated that IL-6 and TNF-α secretion could be down-regulated by the synthesized *N*-glycopeptides. Vaccination of mice with CII glycopeptide fragment **4a** alone or in complex with the class II MHC A^{q} protein [8] has been shown to prevent development of collagen-induced arthritis, a mouse model for RA. This glycopeptide **4a**, which is a fragment from type II collagen, was also recognized by T-cells isolated from a cohort of RA patients [27]. In the activation of TCR, the secretion of IL-2, IL-6 and TNF-α stimulated by glycopeptides might be considered as significant markers. The moderate increased secretion of inflammatory factors was needed for the immunogenicity. The results in Fig. 2 indicated that our peptides **4b-1** and **4b-3** had moderate immunogenic effects and could be developed as therapeutic RA vaccines. Further research will be performed to verify the effect of our *N*-glycopeptides as RA vaccines in the future.

In conclusion, the structure of CII(263–272) was modified to explore the effect of *N*-linked CII glycopeptides on the secretion of pro-inflammatory factors by CIA mouse spleen cells. 30 peptides were synthesized in the yields of 3%–35%. Two *N*-glycosylated CII peptides were identified to have stronger binding ability to DR4 protein and weaker proinflammatory effect than *O*-glycosylated native CII peptide fragment through SPR assay and spleen cell stimulation experiment. The studied glycopeptides could be developed as therapeutic RA vaccines.

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.2023.108762.

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