

Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis

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Immunization of mice with type II collagen (CII) leads to collagen-induced arthritis (CIA), a model for rheumatoid arthritis. T cell recognition of CII is believed to be a critical step in CIA development. We have analyzed the T cell determinants on CII and the TCR used for their recognition, using twenty-nine T cell hybridomas derived from C3H.Q and DBA/1 mice immunized with rat CII. All hybridomas were specific for the CII(256–270) segment. However, posttranslational modifications (hydroxylation and variable O-linked glycosylation) of the lysine at position 264 generated five T cell determinants that were specifically recognized by different T cell hybridoma subsets. TCR sequencing indicated that each of the five T cell epitopes selected its own TCR repertoire. The physiological relevance of this observation was shown by *in vivo* antibody-driven depletion of TCR V α 2-positive T cells, which resulted in an inhibition of the T cell proliferative response *in vitro* towards the non-modified CII(256–270), but not towards the glycosylated epitope. Most hybridomas (20/29) specifically recognized CII(256–270) glycosylated with a monosaccharide (β -D-galactopyranose). We conclude that this glycopeptide is immunodominant in CIA and that posttranslational modifications of CII create new T cell determinants that generate a diverse TCR repertoire.

Received	7/5/98
Accepted	28/5/98

Key words: Autoimmunity / Glycosylation / T cell / Collagen / TCR

1 Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease affecting peripheral joints. Based on the observations that susceptibility to rheumatoid arthritis is associated with genes of the MHC class II (HLA DR4 and DR1) and that activated T cells infiltrate the arthritic joints, it is believed that MHC class II-restricted, Ag-specific CD4⁺ T cells play a crucial role in the disease. The use of animal models has provided possibilities to experimentally address the role of the MHC and T cells in genetically well-defined systems. The most widely used model for RA is type II collagen-induced arthritis (CIA), in which the histopathology of the affected joints mimics rheumatoid arthritis [1, 2]. In mice, the development of

CIA is closely associated with the MHC class II region [1] and in particular with the H-2 A^q molecule [3]. Interestingly, transgenic expression of RA-associated molecules [HLA DR1 (DRB1*0101) or HLA DR4 (DRB1*0401)] in mice conferred susceptibility to CIA [4] and manuscript submitted).

T cell recognition of type II collagen (CII) is believed to be a crucial step in the induction and development of arthritis [5, 6]. It is therefore important to clearly define the T cell determinants within CII. This has been an unexpectedly difficult task because of the nature of CII which is a glycosylated protein. We recently showed that carbohydrates on CII are specifically recognized by T cells [7]. Due to posttranslational modifications of CII, each lysine residue that is situated in an appropriate position (G-X-K) can be in one of four different forms: non-modified; simply hydroxylated (hydroxylysine, Hyl); hydroxylated and further glycosylated with a monosaccharide (β -D-galactopyranose, Gal); or hydroxylated and further glycosylated with a disaccharide [α -D-glucopyranosyl-(1->2)- β -D-galactopyranose, Glc-Gal]. This generates an enormous number of potential T cell determinants on CII.

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Abbreviations: CIA: Type II collagen-induced arthritis CII: Type II collagen Gal: β -D-Galactopyranose Glc-Gal: α -D-Glucopyranosyl-(1->2)- β -D-galactopyranose Hnv: 5-Hydroxy-L-norvaline Hyl: (5R)-5-Hydroxy-L-lysine RA: Rheumatoid arthritis

The role of the different posttranslational modifications of CII in the development of CIA is poorly understood but is clearly important since elimination of the carbohydrates makes CII less arthritogenic [7]. Interestingly, most glycopeptides recognized by T cells so far studied appear to bind with the carbohydrates oriented towards the TCR rather than the MHC [8–11], with some possible exceptions [12, 13]. Consequently, one can expect that posttranslational modifications of CII will influence the TCR repertoire selected for recognition. This is an important question to investigate, not only for the understanding of the selection of TCR by glycopeptides, but also to understand the pathogenesis of CIA which has been proposed to be dependent on TCR repertoire selection [14–16].

In the present study, we have characterized the T cell determinants in CIA by testing the reactivity of CII-specific T cell hybridomas towards synthetic CII peptides exhibiting various posttranslational modifications. We show that the T cell response in CIA is restricted to the CII(256–270) segment. However, posttranslational modifications of a single lysine at position 264 created five T cell determinants recognized specifically by different T cell subsets. The TCR repertoire used for recognition of CII appeared highly diverse and was profoundly affected by posttranslational modifications.

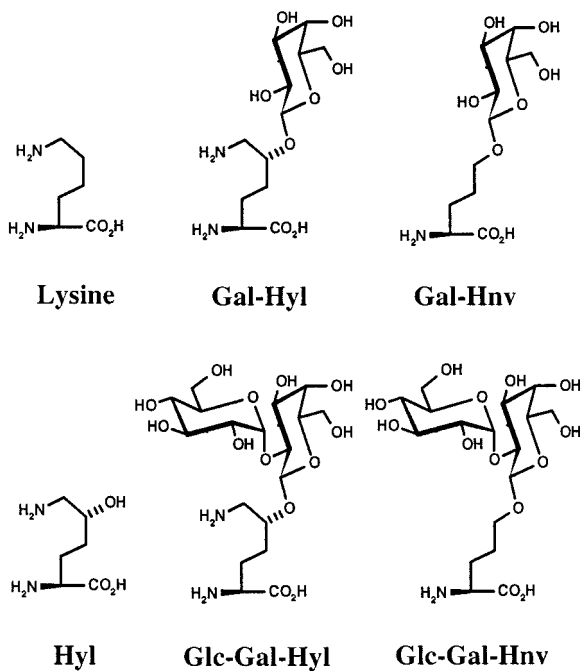


Figure 1. The various forms of lysine residues are shown. Non-modified lysine, Hyl, Gal-Hyl and Glc-Gal-Hyl are four natural variants found in CII. Gal-Hnv and Glc-Gal-Hnv are artificial molecules that lack the aminomethylene group of the corresponding natural form.

2 Results

2.1 Fine specificity characterization of CII-reactive T cell hybridomas

Twenty-nine T cell hybridomas were isolated after immunization of DBA/1 and C3H.Q mice with native rat CII. These T cell hybridomas were selected solely according to their reactivity towards heat-denatured rat CII in culture; *i.e.* without any CII fragment or peptide selection *in vitro*. The fine specificity of the hybridomas was defined by measuring their IL-2 production when cultured together with spleen cells as APC and different peptides representing several posttranslationally modified forms of CII(256–270). We expected CII(256–270) to contain most of the T cell determinants in CII since we and others have reported T cell reactivity towards non-modified CII(256–270) [17, 18]. We have also demonstrated that transgenic expression of type I collagen mutated to express CII(256–270) in the skin of mice leads to a lack of a T cell response to CII and resistance to CIA [19]. The various modifications of the lysines at positions 264 and 270 are illustrated in Fig. 1. It should

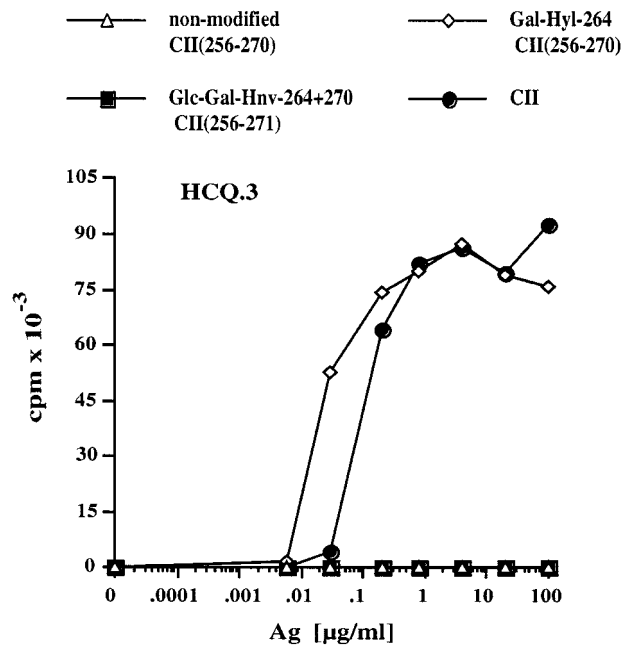


Figure 2. Specific recognition of Gal-Hyl at position 264 of CII by the hybridoma HCQ.3. The hybridoma HCQ.3 was tested for recognition of heat-denatured rat CII and three peptides with various posttranslational modifications at position 264 and 270. The background response (*e.g.* without Ag) in this experiments was 134 cpm. A similar experiment showed that HCQ.3 does not respond to the Gal-Hnv-264 CII(256–270) and Glc-Gal-Hnv-264 CII(256–270) peptides.

Table 1. T cell hybridoma reactivities for CII peptides with various modifications^{a)}

	CII	non-modified CII(256-270)	Hyl-264 CII(256-270)	Gal-Hnv-264 CII(256-270)	Gal-Hyl-264 CII(256-270)	Gal-Hyl- 264+270 CII(259-278) ^{b)}	Glc-Gal-Hnv-264 CII(256-270)	Glc-Gal-Hnv- 264+270 CII(256-271) ^{b)}
Group 1								
HCQ.4	+++	++++	+++		+	-	-	-
HDB.1	+	+++	++		-	-	-	-
HDB.2	+	+++	+		-	-	-	-
HRC.1	+	+++	++		-	-	-	-
HRC.2	++	+++	++		-	-	-	-
HRC.3	+	+++	++		-	-	-	-
Group 2								
HDBR.1	++++	-	++++		-	-	-	-
Group 3								
HCQ.1	++++	-	-	-	+++++	+++	-	-
HCQ.2	++++	-	-	-	+++++	+++++	-	-
HCQ.3	++++	-	-	-	+++++		-	-
HCQ.6	++++	-	-	-	+++++		-	-
HCQ.9	+++	-	-	-	++++		-	-
HCQ.10	+++++	-	-	-	+++++		-	+
HD13.1	++++	-	-	-	+++++		-	-
HD13.2	++++	-	-	-	+++++		-	-
HD13.3	+++++	-	-	-	+++++		-	-
HD13.4	+++	-	-	-	++++		-	-
HD13.5	+++	-	-	-	++++		-	-
HD13.6	++++	-	-	-	+++++		-	-
HD13.7	+++	-	-	-	++++		-	-
HD13.9	+++++	-	-	-	+++++		-	-
HD13.10	++++	-	-	-	+++++		-	-
HM2.1	++++	-	-	-	++++	++++	-	-
HNC.1	+++	-	-	-	+++		-	-
Group 4								
HDC.1	++	-		++	+++		-	
HM1R.1	+++++	-		+++++	+++++		+	++
HM1R.2 ^{c)}	+++++	-		+++++	+++++	++++	++	++
Group 5								
HCQ.11 ^{c)}	++++	-	-	-	-		+++	+++
Group 6								
HM2.2 CII ^{c)}	++++	-	-	-	-	-	-	-

a) The sensitivity of the T cell hybridomas was determined as the amount of Ag required for a CTLL response > 10 times the background (*i. e.* without Ag): -, no reactivity; +, ≈ 100 µg/ml; ++, ≈ 10 µg/ml; +++, ≈ 1 µg/ml; +++++, ≈ 0.1 µg/ml; ++++++, < 0.01 µg/ml. Absence of a symbol (+,-) means "not analyzed". Positions (264 or 270) of Hyl and Hnv residues within the peptides are given.

b) Peptides glycosylated at position 270 were synthesized longer at the C-terminal to permit an optimal T cell recognition of the saccharide at this position.

c) These hybridomas did not react to the CII(256–270) peptide with a non-glycosylated Hnv at position 264.

be noted that the non-natural amino acid hydroxynorvaline (Hnv) was used instead of Hyl for the synthesis of some peptides. As an example, the reactivity of the hybridoma HCQ.3 is shown in Fig. 2.

The hybridomas were divided into six different groups according to their fine specificity (Table 1): Group 1: hybridomas were specific for the non-modified CII(256–270) peptide. They also recognized this peptide when the lysine at position 264 was hydroxylated but with a weaker reactivity. Group 2: hybridoma HDBR.1 specifically recognized CII(256–270) with Hyl at position 264. No crossreactivity was found to the non-modified or to any of the glycosylated peptides. Group 3: hybridomas specifically recognized CII(256–270) with Gal-Hyl at position 264. Reactivity was abolished when Gal was fixed to Hnv instead of Hyl. This shows that both the carbohydrate and the aminomethylene group (on the δ -carbon in the side chain of Hyl) are specifically recognized by these hybridomas (see Fig. 1). Group 4: hybridomas recognized CII(256–270) with Gal attached to either Hyl or Hnv. This means that, in contrast to the previous group, these hybridomas do not need the aminomethylene group for recognition (Fig. 1). Group 5: hybridoma HCQ.11 specifically recognized CII(256–270) with Glc-Gal at position 264. It did not react to the peptide with a monosaccharide (Gal) at the same position. Group 6: hybridoma HM2.2 did not recognize any of the synthetic glycopeptides although it is clearly specific for a glycosylated form of CII(256–270) since it recognized the recombinant collagen pWTCk8 kol 7.11 containing post-translationally modified CII(256–270) [7], but not CII treated with sodium periodate which oxidizes carbohydrates (data not shown). HM2.2 presumably recognizes CII(256–270) with Glc-Gal attached to Hyl at position 264. Unfortunately, all our attempts to synthesize such a glycopeptide have been unsuccessful so far.

The data summarized in Table 1 show that most CII-reactive hybridomas (20/29) were specific for a glycosylated CII(256–270) with Gal at position 264 (groups 3 and 4). The remaining hybridomas (groups 1, 2, 5 and 6) were specific for at least three other T cell determinants representing different forms of CII(256–270), *i. e.* non-modified CII(256–270), CII(256–270) with a Hyl at position 264, or CII(256–270) with Glc-Gal at position 264. In contrast to any modification of the lysine at position 264, glycosylation of the lysine at position 270 with a mono- or a disaccharide had no or very little influence on T cell recognition (Table 1).

2.2 Determination of the TCR structures used for recognition of CII

All T cell hybridomas in groups 1, 2, 5 and 6 as well as 10 out of 20 (randomly chosen) hybridomas recognizing Gal at position 264 (groups 3 and 4) were subjected to TCR DNA sequencing. This revealed fourteen clonally distinct hybridomas (Fig. 3). All TCR β chains were successfully sequenced. Due to the absence of allelic exclusion in TCR α chains, T cells contain two different mRNA for TCR α chains and some of them consequently possess two different functional α chains [20, 21]. It is therefore important to have information on both α chains for each T cell clone or hybridoma when studying a TCR repertoire. We successfully sequenced two α chains in six T cell hybridomas (HCQ.4, HDBR.1, HD13.7, HDC.1, HM1R.2 and HM2.2). For HCQ.10 the in-frame α chain is unknown. For each of the seven remaining hybridomas, only one α chain has been obtained. However, because of amino acid sequence similarities between different hybridomas belonging to the same group (Fig. 3), we believe that at least for six of them (HRC.1, HRC.2, HDB.1, HCQ.6, HCQ.3 and HM2.1), the α chain we sequenced is the one that has been selected for Ag recognition.

As could be expected from their different fine specificities, the TCR used in the different groups of hybridomas appeared to be very different, both in terms of gene segment usage and of amino acid conservation in the complementarity-determining region 3 (CDR3) (Fig. 3). However, strong TCR conservations were observed within the groups containing more than one hybridoma (groups 1, 3 and 4). For instance, the in-frame α chains of the four clonally distinct T cell hybridomas reactive with non-modified CII(256–270) (group 1) were strongly conserved. Their V-J junctional regions (CDR3) were restricted in length and contained a “NNNRI” motif (three asparagines followed by an arginine and an isoleucine) between a V α 2 or V α 4^{5B6} and a J α 24 segment. These structural conservations typical for group 1 hybridomas were not seen in any other group. The five clonally distinct hybridomas specific for CII(256–270) with Gal-Hyl at position 264 (group 3) did not share a common CDR3 motif but tended to use gene segments belonging to the V α 16 family. In addition, group 3 contained two pairs of hybridomas (HCQ.3/HCQ.6, HM2.1/HD13.7) with very similar TCR structures which might reflect the presence of subgroups with similar fine specificity within this group.

Collectively, the TCR selected for recognition of CII appeared to be very heterogeneous. Their in-frame chains were made of nine different V α , eight different V β , eight different J α and seven different J β gene segments;

TCR α chain				TCR β chain		
Hybridoma	V α	CDR3	J α	V β	CDR3	J β
Group 1 (Hybridomas specific for non-modified CII(256-270))						
HRC.1	C A A TGTGCAGCA V α 2	S A N N N N R I AGTGCAAATAACAAACAGAATC	F F G TTCTTTGGT J α 24	C S S TGCTCCAGC V β 20	S Q N S A E T L AGTCAAAACAGTGCAGAAACGCTG	Y F G TATTTTGGC J β 2.3
HRC.2 HRC.3	C A A TGTGCAGCA V α 2	S A N N N N R I AGTGCTAATAACAAACAGAATC	F F G TTCTTTGGT J α 24	C S S TGCTCCAGC V β 20	S P H T G Q L AGTCCGCACACCGGGCAGCTC	Y F G TACTTTGGT J β 2.2
HDB.1 HDB.2	C A A TGTGCAGCA V α 2	S A N N N R I AGTGCAAATAACAAACAGAATC	F F G TTCTTTGGT J α 24	C A S TGTGCAAGC V β 11	S S T T G G A N E R L AGCTCCACGACAGGGGGCCCAACGAAAGATTA	F F G TTTTTCGGT J β 1.4
HCQ.4*	C A L TGTGCTGTG V α 4.5B6	V R N N N N R I GTCCGGAATAACAAACAGAATC	F F G TTCTTTGGT J α 24	C A S TGTGCCAGC V β 12	S L W G N Y A E Q AGTTTATGGGGGAACATATGCTGAGCAG	F F G TTCTTCGGA J β 2.1
Group 2 (Hybridoma specific for CII(256-270) with Hyl at position 264)						
HDBR.1	C A A TGTGCTGCT V α 11.1a	E S S S S F S K L GAGTCATCCTCCTCTCAGCAAGCTG	V F G GTGTTGGG J α 42	C A S TGTGCCAGC V β 8.2	G D A G G E R L GGTGATGCAGGGGGCGAAAGATTA	F F G TTTTCGGT J β 1.4
	C A A TGTGCTGCA V α 19.HDBR.1	S W A G A N T G K L AGTTGGGCTGGAGCTAACACTGGAAAGCTC	T F G ACGTTTGGG J α 44			
Group 3 (Hybridomas specific for CII(256-270) with Gal-Hyl at position 264)						
HCQ.6 HCQ.2	C A M TGTGCTATG V α 16.42H11	S P G T G G Y K V AGCCCCGGGACTGGAGGCTATAAAGTG	V F G GTCTTTGGA J α 10	C A S TGTGCCAGC V β 8.1	G G T A T S Q N T L GGGGGACAGCTACTAGTCAAACACCTTG	Y F G TACTTTGGT J β 2.4
HCQ.3	C A M TGTGCTATG V α 16.H-15.E3	S P G T G G Y K V AGCCCCGGGACTGGAGGCTATAAAGTG	V F G GTCTTTGGA J α 10	C A S TGTGCCAGC V β 8.1	S G T E T S Q N T L AGTGGGACAGAAACTAGTCAAACACCTTG	Y F G TACTTTGGT J β 2.4
HM2.1	C A M TGTGCTATG V α 16.H-15.E3	L N N Y A Q G L CTAAAT AACTAT GCCCAGGGATTA	T F G ACCTTCGGT J α 20	C A S TGTGCCAGC V β 10	S W G G R Q D T Q AGCTGGGGGGGGCCCAAGACACCCAG	Y F G TACTTTGGG J β 2.5
HD13.7*	C A L TGTGCCCTG V α 16.NY7.2	L N N Y A Q G L CTAAATAACTATGCCCAGGGATTA	T F G ACCTTCGGT J α 20	C A S TGTGCCAGC V β 10	S S Q G N Q D T Q AGCTCACAGGGT AACCAAGACACCCAG	Y F G TACTTTGGG J β 2.5
HCQ.10*	HCQ.1 HCQ.9			C A S TGTGCCAGC V β 1	S Q E G Q G F N E R L AGCCAAGAGGGACAGGGATTCAACGAAAGATTA	F F G TTTTTCGGT J β 1.4
Group 4 (Hybridomas specific for CII(256-270) with Gal-Hnv at position 264)						
HDC.1*	C V L TGTGTTCTG V α 4.F5	G H A S S G S W Q L GGTCATGCATCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA J α 17	C A S TGTGCCAGC V β 6	S I D R L S N E R L AGTATAGACAGACTTTCCAACGAAAGATTA	F F G TTTTTCGGT J β 1.4
HM1R.2*	C V L TGTGTTCTG V α 4.F5	V R A S S G S W Q L GTCAGAGCATCTTCTGGCAGCTGGCAACTC	I F G ATCTTTGGA J α 17	C A S TGTGCCAGC V β 12	S P G T G G S A E T L AGCCCTGGGACAGGGGTAGTGCAGAAACGCTG	Y F G TATTTTGGC J β 2.3
Group 5 (Hybridoma specific for CII(256-270) with Glc-Gal at position 264)						
HCQ.11	C A A TGTGCTGCT V α 11.1a	E A S N T D K V GAGGCTTCCAATACCGACAAAGTC	V F G GTCTTTGGA J α 27	C A S TGTGCCAGC V β 8.2	G D W G K R D Q D T Q GGTGATTGGGGGAAGAGGGACCAAGACACCCAG	Y F G TACTTTGGG J β 2.5
Group 6 (Hybridoma specific for a not determined glycosylated form of CII(256-270))						
HM2.2*	C A L TGTGCTTTG V α 8.F32	S L T N A Y K V AGCCTTACAAATGCTTACAAAGTC	I F G ATCTTTGGA J α 23	C A S TGTGCCAGC V β 8.2	E T S Y E Q GAAACCTCCTATGAACG	Y F G TACTTCGGT J β 2.6

Figure 3. TCR V α -J α and V β -D β -J β junctional region nucleotide and derived amino acid sequences of T cell hybridomas specific for rat CII. Nucleotide and deduced amino acid sequences (in single-letter amino acid code) from in-frame sequences of the CDR3 as well as the V and J segments are shown. The D β segments are underlined [61]. The grouping of the hybridomas according to their Ag specificity is described in Sect. 2.1. T cell hybridomas where an out-of-frame TCR α chain could be sequenced are designed with an asterisk (*). The nomenclature for gene segment subfamilies is described in Sect. 4.3. V α 19.HDBR.1 is a new gene segment that differs by a silent base substitution (underlined) from V α 19.2 [62] (...CACAT \underline{I} ACA...). These sequence data are available from EMBL/Genbank/DDBJ under accession numbers X93050–X93077 and X98057–X98062.

the most common gene segments, J α 24 and J β 1.4, were shared by less than one third of the clones. The CDR3 of both in-frame TCR α and β chains showed no obvious overall amino acid conservation (Fig. 3).

2.3 Mouse strain influence on TCR usage

CII-specific T cell hybridomas were derived from two different mouse strains. Hybridomas with the prefix "HCQ" originate from C3H.Q mice; the other hybridomas originate from DBA/1 mice. Both strains have the MHC class II H-2^q haplotype and are highly susceptible to CIA. Both C3H.Q and DBA/1 developed a diverse response to CII, but the TCR sequences indicate that each strain may have its own preferred pool of gene segments and CDR3 structures, with the exception of the V α 16 subfamily which was used rather frequently by both strains (Fig. 3). This strain difference also seemed to affect the highly conserved α chains selected for recognition of the non-modified peptide. The unique C3H.Q-derived hybridoma (HCQ.4) possessed the conserved "NNNRI" motif followed by J α 24 but used a V α 4 segment, while the three DBA/1-derived hybridomas used a V α 2 segment (Fig. 3). An explanation for this observation could be the allelic polymorphism in the V α 2 and V α 4 subfamilies that has been observed between the two strains [22].

2.4 Different TCR repertoires are used for recognition of non-modified versus glycosylated CII(256–270)

The sequence data shown in Fig. 3 indicated a strong TCR α chain conservation for recognition of non-modified CII(256–270) which contrasted with a more diverse repertoire of the TCR specific for posttranslationally modified CII(256–270). In particular the V α 2 gene segment, which was used by all three group 1 hybridomas originating from DBA/1 mice but which was not used in any other group, gave us the possibility to test the physiological relevance of this observation. Depletion of TCR V α 2-positive cells by Ab treatment of DBA/1 mice *in vivo* resulted in a significant inhibition of the T cell proliferative response *in vitro* towards the non-modified CII(256–270) but not towards a glycopeptide with Gal at position 264 (Fig. 4). Control mice were treated with a TCR V α 8-specific mAb. This experiment also confirmed that most CII-specific T cells react to the glycosylated epitope with Gal at position 264, while only a minority of T cells recognize the non-modified CII(256–270). This explains why TCR V α 2 T cell depletion did not significantly affect T cell proliferation with the whole CII molecule (Fig. 4).

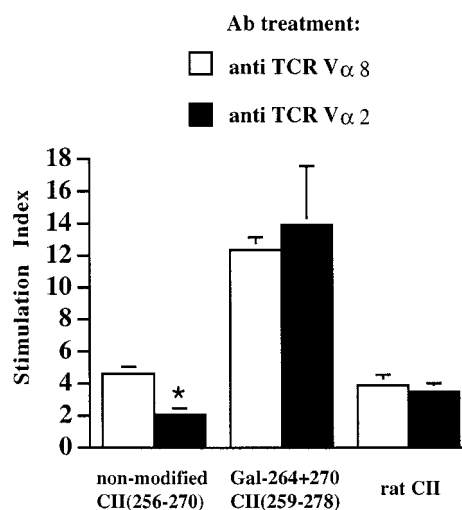


Figure 4. TCR V α 2-positive cells are selected for recognition of the non-modified but not of the galactosylated CII epitope. Five DBA/1 mice were depleted of TCR V α 2 T cells by specific mAb treatment 1 day before immunization with CII+CFA. Four control DBA/1 mice were injected with a similar amount of anti TCR V α 8 mAb. The Ag-specific proliferative response was measured *in vitro* 10 days after the immunization. The figure shows the mean stimulation index \pm SEM of the individual mice. * $p = 0.0143$ (Mann-Whitney).

3 Discussion

Our report shows that CII-specific T cells, which are of critical importance for the development of CIA, predominantly recognize posttranslational modifications of the lysine at position 264 of the CII molecule. Posttranslational hydroxylation and glycosylation of this single amino acid generates five different T cell determinants within the CII(256–270) segment. Importantly, most of the T cell response was directed to the Gal-264 CII(256–270) peptide which is consequently immunodominant. Our work highlights the critical importance of using glycopeptides when looking for T cell determinants within a glycosylated protein. Previous studies on T cell reactivity to CII which were performed with non-modified peptides permitted only the discovery of the non-modified CII(256–270) T cell determinant [17, 18].

It is interesting to note that the presently demonstrated T cell recognition of the immunodominant CII(256–270) glycopeptide is, so far, the only example of MHC-restricted T cell recognition of a naturally selected glycopeptide, in spite of the abundant presence of glycosylation in eucaryotic proteins. However, there are several examples of both MHC class I- and class II-restricted, glycopeptide-specific T cells which have been isolated

after immunization with peptides synthesized with carbohydrates added to amino acids at TCR contact points. Interestingly, T cell recognition has been demonstrated only with carbohydrate side chains of one or two, and in one example of three, saccharides, but not larger [8–11, 13, 23, 24]. Possibly, larger carbohydrates may be difficult for T cells to recognize in an MHC-restricted manner and APC may be unable to process large carbohydrates. Therefore, the limitation of O-linked carbohydrate side chains to one or two saccharides on CII may be a prerequisite for the T cell recognition.

By sequencing fourteen clonally distinct T cell hybridomas (specific for six different T cell determinants), we showed that the anti-CII TCR repertoire in CIA is highly diverse. However, a more conserved pattern of TCR structures was seen within each group of hybridomas specific for one posttranslationally modified form of the epitope. Recent studies have shown that the CII(256–270) segment is bound to the A^q molecule with I260 and F263 as anchors and the lysine at position 264 is a major T cell contact [25, 26]. Thus, it is not surprising that the T cell recognition is profoundly affected by the various modifications of the side chain of this lysine. The apparent restricted usage of TCR used for recognition of each specific form of the epitope is in agreement with earlier data of TCR recognition of both MHC class I- and MHC class II-bound peptides [27, 28]. This finding has been important for earlier studies on autoimmunity because the restricted TCR repertoire used for recognition of the encephalitogenic myelin basic protein MBP(1–11) peptide permitted a successful anti-TCR therapy in a mouse model for multiple sclerosis [28]. In contrast, in the case of CIA, the immunodominant CII segment appears in several different posttranslationally modified forms leading to a very diverse TCR repertoire.

Two groups have previously reported junctional sequences of TCR specific for CII. In both studies, DBA/1 mice were immunized with bovine CII and thirteen [16] or three [29] clonally distinct T cell hybridomas specific for bovine CII in the context of H-2A^q were established. Although not directly shown, it is likely that most, if not all, of those T cell hybridomas recognized the bovine CII(256–270) segment which is identical to rat CII(256–270) except at position 258 (a proline instead of a leucine in the rat CII). Because of this expected similar specificity, we were not surprised to see that the TCR of some of the T cell hybridomas described here (HD13.7, HM2.1 and HDBR.1) were very similar to some of the ones described in those reports. However, they identified ten additional gene segments used for recognition of CII (V α 8.4, V α 11.4, V α 22.1, J α 32, J α 37, V β 4, V β 8.3, J β 1.2, J β 1.3 and J β 1.6), none of which were found in the present study. Hence, the TCR repertoire in CIA is likely

to be even more diverse than the present study indicates. This finding is of importance since it clearly weakens the possibility of depleting T cells carrying certain TCR gene segments to treat arthritis, confirming some earlier *in vivo* experiments [6].

The results concerning the T cell recognition of the immunodominant CII(256–270) segment in CIA may have direct implications for RA. The structure of the peptide binding pocket of the H-2A^q and the RA-associated HLA DR4 (DRB1*0401) and DR1 (DRB1*0101) molecules are surprisingly similar. For example, A^q displays a deep P1 pocket typical of DR molecules. It was possible to predict that the DR4 (DRB1*0401) molecule should bind the CII(261–273) peptide and this was later confirmed in experiments using DR4-transgenic mice [30]. In fact, both DR4 (DRB1*0401)- and DR1 (DRB1*0101)-transgenic mice are susceptible to CIA and predominantly respond to CII(261–273) ([4] and manuscript submitted). The orientation of the CII(261–273) peptide in DR4 has been determined and compared to the orientation of the CII(256–270) peptide in A^q. The P1 position is F263 for binding to DR4 while I260 is used instead for binding to A^q. However, the TCR contact points of CII(261–273) presented by DR4 seem to involve both lysines at positions 264 and 270, depending on the T cell clone ([30] and manuscript submitted). Thus, it is likely that posttranslational modifications of this immunodominant CII segment are also important for T cell recognition in humans.

4 Materials and methods

4.1 Medium and antigens

Cells were cultured in DMEM supplemented with glutamine, streptomycin, penicillin, 2-ME, HEPES and 10% FCS. For *in vitro* stimulation of LN cells, FCS was replaced by 1% normal mouse serum. Rat CII was prepared from the Swarm chondrosarcoma [31] after pepsin digestion [32]. For use in cell cultures, native rat CII was heat-denatured for 30 min at 56°C. Peptides were synthesized essentially as described previously [25, 33]. After cleavage from the solid phase, the glycopeptides were purified by reversed-phase HPLC and structures were confirmed using amino acid analysis, fast-atom bombardment mass spectroscopy and 500 MHz ¹H NMR spectroscopy.

4.2 T cell hybridomas

Male DBA/1 and C3H.Q mice were immunized intradermally in the base of the tail and in the hind footpads with rat CII emulsified in CFA (Difco, Detroit, MI). After 9–11 days, single-cell suspensions of inguinal and popliteal LN were

prepared and stimulated with 25–50 µg/ml rat CII. To generate T cell hybridomas, the cells were fused with the TCR-negative variant of BW5147 [34] after an additional 3–5 days (hybridomas with the prefix HCQ) or after 2–4 cycles of restimulation with CII followed by expansion in IL-2 (hybridomas with the prefix HDB, HRC, HDBR and HD13). Further, established CII-reactive T cell lines were fused to generate hybridomas with the prefix HM2, HM1R, HNC and HDC. The HCQ hybridomas are derived from C3H.Q mice, and the others from DBA/1. Growing clones were tested for reactivity towards CII and pepsin. CII-reactive clones from a total of ten different fusions were subcloned by limiting dilution. For the measurement of T cell hybridoma reactivity, 5×10^4 T cell hybridomas were incubated with 5×10^5 syngeneic spleen cells and Ag in a volume of 200 µl in 96-well flat-bottom plates. After 24 h, 100 µl SN were removed and frozen. To the thawed SN, 10^4 IL-2-sensitive-CTLL [35] were added. The CTLL cultures were incubated for 24 h, after which they were pulsed with 1 µCi [³H]dThd for an additional 15–18 h. The cells were harvested on glass fiber sheets in a Filtermate TM cell harvester (Packard Instruments, Meriden, CT) and the amount of radioactivity determined in a matrix 96™ Direct Beta Counter (Packard). All experiments were performed in duplicate.

4.3 Molecular analysis of the TCR

Total RNA was prepared from T cell hybridomas by extraction with 4 M guanidine isothiocyanate followed by phenol/chloroform extraction and ethanol precipitation [36]. First-strand cDNA synthesis was performed using random hexadeoxynucleotides as primers. The synthesized first-strand cDNA containing TCR α and β chains was then used as template for the PCR amplification reaction. To amplify unknown TCR expressed by the T cell hybridomas, PCR was performed with 36 primers specific for known V α or V β gene segments [37, 38] (a generous gift from Jean-Laurent Casanova) and with the two following primers: “C α 1” (5’TGAACG TTC CAG ATT CCA TGG 3’), which is specific for the unique C α gene segment and “C β 1” (5’GCCAAG CAC ACG AGG GTA GCC 3’), which is a consensus primer specific for both the C β 1 and C β 2 gene segments. The PCR-amplified TCR α or β junctional regions were electrophoresed on a 2 % agarose gel and the DNA corresponding to the predicted size was isolated by use of positive pressure processing (EasyPrep from Pharmacia, Uppsala, Sweden). The nucleotide sequences were determined by the dideoxynucleotide chain termination method using an automated DNA sequencer (Perkin Elmer, Sweden) with the following internal primers: “C α 2” (5’ AAC AGG CAG AGG GTG CTG TCC 3’), which is specific for the unique C α gene segment and “C β 2” (5’ GCA ATC TCT GCT TTT GAT GGC 3’), which is a consensus primer specific for both the C β 1 and C β 2 gene segments.

The nomenclature for V α gene segment subfamilies follows that used in [39]: V α 1–10, [40]: V α 11–13 and [41]: V α 16 and 19. The V α subfamily is separated from the V α gene

segment by a period. V α sequences are as defined in [42]: V α 2, [43]: V α 4.5B6, [44]: V α 4.F5, [45]: V α 8.F3.2, [46]: V α 11.1a, [47]: V α 16.NY7.2, [41]: V α 16.H-15.E3 and [48]: V α 16.42H11. The nomenclature for J α gene segments follows that used in [49], while J α sequences are as in [49]: J α 10, 17, 20, 23, 42 and 44, [37]: J α 24 and [50]: J α 27. The nomenclature for V β gene segment subfamilies follows that previously described [38, 51–53]. V β sequences are as in [54]: V β 1, 6 and 10–12, [53]: V β 8.1 and 8.2 and [38]: V β 20. The J β sequences are from [55] and [56], except for J β 1.4, which is from [37].

4.4 TCR V α 2 T cell depletion experiment

B20.1 (anti-mouse TCR V α 2, [57]) and KT65 (anti-mouse TCR V α 8, [58]) are rat IgG2a mAb. MAR 18.5 is a mouse IgG2a mAb anti-rat kappa chain [59]. mAb were purified from culture SN by affinity chromatography on protein G-Sepharose and the protein content calculated from the absorbance at 280 nm. Primary Ab (120 µg B20.1 or KT65) was injected i.p. into female DBA/1 mice. To increase the depletion capacity [60], 170 µg MAR 18.5 was administered as a secondary Ab 1 h after the primary Ab. One day later, mice were immunized in each hind foot pad with 50 µg rat CII emulsified 1:1 in CFA. To measure the Ag-specific proliferative response, single-cell suspensions of pooled draining LN were seeded in triplicate, 10 days after the immunization, at 7×10^5 cells per well, in 96-well flat-bottom plates, in the presence of 100 µg/ml heat-denatured rat CII or 50 µg/ml peptides. FACS™ analysis was performed the same day with 3×10^5 LN cells stained with biotinylated B20.1 Ab (and FITC-streptavidin) and showed that more than 80 % of the V α 2-positive T cells had been deleted in the B20.1-treated mice. After 72 h, cell cultures (with Ag) were pulsed with [³H]dThd. After an additional 18 h, cells were harvested and the incorporation of [³H]dThd was determined.

Acknowledgments: We wish to thank Mevan Wijetunga for technical assistance and Drs. Andrew Cook and Simon Joscelyne for critical reading of the manuscript. This work was supported by the Swedish Medical Research Council, the Swedish National Association against Rheumatism, King Gustaf V’s 80 year, Osterman, Craaford, the Anna-Greta Craaford Foundation and the Swedish Natural Science Research Council.

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Note added in proof: The reference mentioned as "manuscript submitted" has now been published: **Andersson, E. C., Hansen, B. E., Jacobsen, H., Madsen, L. S., Andersen, C. B., Engberg, J., Rothbard, J. B., McDevitt, G. S., Malmström, V., Holmdahl, R., Svejgaard, A. and Fugger, L.**, Definition of MHC and T cell receptor contacts in the HLA-DR4restricted immunodominant epitope in type II collagen and characterization of collagen-induced arthritis in HLA-DR4 and juman CD4 mice. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 7574–7579.