The major T cell epitope on type II collagen is glycosylated in normal cartilage but modified by arthritis in both rats and humans

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Type II collagen (CII) is a target for autoreactive T cells in both rheumatoid arthritis and the murine model collagen-induced arthritis. The determinant core of CII has been identified as CII260–270, and the alteration of this T cell epitope by posttranslational modifications is known to be critical for development of arthritis in mice. Using CII-specific T cell hybridomas we have now shown that the immunodominant T cell epitope in the normal (healthy) human and rat joint cartilage is O-glycosylated at the critical T cell receptor recognition position 264 with a mono- or di-saccharide attached to a hydroxylysine. In contrast, in the arthritic human and rat joint cartilage there are both glycosylated and non-glycosylated CII forms. Glycosylated CII from normal cartilage could not be recognized by T cells reactive to peptides having only lysine or hydroxylysine at position 264, showing that antigen-presenting cells could not degrade the O-linked carbohydrate. Thus, the variable forms of the glycosylated epitope are determined by the structures present in cartilage, and these vary during the disease course. We conclude that the chondrocyte determines the structures presented to the immune system and that these structures are different in normal versus arthritic states.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease primarily affecting peripheral joints and believed to be initiated by the dysfunction of peripheral tolerance. RA is characterized by hyperplasia of the synovium and subsequent cartilage destruction and bone erosion. Since type II collagen (CII) is the major protein component of articular cartilage and since
several studies have demonstrated T and/or B cell responses against CII in RA patients [1–3], it has been suggested to be an autoantigen associated with the development and pathogenesis of RA. CII is a cartilage-specific homotrimeric fibrilar protein composed of three \( \alpha 1(II) \) chains. It contains posttranslational modifications since the lysines can be hydroxylated and then glycosylated.

Collagen-induced arthritis (CIA) is a widely used animal model for RA. CIA in mice is induced by immunization with CII in adjuvant. Like RA – which is genetically linked to HLA-DR class II molecules [DR1 (DRB1*0101, DRB1*1001) and DR4 (DRB1*0401, DRB1*0404, DRB1*0405)] [4, 5] – susceptibility to CIA in mice is associated with the expression of specific MHC haplotypes [6] and one of the underlying genes has been identified as the class II gene coding for \( \beta 1 \) [7].

Interestingly, the human class II molecules associated with RA – DR4 and DR1 – present the same peptide, and transgenic mice expressing \( \beta 1 \), DR4 or DR1 are susceptible to CIA [7–9]. The T cell response is directed towards the same immunodominant core, CII residues 260–270 [9, 10]. This T cell determinant contains lysine residues at position 264 and 270, which can become posttranslationally modified by hydroxylation and further galactosylated and glucosylated, creating many different epitopes containing hydroxyl, mono- and disaccharide groups in both RA and CIA. We have previously shown that autoreactive T cells predominantly recognize this epitope when it is posttranslationally modified at positions 264 and 270 by hydroxylation and further glycosylation [11–13]. The dominant T cell epitope is the side-chain of lysine at position 264 (K264) when the peptide is bound to \( \beta 1 \), but when bound to DR4 the T cells recognize either position K264 or K270, although position 264 seems to be the more dominant of the two in this case.

The degree of posttranslational modification on CII is believed to be dependent on the time between synthesis of the \( \alpha \)-chains in the endoplasmatic reticulum and formation of the triple helix [14]. In the APC it has been shown that carbohydrates can play a role in protein assembly and folding, can provide protease protection and can also determine the orientation and location of the binding sites of the proteins [15–17]. In RA and CIA, cartilage proteins (including CII) typically undergo degradation by matrix metalloproteinases, leading to the generation of peptides that can bind to MHC II and then cause T cell activation. In the present report, we have investigated the posttranslational modification differences in the immunodominant T cell epitope CII260–270 derived from arthritic and normal joints and how the glycosylated CII is handled by various APC.

**Results**

The T cell epitope CII260–270 is completely glycosylated in normal but not in arthritic human and rat joint cartilage

To investigate the T cell response against CII derived from arthritic and normal joints, we used \( \beta 1 \)-restricted and DR4 (DRB1*0401)-restricted CII-specific T cell hybridomas (Table 1). These T cell clones are highly specific for various different forms of posttranslational

<table>
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<th>Table 1. List and specificity of the T cell hybridomas used in this study</th>
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<tr>
<td><strong>Aβ restricted</strong></td>
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<td>HCQ.3</td>
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<td>HDQ.9</td>
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<td>HCQ.11</td>
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<td>HCQ.10</td>
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<td>HRC.2</td>
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<td>HDBR.1</td>
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<td><strong>DR4 restricted</strong></td>
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<td>mDR-1.1</td>
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<td>QDR-4</td>
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<td>hDR-1.1</td>
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modification at position 264: clone HDQ.9 sees peptide K264 [with a non-modified lysine at position 264] bound to A^8; HDBR.1 sees the hydroxylated peptide HK264 [with (5R)-5-hydroxy-L-lysine at position 264] bound to A^8; HCQ.3 sees the O-linked β-galactosylated peptide Gal-HK264 [with a β-D-galactopyranosyl residue on L-hydroxylysine at position 264] bound to A^8; HCQ.11 sees the O-linked β-galactosylated-glucosylated peptide Glc-Gal-HK264 [with β-D-glucopyranosyl-(1->2)-β-D-galactopyranose at position 264] bound to A^8; QDR.4 sees the non-modified-lysine peptide bound to DR4; and mDR-1.1 sees an O-linked β-galactosylated peptide bound to DR4 (Fig. 1a).

For the comparative analysis, we prepared rat CII from arthritic joint cartilage (AC), normal joint cartilage (NC) and Swarm chondrosarcoma (SC). As shown in Table 2 and Fig. 2a, HCQ.3 hybridoma, which is specific for galactosylated CII260–270, responded to all rat CII preparations, whereas hybridoma HDQ.9, specific for non-modified CII260–270, reacted only to the rat CII derived from AC and SC, but failed to react with rat CII derived from NC. The more common form of the modification seems to be the O-linked galactosylation; however, a disaccharide with both galactose and glucose was identified by the T cells (HCQ.11 hybridoma, Table 2). These data demonstrate that the immunodominant T cell epitope CII260–270 in the normal joint cartilage is completely O-glycosylated at position 264, whereas in the arthritic joint cartilage both glycosylated and non-glycosylated (possibly de-glycosylated) epitopes are presented. Rat CII derived from SC contains CII epitopes with different posttranslational modifications at position 264 as all of the used hybridomas responded to this collagen (Table 2, Fig. 2). Similar responses to the rat CII preparations were obtained from DR4-restricted T cell hybridomas (Table 2, Fig. 2b). HDBR.1 hybridoma cells specific for the hydroxylated epitope (HK264) responded neither to normal nor to arthritic rat CII (Table 2).

To determine whether humans show the same cartilage composition of the T cell epitope, we prepared and tested human CII derived from normal as well as from RA and osteoarthritis (OA) cartilage. Our results (Table 2, Fig. 3) confirm that in both rats and humans, the T cell epitope CII260–270 is completely glycosylated with a mono- or di-saccharide at position 264 in normal joint cartilage. In contrast, arthritic cartilage contains both glycosylated and non-glycosylated epitopes. There was no difference between CII derived from RA and OA cartilage. A response of HK264-specific hybridomas to human CII (normal, OA and RA) was also, as for rat collagen, not detectable (Table 2). In addition, we tested all rat and human CII samples with a DR4-restricted T cell hybridoma specific for galactosylation at position 270 instead of 264 (hDR-1.1), but none of them was able to stimulate these T cells (Table 2).

To investigate whether glycosylated CII from normal cartilage could be de-glycosylated, we periodate (NaIO_4) treated CII and compared the T cell response with the untreated CII from the same source using the same A^8 - and DR4-restricted T cell hybridomas. As shown in Fig. 4, HDQ.9 and QDR.4 reacted to periodate-
treated (de-glycosylated) CII, whereas the response of HCQ.3 and mDR-1.1 was abolished by periodate treatment of CII. These data confirmed our finding that there are not enough accessible non-glycosylated CII260–270 determinants in the normal joint cartilage and that this particular T cell epitope is predominantly glycosylated. In contrast, in the arthritic joint cartilage Gal-HK264 CII260–270 as well as non-glycosylated CII260–270 determinants were detected. The results also showed that the glycosylated side chain in the T cell epitope could not be processed in the APC used (spleen cells). As this is an important point we wanted to confirm it using triple-helical peptides (THP), which are potent and defined antigens, and to specify whether any APC type could process the glycosylated side-chain.

Short, synthetic homotrimeric THP are processed by APC

To mimic the triple-helical structure of CII within the immunodominant T cell epitope and to determine whether this triple-helical part needs intracellular processing for T cell activation, we used short synthetic homotrimeric THP that included either native or glycosylated sequences of the T cell epitope CII259–273 (Fig. 1b) [18]. The THP are covalently bound in one end of the triple helix and closed by a series of G–P–OHP in the other end. Non-modified single peptides and CII259–273 glycosylated at position 264 were used as controls.

Table 2. T cell hybridoma responses to rat and human CII

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<th>Aα restricted hybridomas</th>
<th>DR4 restricted hybridomas</th>
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<tr>
<td></td>
<td>HDQ.9 (K264)</td>
<td>HDBR.1 (HK264)</td>
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<tr>
<td></td>
<td>HCQ.3 (Gal-HK264)</td>
<td>HCQ.11 (Glc-Gal-HK264)</td>
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<tr>
<td>Rat CII</td>
<td></td>
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<tr>
<td>Normal cartilage</td>
<td>1.1±0.3</td>
<td>0.9±0.6</td>
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<tr>
<td>Arthritic cartilage</td>
<td>4.8±0.4</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>Swarm chondrosarcoma</td>
<td>8.1±2.7</td>
<td>20.1±5.4</td>
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<tr>
<td>Human CII</td>
<td></td>
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<tr>
<td>Normal cartilage (#1)</td>
<td>1.1±0.2</td>
<td>0.8±0.6</td>
</tr>
<tr>
<td>Normal cartilage (#2)</td>
<td>1.0±0.4</td>
<td>0.6±0.6</td>
</tr>
<tr>
<td>OA cartilage (#1)</td>
<td>20.5±2.8</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>OA cartilage (#2)</td>
<td>15.2±3.2</td>
<td>0.7±0.8</td>
</tr>
<tr>
<td>RA cartilage (#1)</td>
<td>22.8±4.6</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>RA cartilage (#2)</td>
<td>27.6±3.8</td>
<td>0.8±0.5</td>
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**a) T cell hybridoma cells (5×10⁴) were incubated with syngeneic spleen cells (5×10⁵) as APC in the presence of 50 μg/ml rat or human CII for 24 h. IL-2 production in the supernatants was assayed by sandwich ELISA using the DELFIA system. Data are expressed as mean stimulation index ± SEM from three independent experiments, each with triplicate wells. The positive responses compared with a medium control are indicated in bold.**
As seen in Fig. 5, paraformaldehyde-fixed spleen cells (APC) are unable to present THP to the CII-specific T cell hybridomas, demonstrating that the triple-helical structure of CII requires intracellular processing for generating single peptides (immunodominant T cell epitopes). When the antigen concentration was high, some – albeit poor – presentation of non-glycosylated THP was observed on paraformaldehyde-fixed APC (Fig. 5b). This finding was not valid for glycosylated THP (Fig. 5a). Similar results were obtained with chloroquine-fixed APC (data not shown). When we used viable APC, the strongest T cell response was observed against THP (glycosylated or non-modified), whereas the single peptides were weaker activators of the T cell hybridomas, even when the antigen concentrations were the same (Fig. 5). In fact, presentation by spleen cells of THP was approximately 10-times more efficient when compared with the single peptides or with CII when the molar concentrations of the epitope were the same (data not shown). This shows that THP are more efficient for T cell activation than single peptides or CII are.

DC and macrophages, but not naive B cells, present short THP

In order to investigate which APC could process and present the synthetic glycosylated collagen to T cells, we co-cultured purified Gal-THP-stimulated skin DC, naive B cells or macrophages with CII-specific T cell hybridomas HCQ.3 and HDQ.9. Fig. 6a shows that DC and macrophages efficiently present glycosylated THP to glyco-specific T cells, whereas naive B cells do not. Naive B cells present only the single Gal-HK264 CII259–273 peptide. Macrophages present THP and single peptides equally well, whereas DC present single peptides better than they present THP (Fig. 6a, Table 3). No cross-reactivity of HCQ.3 and HDQ.9 hybridomas was detected (Fig. 6a, b).
To pinpoint whether the different APC were able to process the carbohydrates on CII, we used the same purified APC and antigens, but this time a T cell hybridoma with specificity for non-modified CII259–273 peptide instead of specificity for the glycopeptides (Fig. 6b, Table 3). A weak presentation of high doses of glycosylated THP to T cell hybridomas specific for non-glycosylated epitopes was observed for DC and macrophages, which could suggest that the linked carbohydrate is partially processed intracellularly by APC, but could also suggest that the synthetic THP are not completely glycosylated because of incomplete incorporation of glycosylated hydroxylysine during their preparation. Since the T cell response towards the glycosylated THP is much stronger, it is most likely that the CII glycopeptides with O-linked carbohydrate survive antigen processing intact.

In addition, to confirm that there is no crossreactivity of the THP because of the C-terminal branching element (a lysine dimer with 6-amino hexanoic acid), we tested non-glycosylated THP with different T cell hybridomas specific for either non-glycosylated CII259–273 epitope (HRC.2, HCQ.4) or epitope glycosylated at position 264 (HCQ.3, HCQ.10). No crossreactivity was detected (data not shown).

Fig. 5. T cell hybridoma responses to glycosylated and non-glycosylated single and triple-helical CII peptides after paraformaldehyde fixation of APC. (a) HCQ.3 and HCQ.10 hybridoma cells specific for Gal-HK264 CII260–270. (b) HRC.2 and HDQ.9 hybridoma cells specific for K264 CII260–270. T cell hybridoma cells (5×10⁴) were incubated with paraformaldehyde-fixed (closed symbols) or viable (open symbols) syngeneic spleen cells (5×10⁵) as APC in the presence of titrated concentrations of single CII259–273 peptide or THP for 24 h. IL-2 production in the supernatants was assayed by sandwich ELISA using the DELFIA system. Data are represented as mean values ± SE of triplicates.

Fig. 6. An example of T cell hybridoma responses to glycosylated THP, rCII (SCh) and single peptides presented from different APC. (a) HCQ.3 hybridoma cells specific for Gal-HK264 CII260–270. (b) HDQ.9 hybridoma cells specific for K264 CII260–270. T cell hybridoma cells specific for K264 CII260–270. T cell hybridoma cells (5×10⁴) were incubated with 1×10⁵ purified B cells, DC or macrophages in the presence of 5 µg/ml glycosylated THP or 50 µg/ml rCII for 24 h. Single peptides (5 µg/ml) were used as a control. IL-2 production in the supernatants was assayed by sandwich ELISA using the DELFIA system. Data are represented as mean values ± SE of triplicates.
Primed B cells can present THP, but cannot efficiently process the carbohydrate

We have earlier shown that antigen-primed B cells are more efficient as APC than naive B cells are [19]. Since naive B cells do not present the synthetic triple-helical analogues of CII (THP) to CII-specific T cell hybridomas (Fig. 6, Table 3), we wanted to address whether activated B cells presented THP more efficiently. Therefore, we immunized (B10.Q × DBA/1)F1 mice with non-glycosylated THP as well as native CII (derived from rat SCh) in CFA and, 12 days later, B cells from draining LN were purified as described later. These purified and differently antigen-primed B cells were incubated with titrated amounts of THP and co-cultured with T cell hybridoma cells specific for non-modified CII259–273 peptide. Our data show that primed B cells are efficient presenters of the synthetic collagen analogues to antigen-specific T cells and that this capacity is independent of the antigen used for priming (Fig. 7). This effect can be explained since the T cell epitope in its triple-helical form is a B cell epitope independently of whether it is glycosylated or not (unpublished data). These data support our earlier observations that antigen-primed B cells present CII more efficiently than naive B cells do [19].

To examine whether antigen-primed B cells can process the sugar, we co-cultured the same purified CII-primed B cells or DC isolated from the same mice with HCQ.10 (Fig. 8a) or HCQ.4 (Fig. 8b) hybridomas in the presence of glycosylated THP. Results were similar to those obtained earlier for DC and macrophages (Fig. 6). Thus, primed B cells can present THP, but in similarity with the DC and macrophages they cannot efficiently process the carbohydrate.

![Fig. 7. Presentation of non-glycosylated THP by native-CII-primed B cells and THP-primed B cells. Primed B cells were prepared and purified from draining LN of previously immunized mice. Positively selected B cells (1 × 10^6) were incubated with HCQ.4 hybridoma cells (5 × 10^5) in the presence of titrated concentrations of THP for 24 h. The level of IL-2 production was determined as proliferation of CTLL cells, and cpm were counted. Data are represented as mean values ± SE of triplicates.](image-url)
Discussion

Joint-inflammation with subsequent destruction and remodeling of the cartilage and other joint-associated tissues are typical features for RA and CIA. It is well known that during the arthritic disease course CII undergoes degradation by different enzymes. This results in smaller CII fragments being picked up by APC and presented by MHC class II. Previous studies from our laboratory showed that CII-specific T cells predominantly recognize glycosylation at position 264 in the CII260–270 epitope and that glycosylated CII is more arthritogenic than non-glycosylated CII is [3, 11, 13, 20]. In addition, adoptive transfer of glycopeptide-specific T cells into CII-immunized DBA/1 mice increases the incidence and severity of arthritis as well as the production of CII-specific antibodies [21]. Yet little is known about changes in the posttranslational modifications of CII during the inflammation and disease activity.

In this study, we demonstrate that the immunodominant CII260–270 epitope is glycosylated at position 264 in the normal joint cartilage, but to a lesser extent in the arthritic one, which suggests an important role for changes in the posttranslational modifications of CII in the development of RA, OA and CIA. All variants of the epitope were found to be present in CII derived from the Swarm rat chondrosarcoma. This was expected, as this is a tumor tissue in which chondrocytes proliferate rapidly and there is not enough time for complete posttranslational modification (hydroxylation and glycosylation). Using synthetic THP to mimic the triple-helix structure of CII within the immunodominant T cell epitope (260–270), we have shown that triple-helical CII or THP are processed intracellularly by APC and cannot be presented directly via surface MHC II molecules. Also, we found that APC do not efficiently process the sugar that is O-linked to THP.

In agreement with earlier findings we found that macrophages and antigen-activated B cells are efficient presenters of CII, whereas skin DC (Langerhan’s cells) are surprisingly inefficient [19, 22, 23]. However, although skin DC present CII poorly, they do present and process triple-helical CII analogues (THP) if they are short enough. Thus, the role of DC for priming responses to CII is still unclear and it is possible that macrophages are important for priming the T cell response to CII. Partial extracellular degradation of CII by matrix metalloproteinases has been reported to generate short triple-helical CII fragments, which are suitable for DC internalization or processing [24]. The same authors also showed that highly purified native CII α-chains were presented poorly by DC, whereas large CNBr fragments of CII were presented as efficiently as peptides were. Primed T cells can then activate B cells, leading to production of arthritogenic CII-specific antibodies with subsequent tissue destruction.

The most surprising finding of this study was that CII prepared from healthy joint cartilage from both rats and humans was glycosylated at position 264 of CII260–270 and showed no reactivity with clones specific for hydroxylated or non-glycosylated forms at the same position. In contrast, in CII from arthritis-affected cartilage – irrespective of whether these were derived from human RA, human OA or arthritic rats – both non-glycosylated and glycosylated CII260–270 were detected. As the CII was prepared from adult cartilage it represents only a minor fraction of all CII but there is no reason to believe that the pepsin-extracted CII is different from the non-extracted CII, although this possibility remains to be investigated. Nevertheless, the predominant glycosylation of the immunodominant epitope indicates that chondrocytes that are affected by an arthritic process in the cartilage switch to produce CII without posttranslational modifications at position

Fig. 8. T cell hybridoma responses to glycosylated THP, rCII (Sch) and single peptides presented from primed B cells and DC. (a) HCQ.10 hybridoma specific for Gal-HK264 CII260–270. (b) HCQ.4 hybridoma specific for K264 CII260–270. T cell hybridoma cells (5×10⁴) were incubated with 1×10⁵ purified antigen-primed B cells or DC in the presence of 5 μg/ml glycosylated THP or 50 μg/ml rCII for 24 h. Single peptides (5 μg/ml) were used as a control. IL-2 production in the supernatants was assayed by sandwich ELISA using the DELFIA system. Data are represented as mean values ± SE of triplicates.
264. Alternatively, the carbohydrates on CII may be degraded extracellularly.

These findings raise several questions, such as which forms of posttranslational modification induce tolerance and/or which forms are of importance for triggering of arthritogenic T cells. The issue is complicated by the fact that immune tolerance to CII, in both mice and humans, is more pronounced to non-glycosylated lysine at position 264 than to glycosylated CII [3, 25]. It is therefore likely that, in spite of the lack of non-glycosylated lysine at position 264 CII in normal adult cartilage, exposure has occurred earlier or in other tissues. CII is known to be more actively synthesized and have a more widespread expression during fetal and neonatal development [26]. In addition, it has not been excluded that CII is expressed in the thymus at some stages. However, if CII is expressed in cells other than a chondrocyte, or at a faster rate, it is less likely that it is glycosylated. Nevertheless, tolerance to non-glycosylated epitopes is not complete and the appearance of such epitopes in the arthritic cartilage might reveal a neo-epitope of crucial importance for driving a chronic relapsing disease.

Since the carbohydrates may play a protective role against proteolytic degradation of proteins [17], de-glycosylation could also be involved in the creation of cleavage sites for proteases, leading to an enhanced extracellular degradation of CII. To address the question of whether de-glycosylation of CII plays a role in driving chronic relapsing arthritis due to epitope shift in the T cell recognition, we are currently investigating the changes in posttranslational modifications of CII260–270 and T cell response at different time points of CIA.

In conclusion, we have demonstrated that the immune system can only recognize what the chondrocytes have produced and, interestingly, in normal cartilage the dominant form on the immunodominant T cell epitope is glycosylated at position 264. The formation of neoepitopes by the appearance of de-glycosylated CII260–270 in disturbed cartilage, as in RA, could possibly play a role as immune stimulus to drive a chronic disease. Thus, T cells specific for different posttranslational modifications may play unique regulatory or disease-mediating roles at different stages of disease development.

Materials and methods

Antigens

Rat CII was prepared from knee-joint cartilage of 30 healthy DA rats, from arthritic knee-joint cartilage of 30 DA rats in a chronic phase of CIA (day 40 after immunization) or from rat Sch. Human CII was obtained from joint cartilage after replacement surgery in RA and OA patients (females, between 48 and 77 years old) at an advanced stage of disease or from healthy individuals undergoing surgery because of hip trauma (female and male patients, 85 and 77 years old, respectively) without any clinical manifestations of RA or OA.

All rat and human CII samples were prepared by limited pepsin digestion, and further purified as previously described [27]. Sodium periodate oxidation of CII (leading to de-glycosylation) was performed by incubating CII (5 mg/ml) in 0.05 M sodium acetate, pH 4.5, containing 0.02 M sodium periodate (Sigma-Aldrich Chemie GmbH) for 24 h at 4°C in darkness, after which a molar excess of glycol was added and the material was dialyzed against 0.1 M acetic acid. The synthetic CII single peptides [non-glycosylated CII259–273 (i.e. K264), sequence GIAGFKGEOQPKGEPE; hydroxylated CII259–273 (i.e. HK264), sequence GIAGFK(OH)EOQPKGEP; Gal-Hyl264 CII259–273 (i.e. Gal-HK264), sequence GIAGFK(Gal-Hyl)EOQPKGEP] and THP (Fig. 1) were synthesized, purified and characterized as previously described [28]. All collagens and synthetic peptides were dissolved and stored in 0.1 M acetic acid at 4°C.

Animals and immunization

DBA/1 mice were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and B10.Q mice from Prof Jan Klein, Tübingen, Germany. We also used (B10.Q × DBA/1)F1 mice, 8–10 weeks of age. They were fed standard rodent chow and water ad libitum. For B cell priming, mice were immunized with either 50 μg of rat CII (derived from Sch) or 10 μg TNP, emulsified 1:1 in CFA H37Ra (Difco, Detroit, MI, USA) in the hind footpads and at the base of the tail in a total volume of 100 μl.

Preparation of APC

LN cells were prepared from non-immunized (B10.Q × DBA/1)F1 mice or from mice immunized 11–12 days previously with 50 μg of CII or 10 μg TNP emulsified in CFA H37Ra. B cells were separated from LN cells using B220 RA3-6B2 (anti-B220) microbeads and a magnetic cell sorter (MACS) as described by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Peritoneal cells were collected by peritoneal lavage. Since peritoneal exudates contain 40–50 % B cells, we used anti-B220 microbeads to deplete B cells and the remaining cell population is referred to as macrophages. Langerhan’s cells were prepared from the ears [23] and we refer to them as DC as they showed no contamination by macrophages. The purity of the B cells, DC and macrophages was confirmed by flow cytometry, after staining with CD45R/B220 (RA3-6B2)–Cy-Chrome, CD11c (N418)–biotin or CD11b (Mac I)–PE antibodies, respectively, using FACS™ and Cellquest™ software (BD PharMingen, San Diego, CA, USA). For the fixation of APC we used a final concentration of 1% paraformaldehyde (MERCK, Darmstadt, Germany) in the cell cultures. The cells were fixed during 15 min at room temperature and were washed with complete medium containing 10% FCS before they were stimulated with antigen and co-cultured with antigen-specific T cell hybridomas.
T cell hybridomas and T cell activation assay

A4-restricted and DR4 (DRB1*0401)-restricted T cell hybridomas specific for different posttranslational modifications of CII260–270 were used (Table 1). The hybridomas were generated as described previously [3, 13]. Hybridomas cells (5 × 10⁶) were co-cultured with corresponding CII antigens and syngeneic APC (5 × 10⁵) in DMEM supplemented with 10% FCS, 100 IU penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂ in air and high humidity for 24 h. Production of IL-2 in the supernatants was determined either with a sandwich ELISA using the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) system (Wallac, Turku, Finland) and recombinant mouse IL-2 as a positive control or with a CTLL assay [3].

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