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A Transient Post-Translationally Modified Form of Cartilage Type II Collagen Is Ignored by Self-Reactive T Cells

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Lysine residues in type II collagen (CII) are normally hydroxylated and subsequently glycosylated in the chondrocyte. The immunodominant T cell epitope of CII involves such post-translationally modified lysine at position 264 that has been shown to be critical in the pathogenesis of murine collagen-induced arthritis and also in human rheumatoid arthritis. In this study we identified a line of transgenic mice expressing a TCR specific for hydroxylated rat CII epitope. They were crossed with transgenic mice expressing the rat CII epitope, either specifically in cartilage (MMC mice) or systemically (TSC mice), to analyze T cell tolerance to a post-translationally modified form of self-CII. The mechanism of T cell tolerance to the hydroxylated CII epitope in TSC mice was found to involve intrathymic deletion and induction of peripheral tolerance. In contrast, we did not observe T cell tolerance in the MMC mice. Analysis of CII prepared from rat or human joint cartilage revealed that most of the lysine 264 is glycosylated rather than remaining hydroxylated. Therefore, we conclude that the transient post-translationally modified form of cartilage CII does not induce T cell tolerance. This lack of T cell tolerance could increase the risk of developing autoimmune arthritis. The Journal of Immunology, 2004, 173: 4729–4735.

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1 Abbreviations used in this paper: RA, rheumatoid arthritis; CI, type I collagen; CIA, collagen induced arthritis; CII, type II collagen; HK264 peptide, CII256–270 with a 5R-hydroxy-L-lysine at position 264; Gal-HK264 peptide, CII256–270 with a β-D-galactopyranosyl residue on L-hydroxylysine at position 264; K264 peptide, CII256–270 with a nonmodified lysine at position 264; LN, lymph node; MMC, mutated mouse collagen; TSC, T cell epitope in systemic collagen.

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of heterologous CII (MMC mice) (21). In these mice the heterologous CII epitope is expressed as a cartilage-specific self-Ag. MMC mice have a lower incidence of arthritis after immunization with rat CII than normal mice. The T cell response to rat CII was also reduced, but not completely diminished, in MMC mice. The T cell tolerance in MMC mice was found to be independent of the presence of the thymus (22). These results suggest that T cell tolerance to CII is indeed induced by the self-Ags expressed in the cartilage, although we have only limited information on the mechanism of the T cell tolerance.

We also generated another line of transgenic mice expressing mutated type I collagen (CI) with the heterologous CII epitope (TSC mice) (21). Consequently, in these mice the CII epitope is expressed systemically (i.e., skin). In contrast to MMC mice, none of the TSC mice developed arthritis after immunization with heterologous CII. There was virtually no T cell response to rat CII in vitro. The result indicates that T cells specific for the heterologous CII epitope are completely tolerized when the epitope is expressed systemically. However, as in the case of MMC mice, the mechanism of T cell tolerance in TSC mice is also unclear.

Studies using TCR transgenic mice have contributed greatly to revealing the mechanisms of T cell tolerance to various self-Ags. The tolerance mechanisms include clonal deletion, clonal anergy, and active suppression (23–28). Self-ignorance is an alternative mechanism preventing the development of autoimmunity, even though the self-reactive T cells are not tolerized and maintain their potential to induce the disease in this case (29). In this study we used a line of CII-specific TCR transgenic mice (30) to clarify the mechanism of T cell tolerance to the heterologous CII epitope in either MMC mice or TSC mice. It was revealed that the post-translational modification of CII influences T cell tolerance to self-CII.

Materials and Methods

Mice

The B10.Q mice originated from Dr. J. Klein (University of Tübingen, Tübingen, Germany) and have been maintained as an inbred strain in our animal facility. The MMC-1 (in this work referred to as MMC) and TSC transgenic mice have previously been described (21). Briefly, the MMC transgene is a mutated mouse CII gene in which position 266 has been changed from an aspartic acid (D) to a glutamic acid (E), thereby expressing the heterologous CII256–270 epitope in a CII-restricted fashion. TSC mice express the heterologous CII256–270 within mouse CII sequence. MMC and TSC transgenic mice were backcrossed 10 generations onto the B10.Q background. The anti-CII TCR transgenic founder mouse was provided by Dr. W. C. Ladiges (University of Washington School of Medicine, Seattle, WA) (30). Mice were originally of the DBA/1 background, but were backcrossed eight generations onto the B10.Q background. The TCR transgenic mice were also crossed with MMC or TSC mice. The offspring were screened for the expression of the transgenes by genomic PCR (21, 30) and then used for the experiments. All animals were bred and kept in our animal facility (http://net.inflam.lu.se/).

Antigens

CII was prepared from rat chondrosarcoma (Swarm), mouse xiphoid, rat knee joint cartilage, and human hip joint cartilage (obtained from replacement surgery). For all these samples, CII was solubilized with pepsin digestion and was further purified as described previously (19). The following rat CII256–270 peptides, with or without modifications of the lysine residue at 264, were used: K264 peptide (with a nonmodified lysine at position 264), HK264 peptide (with (5R)-5-hydroxy-L-lysine at position 264), and Gal-HK264 peptide (with a D-galactopyranosyl residue on L-hydroxyllysine at position 264). The CII peptides were synthesized, purified, and characterized as previously described (31). Both CII and peptides were dissolved and stored in 0.1 M acetic acid at 4°C.

Lymphocyte assays

Lymph node (LN) cells (1 × 10⁶) were cultured with various doses of Ags for 72 h in flat-bottom, 96-well plates (Nunc, Roskilde, Denmark). One microcurie of [³H]Tdr was added to each well for an additional 15–18 h. Cell proliferation was measured by counting the incorporation of [³H]Tdr. The amount of IFN-γ in the culture supernatant was measured by ELISA, as described previously (32).

Flow cytometry

FITC-conjugated Vβ8.2 mAb (F23.2) and PE-conjugated anti-TCR β-chain mAb (H57-597; BD Pharmingen, San Diego, CA) were used. The cells were stained with mAbs and analyzed by FACSort flow cytometer with the CellQuest Pro program (BD Bioscience, Mountain View, CA). We added propidium iodide (1 µg/ml) to the cell suspensions before running them on the cytometer to exclude dead cells.

T cell transfer experiment

LN cells were prepared from naive TCR single transgenic mice and pooled. The cells were labeled with a cytoplasmic dye, CFSE (Molecular Probes, Leiden, The Netherlands). Briefly, the cells were incubated 10 min with 5 µM CFSE in PBS at 37°C. After stopping the reaction by adding an equal volume of FBS, the cells were washed three times with PBS. Then the CFSE-labeled cells (1 × 10⁷) were injected i.v. into naive normal BQ mice, MMC transgenic mice, or TSC transgenic mice. In some experiments, the recipient mice were immunized with 100 µg of CII peptide 256–270 with hydroxylysine at 264 emulsified with an equal volume of IFA (Difco, Detroit, MI) 1 wk after the transfer. Inguinal LN cells of the recipients were harvested and stained with PE-conjugated anti-TCR β-chain mAb (H57-597). Then the cells were analyzed with a flow cytometer. The rest of the LN cells were tested for in vitro response to the CII peptide.

T cell hybridoma assays

T cell hybridoma cell clones specific for a different post-translationally modified form of CII256–270 were generated as described previously (16). The hybridoma cells (5 × 10⁴) were stimulated with corresponding CII peptides, rat chondrosarcoma-derived CII, rat joint cartilage-derived CII, or human joint cartilage-derived CII in the presence of irradiated syngeneic spleen cells (5 × 10⁵) in flat-bottom, 96-well plates. The responses of the hybridoma cells were determined by measuring IL-2 production in the supernatants by ELISA.

Results

Fine specificity of the CII-specific transgenic TCR

The TCR transgenic mouse originated from a TCR Vβ8.2+/Vo11.1a cDNA double-transgenic founder and was backcrossed to B10.Q mice. The TCR genes were derived from a DBA/1 T cell specific for bovine CII (30). However, the epitope specificity and its potent cross-reactivity to mouse CII were originally not determined. Therefore, we first examined the response of T cells in the TCR transgenic mice to rat or mouse CII (Fig. 1a). LN cells from the naive TCR transgenic mice responded to heterologous rat CII; at the same time they showed no cross-reactivity with mouse CII. Because the CHI256–270 peptide is the immunodominant peptide in the IA₄-restricted T cell response, we investigated the response to the various modified forms of this peptide (depicted in Fig. 2). As shown in Fig. 1b, T cells in the TCR transgenic mice recognized the CHI256–270 peptide when the lysine residue at position 264 was hydroxylated. They did not respond to the peptide when the hydroxylated lysine was further glycosylated. Thus, it was revealed that the transgenic TCR is specific for heterologous CII peptide 256–270 with hydroxylated lysine at 264. These results indicate that the TCR transgenic mice can be used as a suitable tool for analyzing T cell tolerance in MMC or TSC mice that express the T cell epitope of heterologous CII. To avoid polyclonal T cell responses to various post-translationally modified forms of CII, we used the hydroxylated CHI256–270 peptide instead of whole CII molecules for stimulation of the T cells.
transgenic mice and TCR arthritis or, in fact, any other autoimmune disease. IFN-γ interferon production to the heterologous CII peptide, indicating complete tolerance of T cells to the systemically expressed self-Ag. double-transgenic mice showed no proliferative response or cytotoxicity by FACS analysis. Unfortunately, as mAb specific to the TCR α-chain (Vα11.1a) is not yet available, we were only able to measure the number of T cells expressing the transgenic TCR β-chain (Vβ8.2). Consistent with the data of T cell response, we observed no significant difference in the number of Vβ8.2-expressing T cells from lymph nodes between the TCR single-transgenic mice and TCR × MMC double-transgenic mice. In contrast, there was a significant reduction of Vβ8.2-expressing T cells in the LN of TCR × TSC double-transgenic mice compared with TCR single-transgenic mice (p < 0.05). The reduction of Vβ8.2+ T cells in TSC mice was also observed in the thymus, but there was no difference between TCR single-transgenic mice and TCR × MMC double-transgenic mice (Fig. 6b). Thus, central deletion may be involved in the mechanisms of T cell tolerance in TSC mice, but such a deletional mechanism does not operate in MMC mice.

No evidence of in vivo recognition of CII by the TCR transgenic T cells in MMC mice
Because the T cell tolerance observed in MMC single-transgenic mice is not complete (21), it is possible that a too high frequency of Ag-specific T cells in TCR transgenic mice overcomes the relatively weak tolerance induction in MMC mice. To test this possibility, we transferred a limited number of T cells from naive TCR single-transgenic mice to MMC or TSC single-transgenic mice. We then analyzed the proliferative response of the transferred T cells to the CII peptide in vitro and in vivo. To detect the transferred T cells and also to visualize in vivo division, cells were labeled with a cytoplasmic dye, CFSE, before the transfer.

After the transfer into TSC mice, TCR transgenic T cells were clearly detected, because they had spontaneously divided in vivo (Fig. 5a). Subsequently, they lost their ability to respond to restimulation with the Ag in vitro (Fig. 5b). This indicates that the T cell epitope is expressed in the peripheral part of the immune system of TSC mice as well as in the thymus. It also reveals that induction of anergy may work as a T cell tolerance mechanism in these mice. In contrast, the T cells transferred to MMC mice showed no in vivo division similar to those transferred to normal mice. In addition, the T cells in MMC recipient mice clearly responded to the CII peptide in vitro. There was no significant difference in either the proliferation or the number of transferred T cells between MMC and normal recipient mice (0.63 ± 0.09 and 0.71 ± 0.14% in TCRαβ+ cells, respectively).

In an additional experiment, we immunized the recipients with the hydroxylated CII peptide 1 wk after transfer and subsequently determined the number of dividing T cells in the draining LNs (Fig. 6a). However, we found no differences between MMC and normal mice in either dividing or nondividing cells (Fig. 6b). The
T cells transferred to MMC mice responded to the CII peptide in vitro similar to those in normal mice (Fig. 6c). Therefore, there was no evidence of induction of tolerance in the heterologous CII-specific TCR transgenic T cells in MMC mice, even when the frequency of the Ag-specific T cells was <1% of the total T cells. Thus, T cells specific for hydroxylated CII \(_{256-270}\) do not recognize their Ag in cartilage in vivo. In contrast to the T cells transferred to MMC mice, T cells in TSC mice did not respond to the CII peptide after in vivo immunization (data not shown).

Lack of hydroxylated lysine in the dominant epitope of CII in joint cartilage

From these data we concluded that T cells specific for hydroxylated CII are immunologically ignorant of CII in cartilage. However, this is incompatible with previous data obtained from MMC single-transgenic mice, where T cells responded poorly to heterologous CII (21). One of the differences between these systems is that the CII-specific T cells are monoclonal in TCR \(_{×}\)MMC double-transgenic mice, whereas the responding T cells are more polyclonal in MMC single-transgenic mice. As shown in Fig. 1, the transgenic TCR is specific for hydroxylated CII peptide. One possible explanation for the discrepancy is a selective lack of hydroxylated CII in cartilage. Because the rat CII used in the previous experiment was prepared from a rat chondrosarcoma, it was of interest to examine whether natural joint cartilage-derived CII also stimulates T cells specific for the hydroxylated CII peptide. However, it is difficult to collect enough joint cartilage CII from MMC mice. Therefore, we compared various T cell hybridoma clones with different specificities for post-translational modification of CII in their responses to rat CII derived from joint cartilage or chondrosarcoma. T cell clones specific for the glycosylated peptide responded to both chondrosarcoma-derived and cartilage-derived rat CII (Fig. 7c). However, the hydroxylated peptide-specific T cells responded only to chondrosarcoma-derived, not to cartilage-derived, rat CII (Fig. 7b). Therefore, it is likely that the lack of T cell tolerance in the TCR \(_{×}\)MMC double-transgenic mice is explained by the absence of hydroxylated lysine 264 in cartilage CII in vivo. We also tested CII prepared from the skin of TSC mice using a similar assay. In this case, hydroxylated peptide-specific clone responded to CI from TSC mice (data not shown), which is consistent with the induction of tolerance to the TCR transgenic T cells in TSC mice.

We also prepared human CII from the joint cartilage of six different individuals and tested the responses of T cell hybridomas to these samples. In this experiment we used heterologous CII peptides with different modifications of lysine 264 as controls. It should be noted that there is no difference in the amino acid sequence between human and rat CII within this T cell epitope. All the T cell clones responded well to the corresponding CII peptides, indicating that there is almost no difference in the binding capacities of different peptides to class II molecules. However, there was a large difference in the response to human cartilage CII between the T cell clones. Nonmodified peptide-specific clone responded weakly to human CII (Fig. 8a), whereas the glycosylated peptide-specific clone responded vigorously to all human CII samples (Fig. 8c). In striking contrast, T cells specific for the hydroxylated peptide did not respond to human CII (Fig. 8b). All six different human cartilage samples showed the same pattern, indicating an invariable glycosylation at position 264. These data indicate that most of the lysine residue at 264 of CII, even in human joint cartilage, is glycosylated, whereas there are almost no CII molecules with hydroxylated lysine at 264.

Discussion

We have previously reported (21) that transgenic mice expressing heterologous CII epitope specifically in cartilage (MMC mice) or
systemic (TSC mice) display T cell tolerance to the immunodominant CII epitope. In this study we used heterologous CII-specific, TCR transgenic mice to clarify the mechanism of T cell tolerance in these mice.

We found that the T cell tolerance in TSC mice involves central tolerance (intrathymic deletion) as well as peripheral tolerance (induction of anergy). The latter may serve as a failsafe mechanism of self-tolerance for T cells that escape the central tolerance. The results seem reasonable, because in TSC mice the heterologous CII epitope is expressed on CI, which is a major component in connective tissues, and is expressed systemically, thus including the thymus. Previously, we had confirmed the expression of mRNA of the transgenic TSC CI in the thymus (21), although we did not know where the CII epitope was actually presented to T cells and, therefore, did not know the mechanism of T cell tolerance in these mice. To our knowledge, this is the first report showing in vivo presentation of the transgenic CII epitope in the thymus, as evidenced by the deletion of Ag-specific T cells in the thymus. This is possible through the use of transgenic TCR mice, because these enabled visualization of Ag-specific T cells. We also observed the expression of TSC CI mRNA in other organs, such as spleen and skin, that may be involved in the induction of peripheral anergy in vivo. These results are of importance in helping to understand the generation of T cell tolerance to systemically expressed extracellular matrix proteins.

In contrast, we did not observe any evidence of T cell tolerance in TCR × MMC double-transgenic mice. Tolerance of the TCR transgenic T cells was not observed in a T cell transfer experiment. This transfer of cells is a widely accepted method to reduce the unnaturally high frequency of Ag-specific T cells in TCR transgenic mice (33). These data are in contrast with the results from MMC single-transgenic mice, in which T cell tolerance to the CII epitope was observed (21, 22). The T cell tolerance in MMC single-transgenic mice indicates that the heterologous CII epitope is actually presented to T cells in vivo. Similar to MMC mice, we have also observed T cell tolerance to heterologous CII in human CII transgenic mice (34). One of the major differences between these studies is the clonality of the responding T cells; in the TCR × MMC double-transgenic system the responding T cells are monoclonal, whereas there is a polyclonal T cell response to CII in MMC single-transgenic mice or human CII transgenic mice. At this point, post-translational modification of CII molecules would play an important role. T cell clones with different specificities for the post-translational modification of the immunodominant epitope respond to CII in MMC single-transgenic mice (15, 16), whereas the TCR transgenic mice that we used in this study are specific only for the hydroxylysine at position 264. The lysine at position 264 of CII in joint cartilage is revealed to be glycosylated rather than remaining hydroxylated. Therefore, it is reasonable that the TCR transgenic T cells specific for hydroxylated CII do not recognize the transgenic CII in vivo (Figs. 5 and 6) and consequently are not tolerized. In addition, we have observed, using another line

![FIGURE 5](image-url) Induction of tolerance in TCR transgenic T cells transferred to TSC mice. LN cells (1 × 10⁷) from naive TCR transgenic mice were stained with CFSE and transferred into normal BQ, MMC, or TSC mice. One week after the transfer, CFSE⁺ cells in inguinal LN of the recipients were detected by a flow cytometer (a). The proliferative response of the LN cells was also measured after stimulation with 10 μg/ml heterologous hydroxylated CII256–270 peptide (b).

![FIGURE 6](image-url) In vivo and in vitro responses of the TCR transgenic T cells transferred to MMC mice. As described in Fig. 5, CFSE-labeled LN cells were prepared and transferred to naive BQ mice or MMC mice. One week after the transfer, the recipients were immunized with either the hydroxylated CII peptide or PBS emulsified with IFA. After 1 wk, CFSE⁺ cells were detected in the draining LN by flow cytometry (a). The percentages of the in vivo divided cells (R1) and nondivided cells (R2) were calculated (b). The proliferative response of the recipient LN cells to the CII peptide was also measured (c).
of TCR transgenic mice, that glycosylated CII-specific T cells are

tolerized in MMC mice (35). This suggests that the glycosylated

CII epitope is indeed expressed in vivo. Even though the variable

post-translational modification of lysine residues in CII molecule

is known (13, 14), it is difficult to establish by biochemical meth-

ods the extent of modification of a lysine residue at a particular

position. The use of T cell clones specific for different post-translationally modified forms of rat

CII256–270 makes it easy to solve this problem (Figs. 7 and 8). We revealed that most of the lysine in rodent as

well as human cartilage CII is glycosylated in vivo. In our view

this is the first study to show the predominance of a particular form

of post-translational modification of CII molecule in natural

cartilage.

This result provides important information on the role of post-

translational modification of CII in the pathogenesis of autoim-

mune arthritis. Firstly, glycosylated forms of CII have been sug-

gested to be the major target of immune responses to cartilage CII. Neonatal treatment with glycosylated CII peptides was superior to

CII peptides with other modifications in protecting mice from CIA

development (17). The T cell response to CII is skewed toward the

glycosylated form of CII peptide in “humanized” transgenic mice

that express DR4, human CD4, and human CII (12). These results

seem reasonable, because most of the cartilage CII is in glycosy-
lated form. Importantly, this is not only observable in murine mod-
els of arthritis, but also in human RA. We have recently shown that in RA patients with the arthritis-susceptible DR4 (DRB1*0401) or

DR1 (DRB1*0101) alleles, T cells recognize mainly glycosylated

CII and that DR4-restricted T cell clones predominantly recognize

the side chain of lysine 264 (12). The predominance of glycosy-
lated lysine at position 264 of human cartilage-derived CII (Fig. 8)
is consistent with these data. Secondly, the lack of hydroxylysine in the T cell dominant epitope of CII molecules and the resulting

lack of T cell tolerance suggest that T cells are immunologically

ignorant of the presence of the self-Ag transiently expressed on
cartilage. Consequently, these T cells are easily activated and may

induce an autoimmune attack on joint cartilage once the epitope is

generated and exposed at a certain level. Immunological self-ig-
norance was first demonstrated more than a decade ago in a study

using transgenic mice expressing a viral Ag in the pancreas (29).

The mice suffered from autoimmune insulitis after an infection

with the virus, followed by activation of the neo-self-Ag-specific T

cells. This clearly indicates a possible development of autoimmune
disease by self-ignorant T cells. It is of note that such T cell ig-
norance is observed only when self-Ags are expressed at lower

than certain levels (36). Higher dose of self-Ags induces T cell

FIGURE 7. Expression of different post-translationally modified forms of the CII256–270 epitope in rat chondrosarcoma- or cartilage-derived CII. T cell hybridoma cells specific for different post-translationally modified forms of rat

CII256–270 were cultured with rat chondrosarcoma-derived CII (C) or rat joint cartilage-derived CII (●). After 24 h, IL-2 content in the supernatants was measured. The Tc1 clone was generated from the TCR transgenic mice and

expressed both the α- and β-chains of the transgenic TCR.

FIGURE 8. Expression of different post-translationally modified forms of the CII256–270 epitope in human cartilage-derived CII. T cell hybridoma cells specific for different post-translationally modified forms of rat CII256–

270 were cultured with either corresponding CII peptides (○) or human joint cartilage-derived CII from six different individuals (●). After 24 h, IL-2 was measured in the supernatants by ELISA. Note that there is no difference in amino acid sequence between rat and human CII256–270.
tolerance. Lysine residues in CII molecule are first hydroxylated, thereafter galactosylated (13, 14). These modifications occur in the chondrocyte before the triple helical structure is formed. At present, we do not know under what conditions the levels of post-translational modification of CII changes in vivo and the resulting outcomes. Additional studies are required on the role of changes in post-translational modification of CII in T cell tolerance and the development of arthritis.

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References


