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Therapeutic Vaccination of Active Arthritis with a Glycosylated Collagen Type II Peptide in Complex with MHC Class II Molecules

Balik Dzhambazov,* Kutty Selva Nandakumar,* Jan Kihlberg,† Lars Fugger,‡ Rikard Holmdahl,∗ and Mikael Vestberg∗

In both collagen-induced arthritis (CIA) and rheumatoid arthritis, T cells recognize a galactosylated peptide from type II collagen (CII). In this study, we demonstrate that the CII259–273 peptide, galactosylated at lysine 264, in complex with Aq molecules prevented development of CIA in mice and ameliorated chronic relapsing disease. In contrast, nonglycosylated CII259–273/Aq complexes had no such effect. CIA dependent on other MHC class II molecules (A/E) was also down-regulated, indicating a bystander vaccination effect. T cells could transfer the amelioration of CIA, showing that the protection is an active process. Thus, a complex between MHC class II molecules and a postranslationally modified peptide offers a new possibility for treatment of chronically active autoimmune inflammation such as rheumatoid arthritis. The Journal of Immunology, 2006, 176: 1525–1533.

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the articular synovial tissues initiated by leukocyte infiltration (mainly neutrophils, macrophages, and T cells) and secretion of inflammatory cytokines (TNF-α, IFN-γ, IL-1, IL-6), chemokines, and destructive enzymes such as matrix metalloproteases. Activation of T cells is believed to be an important pathogenic factor in the disease, although its exact role and potential as a therapeutic target have not yet been identified. The abnormal activation of T cells does, however, most likely occur years before the clinical diagnosis of the disease, as T cell-dependent IgG Abs specific for Ig Fe (rheumatoid factors) and citrullinated protein epitopes are highly predictive for disease (1, 2). Importantly, the risk for developing arthritis is increased in individuals who have both such Abs and express certain MHC class II (MHC II) molecules that share a specific peptide pocket, the so-called MHC shared epitope (3, 4). The MHC II region is also the strongest known genetic factor associated with RA. Taken together, these findings argue for a pathogenic role of MHC II-restricted autoreactive T cells. It has, however, been difficult to identify a single specificity of such T cells, although T cell reactivity to several autoantigens such as Bip, RA33, and GpI, and also joint-specific Abs such as type II collagen (CII), has been reported (5–8). CII is of particular interest, as an autoimmune response to this protein leads to collagen-induced arthritis (CIA) in mice, rats, and primates.

CIA, which is the most widely accepted mouse model of RA, is also genetically associated with the MHC II gene region (9), and one of the MHC II genes, Aq, has been identified to control development of arthritis (10). Interestingly, transgenic mice expressing the human MHC II shared epitope molecules, DR1 and DR4, also develop CIA in a manner strikingly similar to Aq-expressing mice (11–13). The Aq-restricted immunodominant CII peptide (CH263–271) has been identified (14, 15), and its binding to both MHC II molecules and TCRs has been characterized (16, 17). This has revealed several unique properties of the immune recognition in both RA and CIA that could provide clues to a further understanding of the disease process as well as providing new possibilities for treatment. In both CIA and RA patients, autoreactive T cells are directed against the same recognition sites on the shared immunodominant CII260–273 peptide (8, 13, 14, 16, 18–21). The lysine at position 264 (K264) can be hydroxylated and further glycosylated with mono- or disaccharides. Interestingly, the T cell recognition of CII-glycosylated peptides plays a major role in the development of autoimmune arthritis (16, 22–25).

Both oral and intranasal administration of CII protein or synthetic collagen peptides have been shown to suppress CIA (26–32). This suppression was associated with down-regulation of IgG2 secretion and an enhanced Th2-type response. Single synthetic peptides are susceptible to proteolytic degradation, and treatment of CIA with such Ags is effective only for a short period. Also, the required quantities of Ag or number of administrations are too high. To allow the use of smaller amounts of Ag and the efficacy of treatment to be longer, we have designed complexes between MHC II molecules and immunodominant peptides.

In this study, we demonstrate that soluble MHC II (Aq) molecules, complexed with the immunodominant CII259–273 galactosylated peptide, have both preventive and therapeutic effects on arthritis and mediate active bystander suppression.

**Materials and Methods**

*Design of the Aq constructs*

The cDNAs for Aa and Ab were amplified from a first strand cDNA reaction (first strand cDNA: Pharmacia). The cDNAs were further modified to include cloning sites immediately upstream of the start codon, and the 3′ end from the transmembrane domain and downstream was replaced by an
in-frame cloning site. Next, DNA for the leucine zipper (33) domain from Jun, including a 3’ end coding for six histidines, was cloned in frame with the β-chain CDNA. The DNA for the leucine zipper domain from Fos was added to the α-chain construct. The resulting constructs were cloned separately into pMTAL (Invitrogen Life Technologies) or pRMHa-3 (34) to allow for heavy metal-induced expression in insect cells. pMTAL contains the resistance gene for hygromycin. Where pRMHa-3 was used, a Copia promoter-driven hygromycin gene was used as selection marker.

**Transfection, expression, and purification of soluble Aβ**

The linearized Aβ-α-chain and Aβ-β-chain constructs were cotransfected at equimolar ratios into Drosophila melanogaster SL2 cells (American Type Culture Collection; CRL-1963) using calcium phosphate transfection. Stable transfectants were derived by hygromycin selection and kept under selection in Schneider’s Drosophila medium (Invitrogen Life Technologies) containing 100 μg/ml hygromycin B (Sigma-Aldrich). Large-scale cell cultures were prepared in Fernbach bottles using a magnetic stirrer. For expression of soluble Aβ, transfected cells were grown in serum-free insect express complete medium (PAA Laboratories) at 25°C and induced with 0.7 mM CuSO4 for 3 days, and the supernatants were clarified by centrifugation and filtration. The SL2 cells produced ~2–3 mg of recombinant protein per liter of culture. The expressed soluble Aβ molecules were purified from the clarified medium using Ni-NTA (Qiagen) affinity chromatography and the manufacturer’s recommended protocol. The dialyzed protein fractions were examined by ELISA, SDS-PAGE, and Western blot analysis. Aβ complexes were purified further on a Superdex 200 gel filtration column (Amersham Biosciences), concentrated again by Amicon centrifugal filter devices (Millipore), and stored at 4°C until used.

**Activation of T cell hybridomas**

Peptide/Aβ complexes were diluted in sterile PBS and coated onto plates by incubation at 4°C for overnight or added directly in soluble form to the hybridomas. The coated plates were then washed twice with sterile PBS to remove unbound protein complexes, and 5 × 104 T cell hybridoma 1F3 were added in well in 200 μl of DMEM supplemented with 5% FCS, 100 μM penicillin, and 100 μM/ml streptomycin. T cell hybridoma HCQ3 and HCQ4, specific for GalOK264 and for nonmodified CI259–273 (K264), respectively, were used in the hybridomas. When the mice were bred and used at the animal department of Medical Inflammation Research (www.imm.lu.se) and kept under standardized conditions.

**Antigens**

Rat CII was prepared from the Swarm chondrosarcoma, and bovine CII from bovine cartilage, by limited proteolysis and further purified, as previously described (35). The CII peptides (nonmodified CI259–273, GIAAGKFGQGQPKGE; GalOK264 CI259–273, GIAAGFK(Gal-HyG)GEQGPKGE) were synthesized, purified, and characterized, as previously described (14, 36, 37). The CII was dissolved in 0.1 M acetic acid. Mouse myelin oligodendrocyte glycoprotein MOG79–90 peptide (GKVTLRQNNRF) was purchased from Schauf-N. All peptides were dissolved in PBS. The collagen and peptides were stored at 4°C until used.

**Induction and clinical evaluation of arthritis**

To induce CIA, each mouse was injected with 100 μg of CII (rat CII for B10.Q and bovine CII for B10.Q × B10.RIII) mice, emulsified 1:1 in CFA (Difco) at the base of the tail in a total volume of 100 μl. Thirty-five days later, the mice were given a booster injection of 50 μg of rat CII emulsified 1:1 in IFA (Difco) in a total volume of 50 μl. Development of clinical arthritis was followed through visual scoring of the animals based on the number of inflamed joints in each paw, starting 2 wk postimmunization and continuing until the end of the experiment. An extended scoring protocol (38) ranging from 1 to 15 for each paw with a maximum score of 60 per mouse was used. The mice were examined two to four times per week for at least 70 days after immunization.

The B10.Q/BALB/c × B10.QJ/F mice were immunized with 100 μg of rat CII emulsified in IFA intradermally at the base of the tail on day 0 and boosted on day 35 intradermally with 50 μg of rat CII in IFA. The mice were scored for a minimum period of 202 days for arthritis development. Mice that developed chronic arthritis (mice with severe arthritis for a minimum period of 120 days were considered as chronic), including the ones with clear relapses, were selected for the treatment protocol.

**Measurement of serum anti-CII Ab levels**

Mice were bled at the time of boost immunization (day 35) as well as at the termination of experiment (day 70), and sera were analyzed for anti-CII IgG Ab levels by quantitative ELISA (39). Briefly, 96-well ELISA plates (Nunc) were coated overnight at 4°C with 10 μg/ml native rat CII in PBS. The wells were washed three times with PBS-0.1% Tween 20, and then 100 μl of blocking buffer (5% BSA in PBS) was added to each well and incubated for 1 h at room temperature. After washing, 50 μl of samples in serial dilutions from 1/100 to 1/105 were added and incubated for 2 h at room temperature. After three washes, peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG (for M5/114 Abs) (The Jackson Laboratory) for 1 h. After extensive washing, plates were developed using ABTS (Roche Diagnostic Systems) as substrate, and the absorbance was then measured at 405 nm in a Spectra Max Plus reader (Göteborgs Termometerfabrik). A standard serum from arthritic and nonimmunized syngeneic mice was added to each plate in serial dilutions as positive and negative controls, respectively.

**Pepptide/Aβ complex treatment protocols**

Animals were treated by either i.v. or intranasal administration of purified peptide/Aβ complexes. In the i.v. treatment of CIA model, mice were injected with GalOK264/Aβ, K264/Aβ, or MOG/Aβ complex (100 μg in 200 μl of PBS) on days 20 and 34 postimmunization (for the chronic model on days 7, 11, and 28 after reimmunization). Control mice were injected i.v.
with 200 μl of PBS on the same days. In the intranasal treatment experiments, mice were administrated with 10 μg (in 20 μl of PBS) of peptide/Aα complex on the days mentioned above.

**Histology**

Hind paws were removed after ending the experiment, fixed in 4% neutral buffered formaldehyde overnight, and then decalcified in 5% (w/v) EDTA at 4°C until the bones were pliable. Tissues were then dehydrated in a gradient of alcohols, paraffin embedded, sectioned at 5 μm, mounted on glass slides, and stained with H&E. Serial H&E-stained sections were analyzed microscopically for the degree of inflammation and for cartilage and bone destruction. Analyses were performed in a blinded fashion.

**T cell transfer**

For the T cell transfer experiment, 15 B10.Q mice (recipients) were immunized with CII/CFA (day 0) and boosted with CII/IFA on day 35 using the standard immunization protocol. At the same time (day 0), three groups (five mice each group) of other B10.Q mice (donors) were injected i.v. with 200 μg of GalOK264/Aα in 100 μl of PBS, 200 μg of MOG/Aα in 100 μl of PBS, or 200 μl of PBS alone, respectively. Five days later, erythrocyte-free spleen and lymph node cells from each mouse were passed through 40-μm nylon cell strainer (BD Discovery Labware), and then T cells were purified by negative selection using Abs against MHC II M5/114- and CD11b M1/70-expressing cells (BD Pharmingen) and Dynabeads (Dynal Biotech), followed by magnetic sorting. The purity of the resulting T cells was measured by flow cytometry and was found to be 97% and to be contaminated with <0.3% MHC II-expressing cells. Purified T cells were analyzed by FACS for expression of CD25, CD62L, and NK 1.1 surface markers, but no differences between the individual mice or groups were found. Purified T cells (10^6) from each individual donor were resuspended in a final volume of 200 μl of sterile PBS and transferred i.v. into recipient mice.

**Statistics**

Statistical difference in the incidence of disease between groups of mice was determined using χ² test. To compare nonparametric data for statistical significance, we applied the Mann-Whitney U or Kruskal-Wallis test on all clinical results and in vitro experiments using the StatView program (SAS Institute).

**Results**

**Design and characterization of soluble Aα proteins**

DNA constructs were designed from the extracellular domains of the murine MHC II Aα molecule with a leucine zipper heterodimerization motif from Fos and Jun to the C terminus of the MHC α and β sequences, respectively. Soluble Aα molecules from culture medium of transfected SL2 cells were characterized for proper folding by sandwich ELISA, using specific mAbs for the native α (Y3P)- and the β-chain (7-16.17). Induced cells expressed the recombinant protein, while the noninduced transfected SL2 cells secreted soluble Aα at a low basal level, but still detectable compared with nontransfected cells. Aα proteins appeared to be correctly folded because the conformation-sensitive Ab Y3P captured them. We next tested several anti-MHC II mAbs to establish which of them could be used for Western blot analysis of Aα proteins. The Y3P, 7-16.17, and 7-23.1 Abs bound specifically to Aα heterodimers, whereas M5/114 stained both single β-chain and heterodimers.

**Selective activation of specific T cell hybridomas by soluble peptide/MHC II complexes**

The purified empty Aα molecules were stabilized by adding a specific peptide (GalOK264, K264, or MOG79–90) for 3 days and further purified by ion exchange chromatography and gel filtration. The functional properties of the complexes were investigated using peptide-specific Aα-restricted T cell hybridomas (24). The hybridomas were HCQ-3, which is specific for galactose at position 264 in the CI1259–273 (GalOK264) peptide, and HCQ-4, which is specific for nonmodified CI1259–273 (K264). The T cell hybridomas responded to peptide/Aα complexes in a dose-dependent manner.

The response was efficient, regardless of whether the peptide/Aα complexes were immobilized onto 96-well plates or in soluble form (Figs. 1 and 2). Secretion of IL-2 from hybridoma cells could be blocked by adding anti-Aα Abs (7-16.17), which shows that the stimulation of T cells by soluble peptide/Aα complexes was MHC restricted (Fig. 1). To confirm that the complexes are peptide specific and that there is no cross-reactivity, we performed criss-cross hybridoma tests (Fig. 2). No cross-reactivity of peptide/Aα complexes was detected, as only Aα molecules in complex with a correct peptide could stimulate the hybridoma cells. Thus, the produced soluble peptide/Aα complexes were functional and could activate T cell hybridomas in an MHC-restricted and peptide-specific manner.

**Soluble peptide/Aα complexes specifically ameliorate CIA**

CII-immunized mice were treated with peptide/Aα complexes on days 20 and 34. As shown by data presented in Table I, both i.v. and intranasal administration with GalOK264/Aα complexes significantly delayed the onset and reduced the incidence and clinical score (severity) of CIA. Intravenous administration of peptide/Aα complexes offered better protection, although the intranasal administration was enough to significantly ameliorate the clinical signs of diseases. Reduced serum levels of anti-CII IgG Abs in GalOK264/Aα-treated mice were also observed on days 35 and 70 after the immunization. These results suggested that treatment with peptide/Aα complexes affects both T and B cell responses and specifically down-regulates autoimmune disease.

**FIGURE 1.** Peptide/Aα complexes activate Ag-specific T cell hybridomas. A, HCQ-3 hybridoma, specific for GalOK264 CI1259–273 epitope; B, HCQ-4 hybridoma, specific for nonmodified (K264) CI1259–273 epitope. Flat-bottom 96-well plates were coated with titrated amounts of GalOK264/Aα (A) or K264/Aα (B) complex, followed by incubation with T cell-specific hybridomas. To block the activation of the hybridomas, 5 μg/ml 7-16.17 Abs was added to the immobilized complexes. After 24-h incubation, supernatants were collected and IL-2 production was assayed by sandwich ELISA using DELFIA system.
Galactosylation at position 264 in the CIJ259–273 peptide is essential for the suppression of CIA

To investigate whether the inhibition effect of peptide/A9 complexes is peptide specific, we repeated the CIA experiment, but this time CIA-immunized mice were divided into three groups and treated i.v. with GalOK264/A9, K264/A9, or MOG/A9 complexes using the same treatment protocol. As shown in Fig. 3, only GalOK264/A9 was able to suppress the development of CIA. Treatment with K264/A9 or MOG/A9 complexes had no effect on incidence or severity of arthritis progression (Fig. 3, A and B). In addition, the serum levels of anti-CII IgG in GalOK264/A9-treated mice were lower as compared with K264/A9- and MOG/A9-treated mice (Fig. 3C). Thus, amelioration of CIA by GalOK264/A9 complexes is peptide specific. Most importantly, the glycosylation of the CII peptide played a crucial role, as only the CII259–273 (GalOK264) peptide glycosylated at position 264, but not the non-modified CIJ259–273 peptide, was able to suppress development of CIA. The arthritis scoring data were confirmed by histological analysis of the paws from the end of the experiment. Fig. 4 shows tissue sections of the ankle joints from K264/A9- and GalOK264/A9-treated mice. Ankle joints of the K264/A9-treated mice had inflammatory cell infiltration and destruction of cartilage and bone (Fig. 4A), while in GalOK264/A9-treated mice, no visible evidence of histopathological signs was observed (Fig. 4B). Thus, preservation of the ankle joint structure could only be seen in GalOK264/A9-treated mice.

Gal-K264/A9 complexes reduce arthritis severity in a chronic relapsing stage

To examine whether treatment with GalOK264/A9 complexes will ameliorate already established disease, we have used a chronic relapsing variant of CIA that develops in mice with mixed B10 and BALB/c backgrounds. B10.Q × (BALB/c × B10.Q) F2 mice were immunized with CII in IFA on days 0 and 35 and evaluated for arthritis for a period of 202 days. These are genetically heterogenous mice, and only mice with severe and active chronic relapsing arthritis for a minimum period of 120 days were selected for the experiment. Fig. 5A shows mean arthritis score of the chosen mice for 202 days. These are mean values and reflect an unpredictable variation of relapses in each mouse. To synchronize a relapse of arthritis, the mice were reimmunized with 50 μg of CII in IFA on day 205 (day 0 of the reimmunization) and scored the next 75 days for clinical signs of arthritis. Within 9 days, all mice had signs of relapse of arthritis. Subsequently, on days 7, 11, and 28, the mice received i.v. injection of either PBS or 100 μg of GalOK264/A9 complexes. Treatment with GalOK264/A9 complexes significantly reduced CIA progression and severity of disease (Fig. 5B). We observed no difference in the levels of Ab titers to CII on day 0 (the day of reimmunization) (Fig. 5C), whereas the total anti-CII IgG levels tended to be lower on day 75, as compared with the control, although this did not reach significance (Fig. 5C). Taken together, administration of GalOK264/A9 complexes had therapeutic effects on chronic relapsing arthritis.

Amelioration of CIA by Gal-K264/A9 complexes is mediated by T cells

Because the GalOK264/A9 complex triggers T cells, we next investigated whether these T cells might actively affect T and B cell responses and down-regulate arthritis. Three groups of B10.Q mice (donors, five mice per group) were administrated i.v. once (day 0) with 200 μg of GalOK264/A9 in 100 μl of PBS, 200 μg of MOG/A9 in 100 μl of PBS, or 100 μl of PBS alone, respectively. At the same time (day 0), 15 other B10.Q mice (recipients) were immunized at the base of the tail with CII in CFA. Five days after the injection, we took the spleens and lymph nodes from the donors, purified individually the T cells by negative selection, and transferred them i.v. to the immunized recipients 1:1 (~1 × 106 cells per mouse). Flow cytometric analysis showed no differences between the donor groups in the expression levels of CD25, CD62L, CD45RB, and NK 1.1 surface markers on the purified T cells used for transfer as well as between the ratio CD4+ /CD8+ (data not shown). T cells from the mice injected with GalOK264/A9 complexes were effective tolerogens, preventing the induction and development of arthritis. In contrast, transfer of T cells from MOG/A9- or PBS alone-injected mice had no effects on the incidence and severity of CIA (Fig. 6A). The levels of anti-CII Abs in sera days 35 and 70 were significantly decreased in mice given GalOK264/A9-tolerized T cells, compared with mice injected with MOG/A9-tolerized or naive T cells (Fig. 6B). Therefore, these results demonstrate that the suppressive effect of GalOK264/A9 complexes operates via T cells, which affect the T cell-dependent B cell response to CII.

Having established that the suppression of CIA development by GalOK264/A9 complexes is T cell mediated, we next investigated whether the tolerance was Ag specific or had bystander effects on arthritis regulation. For these experiments, we used (B10.Q × B10.RIII)F1 mice in which both A9 and A9E molecules are present and the arthritis development can be driven from different
T cell epitopes. The major T cell epitope for H2r mice has been identified as the CII442–456 peptide (40) and the bovine CII607–621 peptide (41, 42), whereas the CII260–270 peptide plays no role (40).

Two groups of (B10.Q/H11003 × B10.RIII)F1 mice were immunized once with bovine CII in CFA and treated i.v. with GalOK264/Aq or MOG/Aq complexes on days 20 and 34. The arthritis onset in these mice (Fig. 7A) was several weeks earlier than in B10.Q mice, and the incidence was 100%, indicating that the Ar/Er class II molecules mediated the arthritis. Treatment with GalOK264/Aq complexes 8 days after the arthritis onset blocked the disease progression, while the mice treated with MOG/Aq developed severe arthritis. In this case, the serum levels of anti-CII Abs on day 70 after immunization were not altered by the treatment with GalOK264/Aq complexes (Fig. 7B). Because in this experiment the mice were treated 8 days after the onset when they already had high levels of anti-CII IgG, we next immunized other (B10.Q/H11003 × B10.RIII)F1 mice

Table I. Peptide/Aq complexes suppress development of CIA in B10.Q mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean Day of Onset</th>
<th>Mean Peak Severity</th>
<th>Mean Anti-CII IgG (μg/ml)</th>
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<td>GalOK264/Aq (i.v.)</td>
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<td>N/A</td>
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<tr>
<td>PBS (control) (i.v.)</td>
<td>8/10 (80%)</td>
<td></td>
<td></td>
<td>135.9 ± 30.9</td>
</tr>
</tbody>
</table>

*B10.Q mice (10 mice per group) were immunized with 100 μg of rat CII in CFA on day 0 and boosted on day 35 with 50 μg of rat CII in IFA. On days 20 and 34, mice were treated by i.v. (100 μg in 200 μl of PBS) or intranasal (i.n.) (10 μg in 20 μl of PBS) administration of purified GalOK264/Aq complex. PBS (200 μl) was administrated (i.v.) as a control on the same days. Mice were monitored for clinical signs of arthritis for 70 days. Levels of IgG anti-CII were measured on days 35 and 70 by ELISA. All values are shown as mean ± SD. N/A, not applicable.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** GalOK264/Aq complexes suppress development of CIA. A, Incidence of arthritis (percentage of affected mice); B, mean clinical score of arthritis severity, including both arthritic and healthy mice; C, anti-CII IgG serum levels. B10.Q mice (10 animals per group) were immunized with 100 μg of rat CII in CFA on day 0 and boosted on day 35 with 50 μg of rat CII in IFA. On days 20 and 34 (arrows), mice were treated by i.v. administration of purified peptide/Aq complexes (100 μg in 200 μl of PBS). Sample sera were collected on days 35 and 70 after immunization and incubated in serial dilutions in rat CII-coated wells. Levels of IgG anti-CII Abs were measured by ELISA. All data represent mean ± SE of 10 mice per group. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Histological examination of ankle joints of B10.Q mice treated with peptide/Aq complexes. A, Joint from K264/Aq-treated mice demonstrating cell infiltration and cartilage/bone destruction; B, joint from GalOK264/Aq-treated mice. Mice were immunized and treated, as described in Fig. 3. Sections were stained with H&E.
cells specific for other epitopes and MHC II molecules. Joint bystander fashion as it suppresses arthritis mediated by T cells producing development of specific regulatory T cells operating in mice.

After immunization and treatment with peptide/Aq, the serum of T cell tolerance to this joint-specific self Ag that is exposed in an MHC molecule. Thus, it has the potential to be a major regulator of arthritis.

Animals were reimmunized on day 205 (day 0 of the reimmunization) with 50 μg of rat CII in IFA and scored the next 75 days for clinical signs of arthritis. On days 7, 11, and 28 after reimmunization (arrows), mice were treated by i.v. (100 μg in 200 μl of PBS) administration of purified GalOK264/Aq complexes (10 mice in this group). PBS was administrated (i.v.) as a control on the same days (seven mice in this group). Sample sera were collected on days 0 and 75 after reimmunization and measured by ELISA. Data are represented as mean ± SE.

**FIGURE 5.** GalOK264/Aq complexes reduce arthritis progression in chronic stage. A, Mean arthritis score for 202 days of the chronic mice chosen for treatment; B, mean clinical score of GalOK264/Aq-treated mice after reimmunization; C, anti-CII IgG serum levels. B10.Q(BALB/c × B10.Q)F₁ mice were immunized with 100 μg of rat CII emulsified in IFA on day 0 at the base of the tail and boosted on day 35 with 50 μg of rat CII in IFA. The mice were scored for a period of 202 days for arthritis development. Mice that developed chronic arthritis were selected for the treatment experiment. All selected animals were reimmunized on day 205 (day 0 of the reimmunization) with 50 μg of rat CII in IFA and scored the next 75 days for clinical signs of arthritis. On days 7, 11, and 28 after reimmunization (arrows), mice were treated by i.v. (100 μg in 200 μl of PBS) administration of purified GalOK264/Aq complexes (10 mice in this group). PBS was administrated (i.v.) as a control on the same days (seven mice in this group). Sample sera were collected on days 0 and 75 after reimmunization and measured by ELISA. Data are represented as mean ± SE.

using the same immunization protocol, but this time the mice received a single i.v. injection of 200 μg of GalOK264/Aq or MOG/Aq complexes at the day of immunization (day 0). On day 18 after immunization and treatment with peptide/Aq, the serum levels of anti-CII titers were significantly reduced in mice treated with GalOK264/Aq, compared with control (MOG/Aq-treated) mice (Fig. 7C). Thus, in (B10.Q × B10.RIII)F₁ mice, the arthritis progression can be blocked with GalOK264/Aq treatment even after the disease onset, without affecting the levels of anti-CII Abs, whereas pretreatment reduced anti-CII levels. These data confirm that the suppression of disease using GalOK264/Aq complexes is mediated by T cells, probably inducing development of specific regulatory T cells operating in joint bystander fashion as it suppresses arthritis mediated by T cells specific for other epitopes and MHC II molecules.

**Discussion**

This study demonstrates the potency of posttranslational modification of an MHC II-bound peptide to induce regulatory T cells and ameliorate CIA. The induction of tolerance prevents development of arthritis and an autoimmune response and was highly specific for structures on a galactose moiety bound to the immunodominant type II collagen peptide. Importantly, it is also therapeutic in a chronic relapsing situation, which is clearly relevant for comparisons with RA.

The glycosylation of CII does not only play a role in CIA in mouse strains with the arthritis-susceptible Aq class II gene, but also in transgenic mice expressing human class II molecules associated with RA and in humans with RA (8). When bound to the human DR4 as well as the murine Aq molecule, the glycosylated hydroxylsine side chain is oriented toward the TCR and not to the MHC molecule. Thus, it has the potential to be a major regulator of T cell tolerance to this joint-specific self Ag that is exposed in arthritis-susceptible individuals. Consequently, it has now been shown by several groups that recognition of the glycosylated epitopes on CII is of central importance both for development of CIA and for the induction of tolerance (24, 25, 43). We have previously shown that neonatal treatment with the GalOK264 CII259–273 peptide alone has a protective effect on CIA and reduces the B cell response to CII (43). Because peptides are highly susceptible to proteolytic degradation, the treatment with single peptides could not completely block development of CIA nor protect the adult mice from the disease. In addition, the required amount of peptides for effective treatment was much higher. By using soluble peptide/Aq complexes, these problems are circumvented.

Surprisingly, the vaccinating effect was entirely dependent on the glycosylation of the peptide, as the nonglycosylated peptide had no effect. This is in contrast to both earlier coimmunization with nonglycosylated peptides (31, 32) and to a vaccination using a divalent CII-class II MHC-IgG3 fusion protein (44). In the latter case, the fusion protein was designed as a single chain, which contains native CII257–269 peptide, Aq molecule, and IgG3 Fc moiety that allows TCR cross-linking, although other alternative mechanisms could also operate. As our peptide/MHC II proteins were not designed for cross-linking TCR, it is possible that this did not allow T cells specific for the nonglycosylated epitope to become activated.

If the posttranslational modifications of the target Ag determine the state of the tolerance, it will have a unique role in the development of disease as well as provide unique opportunities for therapeutic tolerance induction. In accordance with our results showing an effect on experimental autoimmune encephalomyelitis using the MOG peptide/Aq protein, it has recently been reported that MOG35–55 covalently bound to human DR2 induce efficient tolerance in DR2 transgenic mice (45). In agreement with the use of
nonglycosylated CII peptide covalently bound to A\(^{β}\) (44), there was no effect on B cell responses. The specificity and bystander effect were not reported (45). We found that GalOK264/A\(^{β}\) complex, but not K264/A\(^{β}\) (in complex with nonglycosylated CII259–273), suppresses CIA and affects both T and B cell responses. Also, in contrast to the divalent fusion protein, our CII\(^{260–270}\), suppresses CIA and affects both T and B cell molecules (47, 48). The predominant expression of the glycosylated form should lead to a higher degree of immune tolerance, although this effect is likely balanced by the deficiencies in presentation of the glycosylated peptide by APCs (48, 49). Surprisingly, however, this seems not to be the case. T cells specific for nonmodified CII are more efficiently tolerized in human CII-expressing mice than T cells specific for the galactose at position 264 (8). An explanation for this observation could be that there are no cells in the thymus able to make the proper posttranslationally modified structures. Thus, presentation of such large lysine side chain structures is unlikely, and T cells specific for glycosylated CII260–270 will not be efficiently negatively selected. This may also explain the observation that glycosylated CII is more arthritogenic than nonmodified CII. Therefore, to explain the therapeutic effects of the treatment with GalOK264/A\(^{β}\) complexes, we propose that in the initial phase these complexes specifically trigger only partially tolerized T cells specific for the glycosylated epitope, inducing a higher degree of tolerance or regulatory state dependent on the context in which they operate (50, 51).

The partially tolerized and regulatory T cells are clearly highly specific for the galactose on the bound CII peptide. Together with the therapeutic effect of the treatment, this observation gives some
hope for transferring the finding to the human situation, as RA is a disease involving a multitude of autoantigens and a variety of different MHC II molecules.

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Disclosures

R. Holmdahl, B. Dzhambazov, J. Kihlberg, and M. Vestberg have applied for a patent on the use of MHC class II molecules linked to galactosylated peptides in the prevention of rheumatoid arthritis.

References


