Induction of tolerance against the arthritogenic antigen with type-II collagen peptide-linked soluble MHC class II molecules

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In murine collagen-induced arthritis (CIA), self-reactive T cells can recognize peptide antigens derived from type-II collagen (CII). Activation of T cells is an important mediator of autoimmune diseases. Thus, T cells have become a focal point of study to treat autoimmune diseases. In this study, we evaluated the efficacy of recombinant MHC class II molecules in the regulation of antigen-specific T cells by using a self peptide derived from CII (CII260-274; IAGFKGQEPKGEPG) linked to mouse I-Aq in a murine CIA model. We found that recombinant I-Aq/CII260-274 molecules could be recognized by CII-specific T cells and inhibit the same T cells in vitro. Furthermore, the development of CIA in mice was successfully prevented by in vivo injection of recombinant I-Aq/CII260-274 molecules. Thus, treatment with recombinant soluble MHC class II molecules in complex with an immunodominant self-peptide might offer a potential therapeutic for chronic inflammation in autoimmune disease such as rheumatoid arthritis. [BMB Reports 2016; 49(6): 331-336]

INTRODUCTION

Rheumatoid arthritis (RA) is a refractory autoimmune disease characterized by chronic synovitis that erodes adjacent cartilage, ultimately producing articular injury and ankylosis (1). Although the etiology of the disease remains largely unknown, there are cumulative findings suggesting an important role of the immune system in the inflammatory phase predominantly involving pathogenic T cell responses (2).

Collagen-induced arthritis (CIA) is an animal model for human RA. CIA can be induced by immunization with type-II collagen (CII), the major protein constituent of articular cartilage (3). In mice, the susceptibility to CIA appears to be associated with MHC class II haplotypes H-2d and H-2r, whereas in humans, susceptibility to RA is associated with MHC class II haplotypes DR1 and DRA4 (4). The main histopathological features of the resulting joint inflammation in CIA are similar to those in RA, including proliferative synovitis, pannus formation, and cartilage or bone erosion (1, 5). Additionally, CIA and RA are both mediated by the activation of helper T cells expressing pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, and IL-17 (6-8).

Activation of naïve CD4+ T cells is a multi-step process initiated by co-ligation of T cell receptor (TCR) and CD4 by the MHC class II/peptide complex presented on antigen presenting cells (APCs) (signal 1) and costimulation through other T cell surface molecules such as CD28 (signal 2) (9). However, stimulation of CD4+ T cells without costimulatory signals induces anergy rather than activation (10-12). Moreover, T cells require multiple TCR engagements with antigen-loaded MHC molecules at the immunological synapse to receive sufficient signals for their activation. Receptor clustering involving multiple engagements between TCRs and MHC molecules induce cross-activation of TCR/CD3 complexes and mediate the propagation of intracellular signals necessary for T cell activation (13, 14). Thus, the engagement of TCRs with monomeric MHC/peptides does not usually provide sufficient activation signals. Rather, it induces anergy of the engaged T cells (15). Based on these findings, antigen-specific activation of pathogenic T cells with monomeric MHC/antigen in the absence of co-stimulatory signals has been considered as an immunotherapeutic approach for treating autoimmune diseases (12, 16, 17). Therefore, it is possible that soluble MHC molecules loaded with specific auto-antigenic peptides can be recognized by autoimmune T cells and induce the suppression or anergy of self-reactive T cell clones.

In this study, we generated a recombinant molecule consisting of a single chain I-Aq MHC class II molecule covalently linked to immunogenic peptides of CII260-274. In vivo delivery of the soluble form of this molecule in CIA-induced mice reduced the incidence and clinical signs of CIA by suppressing pro-inflammatory cytokines IFN-γ, IL-1β, and IL-6. Moreover, the number of CD4+ pathogenic T cells was decreased in mice treated with recombinant soluble I-Aq/CII260-274. This study will improve our current understanding on the role of pathogenic T cells in autoimmune disease. In addition, it might pro-
Suppression of CIA by soluble MHC II Yoon-Kyung Park, et al.

vide a potential clinical use for treating RA in humans.

RESULTS

Recombinant single chain I-A\(^{\alpha}\)/CII260-274 is recognized by CII-specific CD4\(^{+}\) T cells

To evaluate the immunosuppressive effect of soluble monomeric MHC class II molecules, we produced a single chain murine I-A\(^{\alpha}\) molecule consisting of peptide binding domains (\(\alpha_1\) and \(\beta_1\)) and an immunogenic peptide of CII260-274. Vector map for the production of single chain I-A\(^{\alpha}\)/CII260-274 molecule and the amino acid sequences of in vivo and in vitro versions of this molecule are shown in supplementary Fig. 1. Its in vitro version has an additional 6X His tag.

To test whether the recombinant single chain I-A\(^{\alpha}\)/CII260-274 molecule maintained its specificity against CII-specific T cells, an in vitro stimulation of CII-specific T cells was performed using plate-coated I-A\(^{\alpha}\)/CII260-274 to mimic membrane-expressed MHC II molecules. CII-specific T cells were primed by CIA immunization in DBA/1 mice in which CIA was induced as described in Materials and Methods. Plates were coated with various concentrations of I-A\(^{\alpha}\)/CII260-274. CII-specific CD4\(^{+}\) T cells from DBA/1 mice were incubated in these plates for 48 hr. The levels of IFN-\(\gamma\) secreted by activated CD4\(^{+}\) T cells into the supernatant were correlated with the concentration of I-A\(^{\alpha}\)/CII260-274 used to coat the plates (Fig. 1A). This result indicated that CII-specific T cells could recognize and respond to recombinant single chain I-A\(^{\alpha}\)/CII260-274 molecules.

Recombinant soluble single chain I-A\(^{\alpha}\)/CII260-274 suppresses Ag-specific CD4\(^{+}\) T cells

Because it became evident that the plate-bound form of single chain I-A\(^{\alpha}\)/CII260-274 molecules could be recognized by CII260-274-specific T cells, next we determined whether soluble I-A\(^{\alpha}\)/CII260-274 molecules could suppress CII-specific T cells. To evaluate the suppressive effect of soluble I-A\(^{\alpha}\)/CII260-274 molecules, CII-specific T cells were stimulated with gamma-ray (3000 rad) to prepare APCs. CII-specific T cells were primed by CII immunization in DBA/1 mice in which CIA was induced as described in Materials and Methods. Plates were coated with various concentrations of recombinant I-A\(^{\alpha}\)/CII260-274. CII-specific CD4\(^{+}\) T cells from DBA/1 mice were incubated in these plates for 48 hr. The levels of IFN-\(\gamma\) secreted by activated CD4\(^{+}\) T cells into the supernatant were correlated with the concentration of I-A\(^{\alpha}\)/CII260-274 used to coat the plates (Fig. 1A). This result indicated that CII-specific T cells could recognize and suppress CII260-274-specific T cells, next we determined whether soluble I-A\(^{\alpha}\)/CII260-274 molecules could suppress CII-specific T cells. To evaluate the suppressive effect of soluble I-A\(^{\alpha}\)/CII260-274 molecules, CII-specific T cells were stimulated with gamma-ray-irradiated CII peptide-loaded APCs. Various amounts of soluble I-A\(^{\alpha}\)/CII260-274 molecules were then added to the reaction. The levels of IFN-\(\gamma\) in the culture supernatants after 48 hr of incubation were significantly (P < 0.0001) decreased when 2 \(\mu\)g of soluble I-A\(^{\alpha}\)/CII260-274 was added, although up to 1 \(\mu\)g/well of soluble I-A\(^{\alpha}\)/CII260-274 did not show a noticeable suppressive effect (Fig. 1B). We further determined the effect of soluble I-A\(^{\alpha}\)/CII260-274 on the proliferation of CII-specific CD4\(^{+}\) T cells. Soluble I-A\(^{\alpha}\)/CII260-274 by itself did not stimulate the proliferation of CII-specific CD4\(^{+}\) T cells (Fig. 1C). However, when soluble I-A\(^{\alpha}\)/CII260-274 was added to CII-specific T cells activated by plate-bound I-A\(^{\alpha}\)/CII260-274, as low as 0.5 \(\mu\)g/well of soluble I-A\(^{\alpha}\)/CII260-274 suppressed (P < 0.05) the proliferation of CII-specific T cells. Furthermore, treatment with 3 \(\mu\)g/well of soluble I-A\(^{\alpha}\)/CII260-274 completely blocked (P < 0.01) the proliferation of CII-specific T cells to the same level as in un-activated controls (Fig. 1C).

Recombinant soluble single chain I-A\(^{\alpha}\)/CII260-274 treatment ameliorates CIA

To evaluate the effect of soluble I-A\(^{\alpha}\)/CII260-274 on the suppression of CII-specific CD4\(^{+}\) T cell responses in vivo, we adopted a murine model of the human autoimmune disease RA. To induce CIA, mice were immunized with CII at day 0. Mice then received i.v. injections of 100 \(\mu\)g recombinant soluble single chain I-A\(^{\alpha}\)/CII260-274 on day 25, 27, and 29.

Fig. 1. Suppression of Ag-specific T cells by recombinant soluble I-A\(^{\alpha}\)/CII260-274, (A) Bioactivity test of purified I-A\(^{\alpha}\)/CII260-274 molecules. CII-specific T cells were stimulated with plates coated with various concentrations of recombinant I-A\(^{\alpha}\)/CII260-274 molecules (0-2 \(\mu\)g/well in 96-well plate). CII-specific T cells stimulated with APC alone or CII260-274 (IAGFKGEQGPKGEPG) peptide alone were used as negative controls. Culture supernatants were collected and the concentrations of IFN-\(\gamma\) were measured by ELISA. (B) DBA/1 splenocytes were incubated at 37\(^\circ\)C for 24 hr. Plate bound cells were further incubated with CII260-274 peptide for 48 hr at 37\(^\circ\)C followed by irradiation with gamma-ray (3000 rad) to prepare APCs. CII-specific T cells were stimulated with purified APCs for 48 hr at 37\(^\circ\)C in the presence of different concentrations of soluble I-A\(^{\alpha}\)/CII260-274 molecules. Supernatants were collected and the concentration of IFN-\(\gamma\) was measured by ELISA. (C) CII-specific T cells were stimulated with plate-coated I-A\(^{\alpha}\)/CII260-274 as in (A) with various concentrations of soluble I-A\(^{\alpha}\)/CII260-274. Proliferation of I-A\(^{\alpha}\)/CII260-274-specific T cells was measured by using CCK-8 as an indicator of cell activation. One-way ANOVA with Dunnett’s multiple comparison tests were used to compare significant differences among groups: ***P < 0.0001 or P < 0.001; **P < 0.01; *P < 0.05.
CIA-induced mice treated with I-A<sup>q</sup>CII260-274 showed slightly reduced disease incidence (100% vs. 75%) with significant reduction in disease symptoms (P < 0.05) and delayed onset (P < 0.01) compared to mock control (Fig. 2A, 2B). In addition, the histopathological signs of hind limbs at day 40 of disease induction were compared after H & E staining. While ankle joints of hind limbs obtained from PBS treated group showed significant leukocyte infiltration and cartilage erosion, soluble I-A<sup>q</sup>CII260-274 treated group showed no significant sign of inflammation (Fig. 2C). The amelioration of arthritic index in mice treated with soluble I-A<sup>q</sup>CII260-274 correlated with the decreased number of antigen-specific T cells (Fig. 3A, 3B). When splenic T cells were stained with I-A<sup>q</sup>CII260-274 dimer, CIA-induced control mice showed significantly (P < 0.05) increased number of CII-specific T cells. However, lower numbers of T cells were observed in mice treated with soluble I-A<sup>q</sup>CII260-274, similar to those in negative control mice without CIA induction. Furthermore, in vitro CII restimulated splenocytes, soluble I-A<sup>q</sup>CII260-274 treated group did not show any noticeable cell proliferation, unlike the CIA-induced control group (Fig. 3C).

**Recombinant soluble single chain I-A<sup>q</sup>CII260-274 treatment reduces pro-inflammatory cytokine levels**

Next, we analyzed the effect of soluble I-A<sup>q</sup>CII260-274 treatment on cytokine production by CII-specific T cells in vivo. Splenocytes from two experimental groups at day 50 were isolated, re-stimulated in vitro with CII peptide, and analyzed for cytokine production. As shown in Fig. 4A, the levels of pro-inflammatory cytokines IL-1β and IL-6 related to disease severity (18, 19) were significantly (P < 0.05) lower in the single chain I-A<sup>q</sup>CII260-274 treated group compared to those in the control. Intracellular staining also showed a decrease in the number of IFN-γ-producing CD4<sup>+</sup> T cells in mice treated with soluble I-A<sup>q</sup>CII260-274 compared to that in the control (Fig. 4B). In contrast, the frequencies of IL-4- and IL-10-producing CD4<sup>+</sup> T cells were relatively larger (P < 0.05) than those in the control group (Fig. 4B).

![Fig. 2. Suppression of CIA by the treatment with recombinant soluble I-A<sup>q</sup>CII260-274](image)

![Fig. 3. Decreased numbers of Ag-specific T cells after treatment with recombinant soluble I-A<sup>q</sup>CII molecules.](image)
Expressions among 3 groups: ***P<0.0001 or P<0.001; **P<0.01; *P<0.05.

DISCUSSION
Rheumatoid arthritis is an autoimmune disease perpetuated by self-reactive pathogenic CD4+ T cells (1). CIA is an animal model of RA and a model for Th1-mediated autoimmune diseases (6). In this model, Th2-derived anti-inflammatory cytokines have been shown to be able to ameliorate the disease. It has been proposed that rebalancing the immune response by increasing Th2 cells and suppressing Th1 cells could be beneficial for the treatment of CIA (20, 21).

Monomeric TCR engagements excluding the cross-linking of TCRs can induce the apoptosis of T cells without inducing antigen-specific activation (12, 15). It has been demonstrated that partial activation of pathogenic T cells by stimulating Ag-specific TCRs in the absence of costimulation could induce energy (12) or apoptosis (16, 17). Recombinant MHC/peptide antigen complexes (either for MHC class I or class II) can be generated to target antigen-specific T cells (22-24). The use of these MHC/peptide complexes as a direct approach for Ag-driven immunosuppression of autoimmune diseases has been previously studied (9, 23, 25, 26). In this study, we constructed and produced recombinant soluble single chain I-Aq/CII260-274 molecules to suppress activated pathogenic CD4+ T cells in CIA-induced DBA/1 mice, a mouse model of human RA.

The plate-bound form of I-Aq/CII260-274 induced TCR cross-linking of antigen-specific T cells and activated CI-specific T cell clones. In contrast, the soluble form suppressed CI-specific T cell clones. In vivo treatment with the soluble monomeric form of this molecule in CIA-induced mice showed reduced incidence, delayed onset (P<0.01), and decreased (P<0.01 up to day 40) clinical signs of CIA. Histopathological data were also correlated with the clinical signs of animals. We also observed decreases in the production of pro-inflammatory cytokines IFN-γ, IL-1β, and IL-6 and increases in the production of anti-inflammatory cytokines IL-4 and IL-10 by CI-specific CD4+ T cells compared to those of the control CIA-induced mice sacrificed at the end of disease scoring (on day 50). However, in vivo treatment with single chain I-Aq/CII260-274 could not maintain the suppressive effect to a statistically significant level at the end of the scoring period, although the experimental group still showed lower disease score. These results provide an insight for the best therapeutic protocol for a long term suppression of T cell-mediated autoimmune disease by using soluble single chain MHC II molecules, including the duration of treatment effect and the intervals of treatment. Determining the best protocol for the dosages and treatment intervals of single chain I-Aq/CII260-274 molecules in CIA model will provide a basis for the utilization of antigen-linked MHC II molecule as a therapeutic reagent. In conclusion, our results demonstrated that soluble single chain I-Aq/CII260-274 molecules could protect mice against severe CIA by suppressing and anergizing pathogenic CD4+ T cell clones. Conversely, the rebalance of pro- and anti-inflammatory cytokine production by CI-specific T cells through using soluble I-Aq/CII260-274 molecules may explain the ameliorated disease outcomes of CIA-induced mice. Our results suggest that well-designed MHC/peptide monomers can be used therapeutically in humans to treat chronic inflammation associated with autoimmune diseases such as rheumatoid arthritis.

MATERIALS AND METHODS

Mice
DBA/1 mice were purchased from Charles River Laboratories (Japan). These animals were kept under specific pathogen-free conditions. They were used at 7-10 weeks of age. The experimental protocols were approved by the Laboratory Animal Care and Use Committee of Korea University.

Expression of recombinant soluble mouse MHC class II
The gene used for the expression of single chain I-Aq/CII260-274 was constructed by connecting immunodominant CII260-274 (IAGFKGEQGPKGEPG) to beta 1 and alpha 1 domains of I-Aq with linkers TSGGGGSLVPRSGGGGS and VD, respectively. For tetramer staining, another version (6X His tag was added at the end of alpha 1 domain) was constructed. These cloned genes...
were inserted into pET21d+ expression vector and expressed in *Escherichia coli* using 1 mM IPTG at 37°C for 4 hr. Inclusion bodies produced by the bacteria were isolated, denatured, refolded, and purified using methods described previously (27-29).

**Preparation of Antigen-specific T cells, CIA induction, and clinical scoring**

Mice were immunized intradermally (i.d.) at the base of the tail with 100 µg CII (Sigma-Aldrich) emulsified with an equal volume (50 µl) of CFA (Sigma-Aldrich) using standard protocols (30). Mice were booster immunized by i.d. injection of 100 µg CII emulsified with incomplete Freund’s adjuvant (IFA) on day 21. Nine days later, mice were sacrificed to obtain splenocytes. These cells were restimulated with CII peptide and IL-2 in *vivo* to obtain antigen (Ag)-specific CD4⁺ T cells.

Before CIA induction, DBA/1 mice were randomly divided into two treatment groups. Mice were either intravenously (i.v.) injected three times with 100 µg recombinant soluble I-Aq/CII260-274 molecules dissolved in 200 µl of PBS (experimental group) or PBS alone (control group) with 2 day intervals. Treated mice were monitored every other day for onset and development of arthritis until the end of the experiment. The clinical severity of arthritis was graded for each paw according to the following scale: 0 = normal paws, 1 = edema and erythema in only one digit, 2 = progressive edema or erythema in at least two digits, and 3 = severe edema/inflammation and erythema involving the entire paw. The average of macroscopic score was expressed as the cumulative value of all paws. The maximum possible score was 12.

**Tetramer staining and flow cytometric analysis**

CII-specific CD4⁺ T cells were stained with I-Aq/CII260-274 dimers in FACS staining buffer (PBS containing 0.1% BSA and 0.01% sodium azide). Cells were pre-incubated with anti-FcRγ mAbs (2.4G2) and goat anti-mouse IgG-pure antibody to block non-specific antibody binding at 4°C for 20 min. Monomeric I-Aq/CII260-274 (4 µg/sample) containing a 6X His tag was incubated with a mouse monoclonal antibody specific to penta-His (QIAGEN cat. no. 34660) for 30 min. The resulting dimer was added to cells and incubated for 2 hr. After washing the cells, anti-mouse lgG1-PE was added to produce tetramers. The tetramer-stained cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

**Measurement of CII-specific T cell responses in vitro**

To analyze cytokine response of CII-specific T cells, splenocytes were harvested from mice at day 50 after the first CIA immunization. Cells (5 × 10⁵ cells/well in a 96-well flat-bottom plate) were restimulated in triplicates with 100 µg/ml CII or PBS alone (mock). After 48 or 72 hr, the supernatants were collected and assessed for the presence of cytokines by ELISA (OPTEIA Mouse cytokine set; BD Pharmingen).

For intracellular cytokine staining, at day 50 after the first immunization, splenocytes were isolated from immunized mice. Cells (5 × 10⁵ cells/well on 96-well flat-bottom plate) were cultured with 100 µg/ml CII or mock for 48 hr followed by the addition of Golgi stop (BD Pharmingen). After 6 hr, cells were harvested for intracellular cytokine staining. To determine the levels of intracellular cytokines, cells were initially stained with appropriate mAbs, fixed, permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), and stained with APC or R-PE conjugated anti-IL-4, anti-IL-10, or anti-IFN-γ mAbs at 4°C for 45 min. The percentage of cells expressing cytoplasmic IL-4, IL-10, or IFN-γ was determined by flow cytometry.

**Cell proliferation assays**

Quantification of CII-specific T cells were estimated in 96-well plates using Cell Counting Kit-8 (CCK-8; Dojindo) (31) or by treating with 0.25 µCi of [³H]thymidine (NEN). Briefly, cells were incubated with 0.25 µCi of [³H]thymidine for 6 hr prior to β-counting. Incorporation of [³H]thymidine was measured with a MicroBeta 1450 Trilux Liquid Scintillation counter (Wallac). Data were presented as the average count per minute (cpm) from triplicates. All proliferation experiments were repeated at least three times.

**Histology**

Fixed joints were decalcified in formic acid and processed for paraffin embedding. Tissue sections (3 µm in thickness) were stained with hematoxulin and eosin for histological signs. Histological analysis was conducted using AbionCRO (Seoul, Korea).

**Statistical analysis**

Prism software (GraphPad) was used for all statistical analyses. One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests were used to determine significant differences among 3 or more groups. Student’s t-tests were used to determine significant differences between two groups. Throughout the text, figures, and legends, the following symbols were used to denote statistical significance: ***P < 0.0001 or P < 0.001; **P < 0.01; and *P < 0.05.

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