Research article

Open Access

Induction of IL-10-producing $CD4^+CD25^+$ T cells in animal model of collagen-induced arthritis by oral administration of type II collagen

So-Youn Min[1]*, Sue-Yun Hwang[1]*, Kyung-Su Park[2], Jae-sun Lee[1], Kang-Eun Lee[1], Kyung-Wun Kim[1], Young-Ok Jung[2], Hyunk-Jae Koh[2], Ju-Ho Do[2], Haerim Kim[2] and Ho-Youn Kim[1,2]

[1]Rheumatism Research Center, Catholic Research Institute of Medical Science, The Catholic University of Korea, Seoul, Korea
[2]Center for Rheumatic Disease, Kangnam St. Mary's Hospital, The Catholic University of Korea Medical School, Seoul, Korea
*Co-first authors; order can be switched for bibliographic purposes

Corresponding author: Ho-Youn Kim (e-mail: ho@catholic.ac.kr)

Received: 22 Oct 2003 Revisions requested: 2 Dec 2003 Revisions received: 10 Feb 2004 Accepted: 26 Feb 2004 Published: 11 Mar 2004

Arthritis Res Ther 2004, **6**:R213-R219 (DOI 10.1186/ar1169)

Abstract

Induction of oral tolerance has long been considered a promising approach to the treatment of chronic autoimmune diseases, including rheumatoid arthritis (RA). Oral administration of type II collagen (CII) has been proven to improve signs and symptoms in RA patients without troublesome toxicity. To investigate the mechanism of immune suppression mediated by orally administered antigen, we examined changes in serum IgG subtypes and T-cell proliferative responses to CII, and generation of IL-10-producing $CD4^+CD25^+$ T-cell subsets in an animal model of collagen-induced arthritis (CIA). We found that joint inflammation in CIA mice peaked at 5 weeks after primary immunization with CII, which was significantly less in mice tolerized by repeated oral feeding of CII before CIA induction. Mice that had been fed with CII also exhibited increased serum IgG_1 and decreased serum IgG_{2a} as compared with nontolerized CIA animals. The T-cell proliferative response to CII was suppressed in lymph nodes of tolerized mice also. Production of IL-10 and of transforming growth factor-β from mononuclear lymphocytes was increased in the tolerized animals, and $CD4^+$ T cells isolated from tolerized mice did not respond with induction of IFN-γ when stimulated *in vitro* with CII. We also observed greater induction of IL-10-producing $CD4^+CD25^+$ subsets among CII-stimulated splenic T cells from tolerized mice. These data suggest that when these IL-10-producing $CD4^+CD25^+$ T cells encounter CII antigen in affected joints they become activated to exert an anti-inflammatory effect.

Keywords: collagen-induced arthritis, IL-10, oral tolerance, type II collagen

Introduction

Oral tolerance is a state of absent or minimal immune responsiveness to protein antigens that were repeatedly administered by oral feeding [1]. Induction of peripheral tolerance by oral administration of antigen has been applied to the treatment of autoimmune diseases such as rheumatoid arthritis (RA) [2,3], multiple sclerosis, systemic sclerosis, type I diabetes and iritis [4], but the mechanisms by which orally administered antigen can induce peripheral tolerance have not yet been elucidated. Studies conducted in animal models have suggested that the possible mechanism may involve secretion of anti-inflammatory cytokines including IL-4, IL-10 and transforming growth factor (TGF)-β by mucosal T lymphocytes that have differentiated into T-helper (Th)2 or Th3 cells after encountering the antigen [5–7]. However, individual studies often report conflicting findings, depending on the route, dose and timing of antigen administration [8].

Although much of the RA pathogenesis remains to be elucidated, it has been reported that joint proteins, probably type II collagen (CII), play a key role in the instigation of T-cell mediated immune responses.

CFA = complete Freund's adjuvant; CIA = collagen-induced arthritis; CII = type II collagen; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorting; IFN = interferon; IL = interleukin; PBS = phosphate-buffered saline; RA = rheumatoid arthritis; TGF = transforming growth factor; Th = T-helper.

Administration of CII to DBA/1 mice induces polyarthritis with pathological symptoms similar to those observed in human RA [9,10]. In order to investigate the cellular mechanisms that underlie oral tolerance, we studied an animal model of collagen-induced arthritis (CIA), in which mice undergo repeated oral administration of CII, and monitored changes in immune cell function and factors associated with inflammation. We found that serum levels of IgG subtypes, as well as the production of IL-10, TGF-β and IFN-γ, were affected in tolerized mice. We also noticed greater proportions of IL-10-producing $CD4^+CD25^+$ T cells in the Peyer's patch, mesenteric lymph nodes and the spleen of tolerized mice. Production of these cells, exhibiting the characteristics of the Treg subset, was induced to a significant degree by lymphocytes from tolerized spleen when stimulated *in vitro* with CII. We hypothesize that expansion of these suppressor T cells in the periphery might have contributed to the reduced inflammation observed in affected joints.

Materials and methods

Induction of oral tolerance in DBA/1 mice

DBA/1 mice used in this study were fed either with 100 μg bovine CII (a kind gift from Prof. Andrew Kang, University of Tennessee) dissolved in 0.05 N acetic acid at 2 mg/ml (50 μl solution plus 150 μl acetic acid) or with an equal volume of phosphate-buffered saline (PBS) using an oral Zonde needle (Natsume, Japan) every 2 days for 2 weeks. All experimental procedures were examined and approved by the Animal Research Ethics Committee at The Catholic University of Korea.

Induction of CIA and evaluation of arthritis

Bovine CII was dissolved in 0.05 N acetic acid at 2 mg/ml and emulsified with an equal volume of complete Freund's adjuvant (CFA). As primary immunization, 0.1 ml of the emulsion, containing 100 μg CII, was injected into the tails of DBA/1 mice (both tolerized mice and nontolerized control mice; $n = 6$ per group). Two weeks later, a booster injection consisting of 200 μg CII similarly dissolved and emulsified 1:1 with incomplete Freund's adjuvant was injected into a hind leg. Starting from 2.5 weeks (18 days) after primary immunization, three independent observers examined the degree of arthritis three times a week for up to 11 weeks. The severity of arthritis was represented as mean arthritic index on a 0–4 scale according to the following criteria [11]: 0 = no oedema or swelling; 1 = slight oedema and erythema limited to the foot and/or ankle; 2 = slight oedema and erythema from the ankle to the tarsal bone; 3 = moderate oedema and erythema from the ankle to the tarsal bone; and 4 = oedema and erythema from the ankle to the entire leg. The sum of values from three legs, excluding the hind leg into which CII/incomplete Freund's adjuvant was injected, was determined at the time of the second injection. The final values presented in the Results section represent an average of the indices recorded by three independent observers. All experimental procedures were examined and approved by the Animal Research Ethics Committee at The Catholic University of Korea.

Analysis of IgG antibody subtypes

Blood samples obtained from each mouse at 3, 5 and 7 weeks after primary immunization were used to investigate IgG antibody subtype concentrations using the mouse IgG_1/IgG_{2a} ELISA quantitation kit (Bethyl Lab Co., Montgomery, TX, USA). Levels of IgG_1 and IgG_{2a} were measured in mice sera diluted 50,000- to 400,000-fold.

Determination of collagen-specific T-cell proliferative response

The draining lymph nodes and the spleen were removed from each mouse and washed twice with PBS. Tissues were minced and the cells were filtered through a cell strainer and centrifuged at 1500 rpm at 4°C for 10 min. The cell pellet was resuspended in RPMI-1640 medium to a concentration of 1×10^5 cells/ml. Cells were than plated in 96-well microtitre plates at a concentration of 2×10^5 cells/well concentration and cultured with 40 μg/well CII in 0.3 ml Click's medium supplemented with 0.5% mouse serum for 3 days. The same amount of ovalbumin was added instead of CII to control wells. Eighteen hours before the termination of culture, 0.5 μCi $[^3H]$thymidine (NEN, Boston, MA, USA) was added to each well. Cells were harvested onto glass fibre filters and counted on a Matrix-96 direct ionization β counter (Packard Instrument Co., Downers Grove, IL, USA). The degree of T-cell proliferation is presented as the stimulation index, which is calculated by dividing the counts/min in the presence of CII by the counts/min in the presence of ovalbumin.

Analyses of cytokine production by ELISA

Mononuclear cells were isolated from the Peyer's patch and spleen, and cultured at a density of 0.5×10^6 cells/ml in flat-bottomed, 48-well tissue culture plates (Corning, Corning, NY, USA). After 2 days, culture supernatants were harvested and stored at −70°C. To determine the amount of IL-10 and TGF-β in each supernatant, 96-well ELISA plates were coated with rat antimurine IL-10 and TGF-β monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) for 24 hours at 4°C. After incubating with blocking agents, the plates were incubated with previously collected supernatants for 1 hour at room temperature. The plates were then washed and incubated for 1 hour at room temperature with biotin-conjugated rat antimurine IL-10 and TGF-β monoclonal antibodies, followed by an alkaline phosphatase-conjugated goat antibiotin monoclonal antibody (R&D Systems). The fluorescent substrate for alkaline phosphatase (R&D Systems) was used for colour development, and fluorescence was measured using a microtitre plate reader (Dynex, Chantilly, VA, USA) at excitation and

emission wavelengths of 450 nm. The amounts of cytokines present in test samples were determined from standard curves established with serial dilutions of recombinant murine IL-10 and TGF-β.

Fluorocytometric analysis of T cells

Single-cell suspensions were prepared from each lymphoid organ and cultured in 24-well plates at a concentration of 1×10^6/well with or without 40 μg/well CII for 3 days. Golgi Stop (Pharmingen, San Diego, CA, USA) was added 4 hours before the termination of culture. Cells were subsequently washed and resuspended in fluorescence-activated cell sorting (FACS) staining buffer (PBS plus 0.1% bovine serum albumin plus 0.09% sodium azide), and probed with anti-CD4-perCP and/or anti-CD25-FITC (Pharmingen) for 30 min at 4°C. Next, cells were fixed with cytoperm/cytofix (Pharmingen) for 20 min and probed for intracellular cytokines using phycoerythrin-labelled anti-IL-10 antibody, anti-IFN-γ antibody, or isotype control antibody (Pharmingen) for 30 min at room temperature. Finally, cells were washed with PBS and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Experimental findings are presented as mean ± standard deviation. Statistical significance was determined by Student's *t*-test using the SPSS program (version 10.0; SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

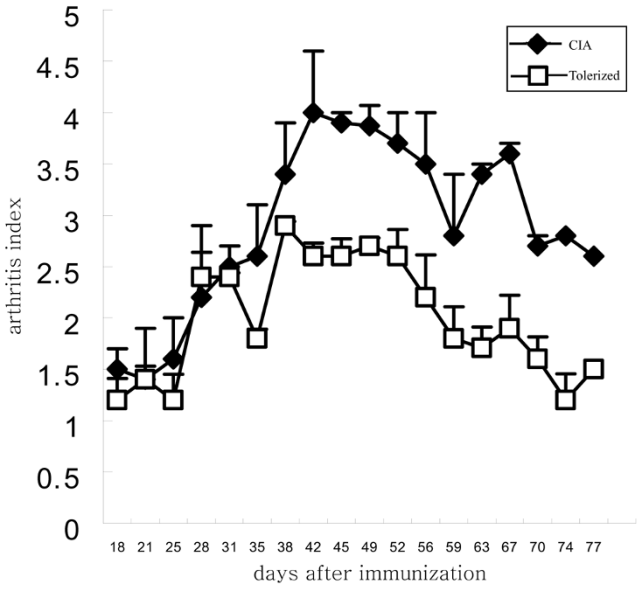
Inhibition of arthritis development in tolerized CIA mice

The severity of arthritis remained low both in the tolerized and nontolerized CIA mice until 4 weeks after primary immunization with CII/CFA (Fig. 1). The average arthritis index in the nontolerized mice began to increase after week 4, reaching a peak between weeks 5 and 6 after primary immunization, and then started to decrease by week 8, and then persisted at an almost constant level until the 11th week. The arthritis index in the tolerized group also peaked between 5 and 6 weeks after primary immunization, but the level was significantly lower than that in the nontolerized CIA mice throughout the period of examination. Mice tolerized by oral CII feeding also exhibited an earlier decrease in their mean arthritis index, which began at week 7 and continued gradually thereafter.

Changes in the serum level of IgG subtypes in tolerized mice

Among three subtypes of IgG, IgG_1 is reported to be associated with anti-inflammatory actions, whereas IgG_{2a} is known to be a mediator of inflammation. Likewise, the level of IgG_{2a} has been shown to be significantly increased in DBA/1 mice with CIA [12,13]. We investigated the change in the concentration of serum IgG subtypes at 3, 5 and 7 weeks after primary immunization (Fig. 2). Serum IgG_1 concentration was greater in tolerized mice than in nontolerized CIA mice (104.6 ± 21 ng/ml versus 76.3 ± 15 ng/ml; $P < 0.05$; Fig. 2a). On the other hand, serum IgG_{2a} concentration was significantly lower in the tolerized group when examined at both 3 weeks and 5 weeks after primary immunization (86.6 ± 17.5 ng/ml versus 114.7 ± 12.2 ng/ml; $P < 0.05$; Fig. 2b). Notably, the average level of IgG_{2a} in the tolerized mice was about twofold lower than in the nontolerized CIA mice at 5 weeks after primary immunization. After week 11, when the mean arthritic index had already dwindled, no further significant differences in IgG subtype concentrations were observed between the two groups (data not shown).

Figure 1

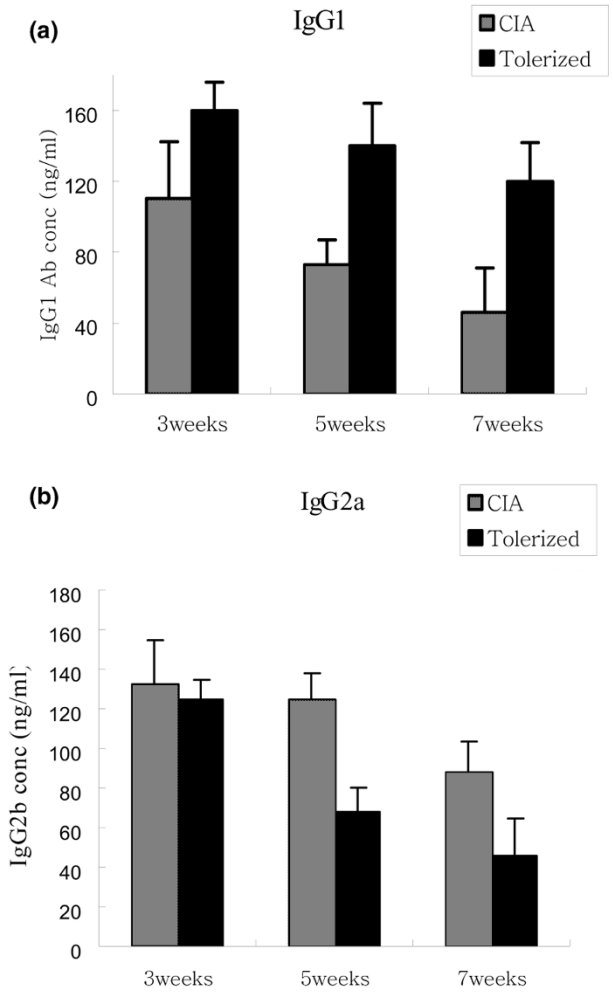


Suppression of arthritis in mice that received oral type II collagen (CII) before arthritis induction. Mice that were given six consecutive administrations of 100 μg CII orally before induction of arthritis exhibited a marked reduction in arthritis index as compared with nontolerized collagen-induced arthritis (CIA) controls throughout the period of examination (i.e. from 2 to 11 weeks after the primary CII immunization for CIA induction). The data represent average values from 15 individual mice in each group.

Proliferation response to CII by T cells isolated from the draining lymph nodes and spleens of tolerized mice

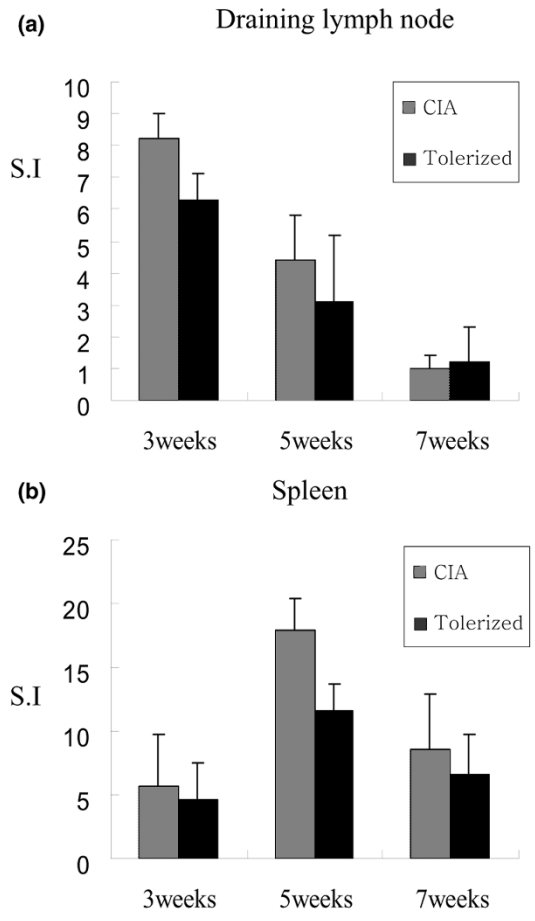
We then investigated the proliferation of T cells isolated from the draining lymph node and spleen on *in vitro* antigenic simulation with CII. At 3 and 5 weeks after primary immunization with CII/CFA, the T-cell proliferative response to CII was about 25% lower in cells isolated from the draining lymph node of orally tolerized mice than in cells from the nontolerized CIA mice (stimulation index 8.2 ± 2.3 versus 6.2 ± 3.5; $P < 0.05$). After week 7 no

Figure 2



Serum levels of **(a)** IgG_1 and **(b)** IgG_{2a} subtypes in tolerized and nontolerized collagen-induced arthritis (CIA) mice. When examined at 3, 5 and 7 weeks after primary immunization, mice that received oral type II collagen (CII) exhibited higher serum levels of IgG_1 than did nontolerized mice at all three time points, whereas the IgG_{2a} level was lower in the tolerized group. The data represent average values from 15 mice in each group.

Figure 3



T-cell proliferative response to antigenic stimulation with type II collagen (CII). T cells were isolated from draining lymph nodes and spleens of mice killed at 3, 5 and 7 weeks after primary immunization, and were cultured for 72 hours in the presence of 40 μg/ml CII. Panels show changes in the stimulation index (SI) of T cells isolated from **(a)** draining lymph node and **(b)** spleen. The data represent average values from 15 mice in each group.

significant difference was noticed between draining lymph node T cells from the two groups (Fig. 3a). Splenic T cells from orally tolerized mice also exhibited a lower proliferative response to CII than did cells from nontolerized CIA mice when examined at 5 weeks after primary immunization. By week 7 proliferation on CII stimulation decreased to approximately 50% of the value observed at week 5 in both groups (Fig. 3b).

Changes in the production of IL-10, TGF-β and IFN-γ by lymphocytes of orally tolerized mice

To examine the effect of oral CII administration on the activity of immune cells, we measured the production of IL-10 and TGF-β by mononuclear lymphocytes isolated from the Peyer's patch and spleen of each mouse. As shown in Fig. 4, production of both IL-10 and TGF-β was increased in lymphocytes isolated from the Peyer's patch and the spleen of tolerized mice as compared with lymphocytes from nontolerized CIA mice or from normal DBA/1 mice. Although the level of TGF-β production in cells from nontolerized CIA mice was similar to that in cells from normal DBA/1 mice, the production of IL-10 was slightly greater in cells from nontolerized CIA than in cells from normal DBA/1 mice.

We then compared the level of IFN-γ production from antigen stimulated $CD4^+$ T cells isolated from the Peyer's patch, mesenteric lymph nodes and spleens of tolerized and nontolerized mice (Table 1) by FACS analyses using

monoclonal antibodies against CD4 and intracellular IFN-γ. When examined *ex vivo*, no apparent differences in IFN-γ production were observed between the two groups. When these cells were stimulated *in vitro* with 40 μg/ml CII for 3 days, the level of IFN-γ synthesis increased up to 10-fold in nontolerized CIA mice but remained virtually unchanged in tolerized animals.

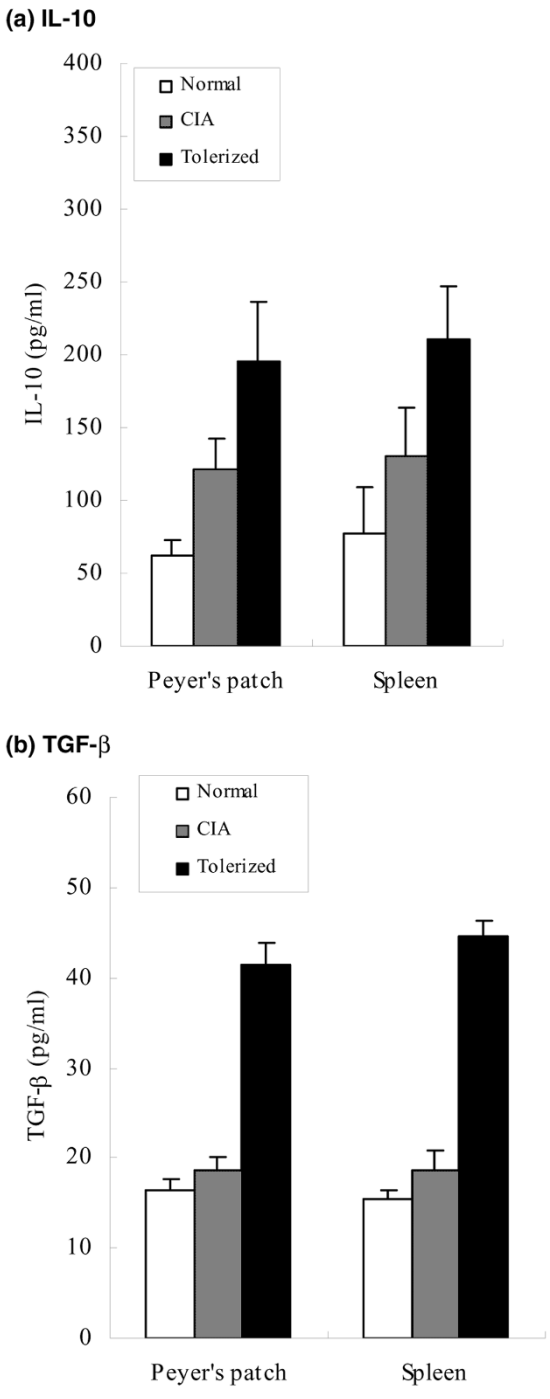
Induction of IL-10-producing $CD4^+CD25^+$ population from splenic T cells of orally tolerized mice by *in vitro* CII stimulation

To identify any possible involvement of particular T-cell subsets that are known to exert immune suppressive functions [6,14], we compared the proportion of IL-10-producing $CD4^+CD25^+$ T lymphocytes between tolerized and nontolerized mice. For this, single-cell preparations from the Peyer's patch, mesenteric lymph node and spleen extracted at 5 weeks after primary immunization were cultured *in vitro* with or without CII stimulation for 3 days, and analyzed using triple-colour FACS. The proportion of IL-10-producing $CD4^+CD25^+$ cells was higher in tolerized mice than in nontolerized CIA mice and normal DBA/1 mice in all three organs examined (Table 2). In normal DBA/1 mice the proportion of IL-10-producing $CD4^+CD25^+$ cells remained unchanged from baseline in all three organs examined, regardless of whether *in vitro* CII stimulation was applied. The proportion of IL-10-producing $CD4^+CD25^+$ cells in tolerized mice exhibited a modest increase in the Peyer's patch and mesenteric lymph nodes when stimulated *in vitro* with CII; however, the degree of enrichment was not significantly different from that in nontolerized CIA mice. On the other hand, *in vitro* CII stimulation induced a much steeper elevation in IL-10-producing $CD4^+CD25^+$ population in the spleens of tolerized mice (from $2.95 \pm 1.62\%$ to $15.5 \pm 4.50\%$) than in the nontolerized group (from $0.15 \pm 0.07\%$ to $3.85 \pm 0.63\%$). Differences between the enrichment in T-cell subsets in the spleen are shown in a representative dot plot (Fig. 5).

Discussion

Treatment of autoimmune diseases by induction of oral tolerance is attractive because of the few side effects and easy clinical implementation of this approach. Although protein antigens administered orally are degraded quickly in the digestive system and their effects do not last long, we previously overcame these drawbacks by encapsulating CII – the major cartilage autoantigen in RA – in a polymer complex [15]. In the present study we attempted to improve our basic understanding of the immune cell functions that participate in the process of induction of oral tolerance. Because most of the previous studies that addressed the mechanism underlying oral tolerance were conducted in transgenic animal models that overexpress T-cell clones that recognize experimental antigens such as ovalbumin, it is difficult to relate their findings to the

Figure 4



Comparisons of **(a)** IL-10 and **(b)** transforming growth factor (TGF)-β production by mononuclear lymphocytes from lymphoid organs of tolerized and nontolerized mice. Organs were extracted from normal DBA/1, and tolerized and nontolerized collagen-induced arthritis (CIA) mice at 5 weeks after primary immunization with type II collagen (CII) for induction of CIA. Mononuclear cells were isolated from the Peyer's patch and the spleen, and production of IL-10 and TGF-β was assessed by sandwich ELISA analyses of culture supernatants. The data represent average values from three independent measurements.

Table 1

IFN-γ production by CD4+ T cells in lymph nodes and the spleen

	% IFN-γ-positive cells	
Site	Without CII stimulation	With CII stimulation
Peyer's patch		
CIA	3.4 ± 0.22	26.6 ± 0.6
Tolerized	4.6 ± 0.1	8.9 ± 0.2
Mesentric lymph node		
CIA	3.3 ± 0.4	34 ± 0.2
Tolerized	2.3 ± 0.5	5.6 ± 0.8
Spleen		
CIA	5.4 ± 0.01	42.6± 0.1
Tolerized	4.5 ± 0.2	5.3 ± 0.02

Values are expressed as mean ± standard deviation, calculated from three independent measurements. CIA, collagen-induced arthritis; CII, type II collagen.

Table 2

Proportion of IL-10-producing CD4+CD25+ T cells in lymph nodes and spleen

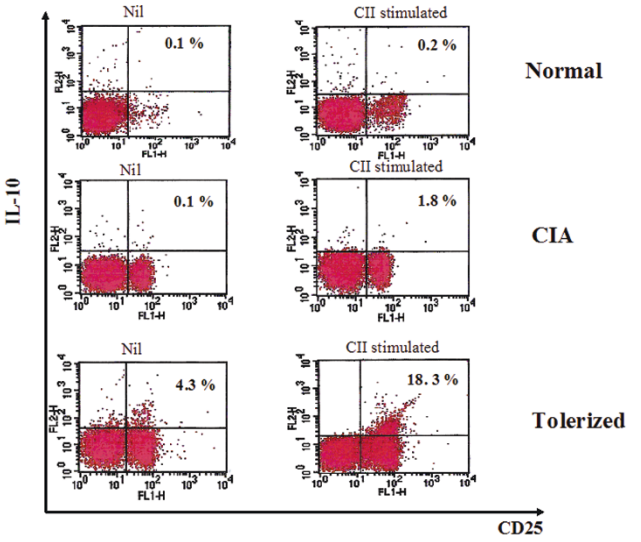
	% CD4+CD25+ IL-10-positive cells	
Site	Without CII stimulation	With CII stimulation
Peyer's patch		
Normal	0.17 ± 0.12	0.14 ± 0.06
CIA	1.6 ± 0.7	3.6 ± 0.2
Tolerized	2.4 ± 1.6	6.8 ± 1.2
Mesentric lymph node		
Normal	0.35 ± 0.35	0.5 ± 0.28
CIA	0.85 ± 0.35	1.8 ± 0.5
Tolerized	2.95 ± 1.06	7.9 ± 1.48
Spleen		
Normal	0.13 ± 0.04	0.25 ± 1.3
CIA	0.15 ± 0.07	3.85 ± 0.63
Tolerized	2.95 ± 1.62	15.5 ± 4.5

Values are expressed as mean ± standard deviation, calculated from three independent measurements. CIA, collagen-induced arthritis; CII, type II collagen.

clinical situation [16]. In the present study we examined the changes in immune function that are involved in the induction of peripheral tolerance in an orally tolerized mouse model of arthritis.

Our findings indicate that the serum levels of pro-inflammatory and anti-inflammatory IgG subtypes are

Figure 5



Induction of IL-10-producing CD4+CD25+ T cells in the spleen of tolerized mice. The proportion of IL-10-producing CD4+CD25+ T cells was analyzed by triple-colour fluorescence-activated cell sorting (FACS) analyses. Single-cell suspension was prepared from the spleens of normal DBA/1, and tolerized and nontolerized collagen-induced arthritis (CIA) mice at 5 weeks after primary immunization. Cells were first gated for CD4+ proportions, and subsequently analyzed for surface expression of CD25+ and for the presence of intracellular IL-10. A representative dot plot chosen from three independent measurements (summarized in Table 2) is shown.

differentially modulated in mice that have undergone repeated oral administration of CII. We also found that, although the production of IFN-γ by CD4+ T cells from tolerized mice on CII stimulation is reduced, the production of IL-10 and TGF-β is increased not only in cells from the site of initial contact with the oral antigen (i.e. the Peyer's patch) but also in cells from lymph nodes and the spleen. That *in vitro* restimulation with CII generated significant enrichment in IL-10-producing CD4+CD25+ T cells among lymphocytes from tolerized spleen is particularly interesting. We hypothesize that by the time these IL-10-producing cells have reached the spleen through peripheral blood, they have also been transferred to the affected joints where CII is accumulated from cartilage degradation and have begun to alleviate inflammation.

Many recent studies have reported that IL-10, a major anti-inflammatory cytokine secreted by Th2 lymphocytes, contributes to the process of peripheral tolerance [1,6]. Our data suggest that the establishment of CII-mediated tolerance to arthritic inflammation involved, among other mechanisms of immunosuppression, participation of CD4+CD25+ T cells and their production of IL-10 in peripheral lymphoid organs. However, roles for Th3 cells (known for their production of TGF-β [17,18]) and for Tr1

cells (which are characterized by low proliferation and high production of IL-10 [19–21]) are equally implicated in the process of induction of peripheral tolerance. We observed elevated production of TGF-β from tolerized lymphocytes as well, and it would therefore be very interesting to investigate the role of Th3 cells. In order to further define the characteristics of cells that exert immunosuppressive functions, we are in the process of examining the expression of CD62L and CD45RB, which are accepted as additional phenotypic markers for regulatory T cells [22,23], and the effect of transferring T cells into nontolerized CIA mice.

Conclusion

We observed a greater proportion of IL-10-producing $CD4^+CD25^+$ T cells in mice that had undergone repeated oral administration of CII. Together with increased production of IL-10 and TGF-β in peripheral lymphoid organs, induction of this suppressor population is believed to block the induction of IFN-γ from CII-stimulated $CD4^+$ cells. Because little is known about the cellular mechanisms that underlie orally induced tolerance in non-transgenic systems, our findings provide important basic information on the generation of peripheral immune tolerance. Once merged with findings on possible intercellular cooperation between CII-specific T cells and other participants such as dendritic cells, these data will provide a basis for more effective application of oral tolerance induction in RA patients.

Competing interests

None declared.

Acknowledgements

This study was supported by the 2002 Specialized Research Center (SRC) grant from the Korean Science and Engineering Foundation (KOSEF) to the Rheumatism Research Center (RhRC) at The Catholic University of Korea.

References

1. Weiner HL, Friedman A, Miller A, Khoury SJ, al-Sabbagh A, Santos L, Sayegh M, Nussenblatt RB, Trentham DE, Hafler DA: **Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens.** *Annu Rev Immunol* 1994, **12:**809-837.
2. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL: **Effect of oral administration of type II collagen on rheumatoid arthritis.** *Science* 1993, **261:**1727-1730.
3. Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH, Trentham DE: **Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial.** *Arthritis Rheum* 1998, **41:**290-297.
4. Strobel S, Mowat AM: **Immune responses to dietary antigens: oral tolerance.** *Immunol Today* 1998, **19:**173-181.
5. Fishman-Lobell J, Freidman A, Weiner HL: **Different kinetic patterns of cytokine gene expression in vivo in orally tolerant mice.** *Eur J Immunol* 1994, **24:**2720-2724.
6. Tsuji NM, Mizumachi K, Kurisaki J: **Interleukin-10 secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance.** *Immunology* 2001, **103:**458-464.
7. Weiner HL: **Oral tolerance: immune mechanisms and treatment of autoimmune diseases.** *Immunol Today* 1997, **18:**335-343.
8. Friedman A, Weiner HL: **Induction of anergy or active suppression following oral tolerance is determined by antigen dosage.** *Proc Natl Acad Sci USA* 1994, **91:**6688-6692.
9. Trentham DE, Townes AS, Kang AH: **Autoimmunity to type II collagen: an experimental model of arthritis.** *J Exp Med* 1977, **146:**857-868.
10. Stuart JM, Huffstutter EH, Townes AS, Kang AH: **Incidence and specificity of antibodies to type I, II, III, IV, and V collagen in rheumatoid arthritis and other rheumatic disease as measured by 125I-radioimmunoassay.** *Arthritis Rheum* 1983, **26:** 832-840.
11. Rosloniec EF, Cremer M, Kang A, Myers LK: **Collagen-induced arthritis.** In: *Current Protocols in Immunology*, vol 3. Edited by Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. New York: Wiley; 1996:15.5.1-15.5.19.
12. Seetharaman R, Mora AL, Nabozny G, Boothby M, Chen J: **Essential role of T-cell NF-kappa B activation in collagen-induced arthritis.** *J Immunol* 1999, **163:**1577-1583.
13. Butler DM, Malfait AM, Maini RN, Brennan FM, Feldmann M: **Anti-IL-12 and anti-TNF antibodies synergistically suppress the progression of murine collagen-induced arthritis.** *Eur J Immunol* 1999, **29:**2205-2212.
14. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H, Shevach EM: **$CD4^+$ $CD25^+$ regulatory T-cells can mediate suppressor function in the absence of transforming growth factor 1 production and responsiveness.** *J Exp Med* 2002, **196:**237-245.
15. Kim WU, Park JS, Ryoo JW, Kim J, Youn J, Kim SH, Min SY, Bae EY, Lee WK, Hwang SY, Park SH, Cho CS, Kim HY: **Suppression of collagen induced arthritis by single administration of poly(lactic-co-glycolic acid) nanoparticles entrapping CII: a novel therapeutic strategy for oral tolerance.** *Arthritis Rheum* 2002, **46:**1109-1120.
16. Gonnella PA, Chen Y, Inobe J, Komagata Y, Quartulli M, Weiner HL: **In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice.** *J Immunol* 1998, **160:**4708-4718.
17. Shi HN, Grusby MJ, Nagler-Anderson C: **Orally induced peripheral nonresponsiveness is maintained in the absence of functional Th1 or Th2 cells.** *J Immunol* 1999, **162:**5143-5148.
18. Oldenhove G, de Heusch M, Urbain-Vansanten G, Urbain J, Maliszewski C, Leo O, Moser M: **$CD4^+CD25^+$ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo.** *J Exp Med* 2003, **198:**259-266.
19. Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H: **Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo.** *Immunity* 2003, **18:**605-617.
20. Levings MK, Bacchetta R, Schulz U, Roncarolo MG: **The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells.** *Int Arch Allergy Immunol* 2002, **129:**263-276.
21. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N, Cottrez F, Tedgui A, Groux H: **Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice.** *Circulation* 2003, **108:**1232-1237.
22. Annacker O, Burlen-Defranoux O, Pimenta-Araujo R, Cumano A, Bandeira A: **Regulatory CD4 T cells control the size of the peripheral activated/memory CD4 T cell compartment.** *J Immunol* 2000, **164:**3573-3580.
23. Jump RL Levine AD: **Murine Peyer's patches favor development of an IL-10 secreting, regulatory T cell population.** *J Immunol* 2002, **168:**6113-6119.

Correspondence

Ho-Youn Kim, MD PhD, Rheumatism Research Center (RhRC), Catholic Institutes of Medical Science, The Catholic University of Korea, 505 Banpo-Dong, Seocho-Ku, Seoul 137-701, Korea. Tel: +82 2 590 2702; fax: +82 2 537 4673; e-mail: ho@catholic.ac.kr