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Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis ☆

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Abstract

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, but its autoimmune mechanisms are not clearly understood. Recently, anti-citrullinated peptide antibodies have been specifically observed in sera of RA patients. Furthermore, we identified RA-susceptible variant in a gene encoding citrullinating enzyme, peptidylarginine deiminase type 4 (PADI4). Therefore, we hypothesized that proteins which are modified in RA synovium by PADI4 act as autoantigens. Subsequently, we obtained human collagen type I (huCI) as one of the autoantigens using a RA synoviocyte cDNA library by immunoscreening. We also investigated that the levels of anti-citrullinated huCI were significantly higher in RA patient sera than in normal control sera with high specificity (99%) and positively correlated with the levels of anti-cyclic citrullinated peptide (anti-CCP) antibodies. We concluded that huCI is a novel substrate protein of PADIs and that citrullinated huCI is a candidate autoantigen of RA.

Keywords: Autoantibodies; Peptidylarginine deiminases; Rheumatoid arthritis; Human type I collagen; Human type II collagen

Rheumatoid arthritis (RA) is a major autoimmune disease and affects ~1% of the world population. Many autoantibodies have been found in sera of RA patients, including antibodies against rheumatoid factor (RF). However, the antibodies that are most specific to RA are autoantibodies against citrulline-containing proteins or peptides. These are the so-called anti-citrullinated protein antibodies, including APF (anti-perinuclear factor), AKA (anti-keratin antibodies), AFA (anti-filaggrin antibodies), and anti-Sa. Anti-CCP antibody is an anti-citrullinated protein antibody that is reasonably sensitive (41–88%) and extremely specific (~98%) for RA, and has been used in clinical applications with high reliability [4,23,24]. The anti-CCP antibody system is also suited to the diagnosis of early RA [33]. Although the highly reliable anti-CCP antibody assay system (second generation) is designed to detect antibodies that recognize a mixture of synthetic peptides containing citrulline, the precise sequences of those peptides are not known. To clarify the pathologic mechanism of anti-citrullinated peptide antibodies in RA, it is important to identify native citrullinated peptides that are recognized by RA sera. Native citrulline-containing peptides are only produced by enzymatic conversion of peptidylarginine to citrulline, because citrulline is a non-coded amino acid in vivo. The enzymes involved in this conversion are

☆ *Abbreviations:* RA, rheumatoid arthritis; PADI, peptidylarginine deiminase; huCI, human collagen type I; huCII, human collagen type II; anti-MC, anti-modified citrulline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; pNPP, *p*-nitrophenyl phosphate.

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peptidylarginine deiminases (PADIs). Five PADI isozymes have been detected in humans, and two of them (PADI2 and PADI4) have been detected in RA synovial tissue [35]. Previously, we found that the PADI4 gene is associated with RA [28]. These facts strongly suggest that citrullination of self proteins (particularly by PADI4) and production of autoantibodies against those citrullinated proteins play pathologic roles in RA. Consequently, identification of the substrates of PADIs is important for investigation of autoimmunity in RA.

PADIs are enzymes that participate in post-translational modification of proteins by catalyzing citrullination of arginine residues. PADIs have five mammalian isoforms, which are expressed in different tissues and different stages of development [37]. All PADIs are dependent on calcium ions for activity. Arginine residues in a variety of proteins are modified by PADIs, but free L-arginine is not a substrate of PADIs [30]. Recently, citrullinated histone was reported to have an ability to antagonize transcriptional induction by regulating histone Arg methylation levels [8,38,41]. Although the physiologic role of peptidylcitrulline is unknown, it has been reported that citrullination is related to several diseases including autoimmune diseases [35,37,40].

Many substrates that are citrullinated by PADIs have been identified; e.g., histones, nucleophosmin/B23 [12], keratins [25], filaggrin [32], vimentin [2,34], myelin basic protein (MBP) [42], and fibrin(ogen) [18]. Not all of those proteins are expressed in RA-specific tissues, such as synovial tissue or joints, but all are recognized by RA autoantibodies. Also, there appears to be cross-reaction with anti-citrullinated antibodies. On the other hand, citrullinated fibrin(ogen) has been detected in synovial membranes of RA patients, and is recognized by RA autoantibodies [18]. However, it is unclear whether citrullinated fibrin(ogen) or other citrullinated peptides are pathogenic autoantigens of anti-citrullinated antibodies in RA. Therefore, we designed a survey to identify further candidate citrullinated proteins that are important in the pathology of RA autoimmunity.

To identify novel substrates of PADIs, which are candidate autoantigens of anti-CCP and anti-citrullinated peptide antibodies, we targeted proteins expressed in RA synovium. Because the anti-CCP antibody assay is designed to detect peptide epitopes, we also focused on citrulline-containing peptides rather than structures or various modifications of proteins. In order to survey proteins expressed by RA synovium regardless of their expression level, we adopted a λ-ZAP library of cDNA from RA synoviocytes as the origin of our proteins, and expressed proteins were citrullinated in vitro.

As a result of immunoscreening, we identified human collagen type I (huCI) peptides as a substrate of PADIs. Collagens, including CI, are structural proteins found in connective tissues of animals, and comprise the main extracellular support system. Type II collagen (CII)-induced arthritis (CIA) is a widely used model of arthritis [7,16,27,39]. Anti-CII antibodies were also commonly found in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. However, there have been no reports of a pathogenic role of CI in RA, although CI is distributed in bone, tendon, vascular tissues, synovial tissues, and skin (where it has functions related to its unique mechanical properties). In the present study, we identified human collagen type I (huCI) peptides as a candidate native substrate of PADIs. We also demonstrated that anti-citrullinated huCI peptide antibody is specific to RA, and that anti-citrullinated huCI peptide antibody appears to share some diagnostic features with anti-CCP antibody, although there are some distinguishing features.

Materials and methods

Human sera and synovial tissues. Human serum was obtained from three groups of subjects: 117 patients diagnosed with RA according to the criteria of the American Rheumatism Association [1]; 47 healthy blood donors (control); 37 patients with non-RA diseases (systemic lupus erythematosus, 19 patients; Sjogren's syndrome, 4 patients; Bechet's disease, 2 patients; other rheumatic disease, 12 patients). All non-RA patient serum and healthy control serum were anti-CCP negative. Mean age of the RA patients was 61.1 years (range, 33–81 years), and 66% of RA cases were positive for rheumatoid factor. No relationship was observed between age and anti-CCP antibody ($R^2 = 0.00001$). Mean age of non-RA patients and the healthy controls was 45.7 years (range, 20–82) and 40.5 years (range, 23–84), respectively. Informed consent was obtained from all subjects.

Preparation of PADIs. His-tagged human PADI4 (hPADI4) was expressed in *Escherichia coli*, BL21-SI, and was purified using a HiTrap protein purification system (Amersham Biosciences, Piscataway, NJ), as previously described [21]. Rabbit PADI2 (rPADI2) was obtained from Sigma. We used hPADI4 or rPADI2 in assays for in vitro citrullination of substrates according to assay condition requirements, after adjusting their enzymatic activity unit for artificial substrates, as described elsewhere [21].

Immunoscreening. A human RA synoviocyte cDNA library in λ-ZAP (Stratagene, La Jolla, CA) was used for the immunoscreening of citrullinated proteins. Induction of protein expression was performed according to the manufacturer's instructions. Briefly, the phage library was plated at 5×10^3 pfu/plate on a series of 100-mm NZY agar plates and was incubated at 37 °C for 5 h. After transfer to nitrocellulose membrane, protein expression was induced on the membranes by incubation with 20 mM IPTG at 37 °C for 4 h. After the membranes were washed, citrullination by hPADI4 was performed on a membrane with 0.075 U/ml hPADI4, 100 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT at 37 °C for 1 h. Detection of citrullinated proteins was performed using an anti-MC detection kit (Upstate, Waltham, MA). The positive clones were converted to a pBluescript II SK (+). The sequence of the inserted DNA was determined using an ABI PRISM 3700 Sequencer (Applied Biosystems, Foster City, CA).

Identification of citrullinated sites of human CI by LC/MS/MS. Human CI was citrullinated by rPADI2, which is generally used for analysis of anti-citrullinated antibodies, in a reaction buffer containing 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT for 12 h at 50 °C. Then, 100 μg huCI, with or without enzyme treatment, was separate by SDS–PAGE, and bands on Coomassie brilliant blue-stained gel were excised and digested with trypsin. The mixture of the digested peptides was analyzed by LC/MS/MS (APROscience, Toku-

shima, Japan). A Q-TOF2 mass spectrometer (Micromass, Manchester, UK) equipped with MAGIC 2002 (Michrom BioResources, Auburn, CA) was also used. Elution of peptides was performed using an acetonitrile gradient in 0.1% formic acid. The MS/MS data were searched against the SWISS-PROT database with protease specificities under consideration of fixed modification (propionamide-Cys) and variable modifications (citrullination of Arg, deamination of Asn and Gln, hydroxylation of Pro, Pyro-glu of N-terminus of Gln, and oxidation of Met) using the Mascot program (Matrix Science, Boston, MA).

Western blotting using human antisera. Citrullinated proteins were separated by SDS–PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). Membrane strips were probed with human sera (1:50) in blocking buffer (5% skimmed milk in T-TBS). Goat anti-human IgG peroxidase-conjugated antibodies were used for the detection of primary antibody, and peroxidase activity was visualized using ECL Western blotting reagents (Amersham).

Detection of citrullination of collagen type I and type II by ELISA. We coated 96-well ELISA plates (Corning, Corning, NY) with 100 μl of acid-extracted huCI (Becton, Dickinson and Company) and huCII (Collagen Research Center, Tokyo, Japan, or Becton, Dickinson and Company) per well in 0.01 M acetic acid at 5 μg/ml, and performed citrullination by rPADI2 at 50 °C. After washing with T-PBS and adding 0.1% ovalbumin in TBS, the plate was treated with 1% glutaraldehyde in PBS. Then, the plate was washed with 0.2 M Tris-HCl (pH 7.8) and distilled water. The modified citrullinated collagen was detected by anti-MC antibody (Upstate) using the standard method.

Detection of anti-citrullinated antibodies in RA patients and normal sera by ELISA. After coating and deimination as described above, anti-citrullinated collagen antibodies were detected by a modification of a previously reported method [29]. Briefly, citrullinated collagen coated plates were blocked with 5% skimmed milk in T-TBS. Then, 100 μl of each diluted serum was added, followed by incubation for 2 h at room temperature. After washing, 100 μl of 1:20000 dilution of goat $F(ab')^2$ anti-human IgG AP (Biosource, Camarillo, CA) was added, followed by incubation at room temperature for 1 h. Plates were developed at room temperature with 100 μl of pNPP substrate (Sigma) per well, and the absorbance at 405 nm was measured using a Fusion plate reader (PerkinElmer, Boston, MA). Standard control serum was analyzed, to assure standardized conditions for ELISA of anti-citrullinated collagen. All tests were run in triplicate. Testing for significant differences between means was performed using Student's *t* test (Microsoft Excel).

Results

Identification and confirmation of (in vitro) citrullination of proteins from synoviocyte cDNA library

To identify novel self molecules that are citrullinated as targets of RA-specific autoantibody, we immunoscreened $\sim 10^6$ pfu lambda of a ZAP cDNA expression library that was constructed using synoviocytes from RA patients. The expressed proteins were citrullinated and expressed proteins that were not treated with the citrullinating enzyme were used as a negative control (Fig. 1A). Three of the positive clones were obtained from a second screening plate and were identical with the huCI α1 gene. To confirm that the huCI was citrullinated, we performed Western blotting (Fig. 1B). We detected citrullination of huCI using ELISA (Figs. 1C and D). Our results indicate that PADIs citrullinated huCI.

After in vitro citrullination of huCI by rPADI2, we performed LC/MS/MS to identify the citrullination site of huCI. The coverage of LC/MS/MS analysis was 45% for huCI α1 precursor and 55% for huCI α2 precursor. We identified 20 citrullination sites of 35 arginine residues in analyzed fragments of huCI α1 and identified 27 citrullination sites of 45 arginine residues in analyzed fragments of huCI α2 (Figs. 1E and F). We did not observe sequence specificity of recognition of targeted arginine residues by the enzyme for conversion from peptidylarginine to peptidylcitrulline. These findings are consistent with those of a previous study [21]. We also confirmed that huCII was citrullinated by PADIs in vitro (data not shown), in addition to their citrullination of huCI.

IgG from RA patients' sera recognized citrullinated human collagen type I in vitro

Reactivity of sera to citrullinated and uncitrullinated huCI was analyzed by Western blotting using 10 RA sera and five normal control sera (Table 1). There was a slight difference in reactivity toward uncitrullinated huCI between sera of RA patients and sera of normal controls. Sera of RA patients were reactive toward the citrullinated huCI.

Additionally, we detected anti-citrullinated huCI by ELISA using serum from a RA patient with a high anti-CCP titer and anti-CCP-negative serum from a healthy control (Fig. 2A). Serum from the RA patient was highly reactive with citrullinated huCI, but serum from the healthy control was not reactive. We also performed time-course analysis (Fig. 2B). The level of anti-citrullinated huCI antibodies detected was increased by citrullination, and was dependent on the enzyme reaction time.

High titers of anti-citrullinated collagen antibody are present in RA sera, but not in normal sera

To investigate whether RA patients, non-RA patients, and normal healthy controls had serum antibodies against citrullinated collagens, ELISA was used to analyze panels of sera from RA patients, non-RA patients, and normal healthy controls.

Next, we measured levels of anti-collagen antibodies in the present subjects. The relative levels of anti-huCII were significantly higher for RA patients than for normal controls ($p = 0.0026$, Student's *t* test; Fig. 3B), and 22 of 56 sera (39%) from RA patients and 1 of 9 sera (11%) from healthy controls were positive for anti-huCII antibodies. However, the relative levels of anti-huCII were significantly higher for non-RA patients than for normal controls ($p = 0.00014$, Student's *t* test; Fig. 3B), and 8 of 13 sera (62%) from non-RA patients were positive for anti-huCII antibodies. In contrast, there

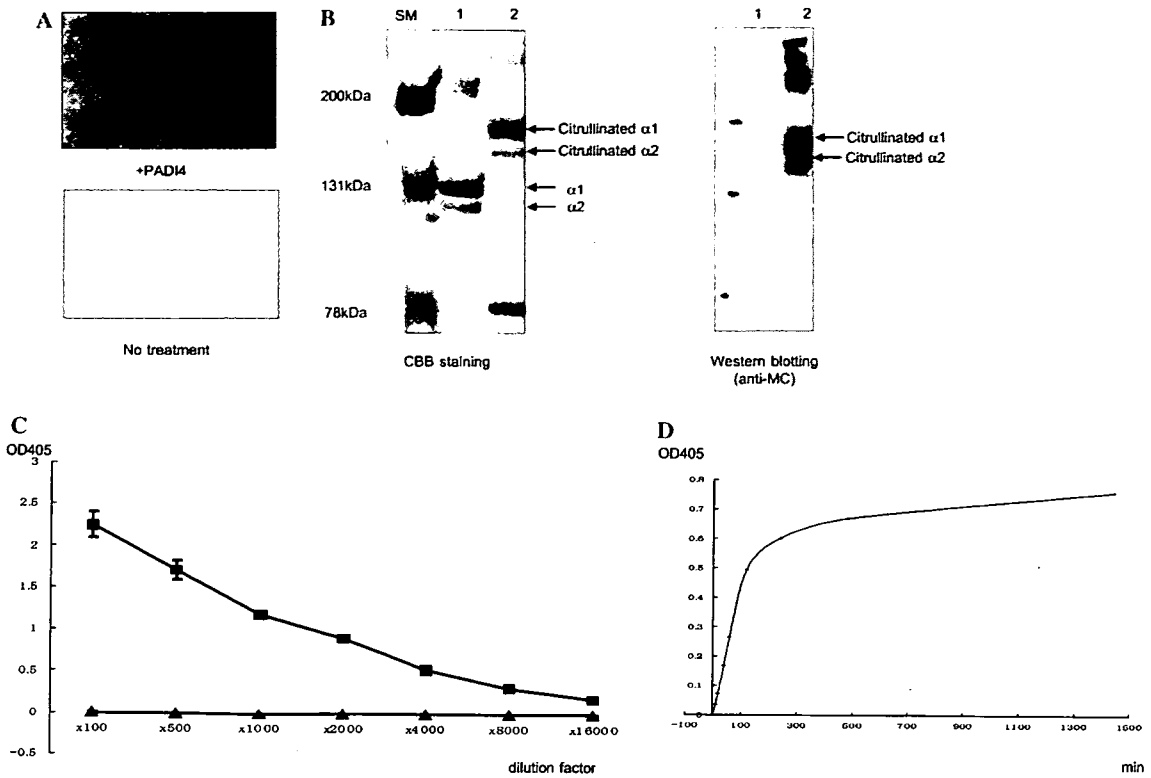

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Fig. 1. Identification and confirmation of citrullinated CI modified by PADIs. (A) Secondary immunoscreening using λ-ZAP cDNA expression library, with detection by anti-MC (1:1000). Three selected positive clones were identical: CI. Citrullination of CI by PADI2 and PADI4. (B) Citrullination by PADI2 was confirmed using Western blotting (SM, size marker; lane 1, CI; lane 2, modified CI). (C) The citrullinated CI was detected by ELISA at each dilution rate of anti-MC. Square indicates reaction with PADI2 and triangle indicates reaction with enzyme reaction buffer. (D) Time course of citrullination of human CI by PADI. The reaction mixtures were incubated at 50 °C for 0, 10, 20, 40, 60, 120, 240, 480, and 1440 min, respectively. Anti-MC (1:1000) was used for detection of citrullination by ELISA. (E,F) Amino acid sequence of procollagens (NCBI database, NP_000079 and NP_000080). Procollagen type I α1 (E) and procollagen type I α2 (F) were digested by peptidase, producing mature collagen. Italic letters indicate digested peptide. Colored amino acid sequence indicates peptide fragments that were analyzed by LC/MS/MS. Red-colored residues are highly citrullinated arginine residues, and green-colored residues are arginine residues that are unlikely to be citrullinated. All arginines are shown in bold letters.

was no difference in the level of anti-huCI antibodies among RA patients, non-RA patients, and normal controls (RA versus normal individuals, $p = 0.87$; non-RA versus normal individuals, $p = 0.88$; Student's t test; Fig. 3A). Five of 117 sera (4%) from RA patients, 1 of 46 sera from non-RA patients (2%), and 1 of 37 sera (3%) from healthy controls were positive for anti-huCI antibodies.

Table 1
Summary of Western blotting using antisera from RA patients and normal controls

	Citrullinated CI	Non-citrullinated CI
RA patients ID		
RA_1	+	+
RA_2		
RA_3	+	
RA_5	+	
RA_6	+	+
RA_7	+	
RA_8		
RA_9	+	
RA_10	+	
RA_11		
	7/10 (70%)	2/10 (20%)
Healthy controls ID		
HC_2		
HC_3		
HC_4		
HC_5		
HC_11		+
	0/5 (0%)	1/5 (20%)

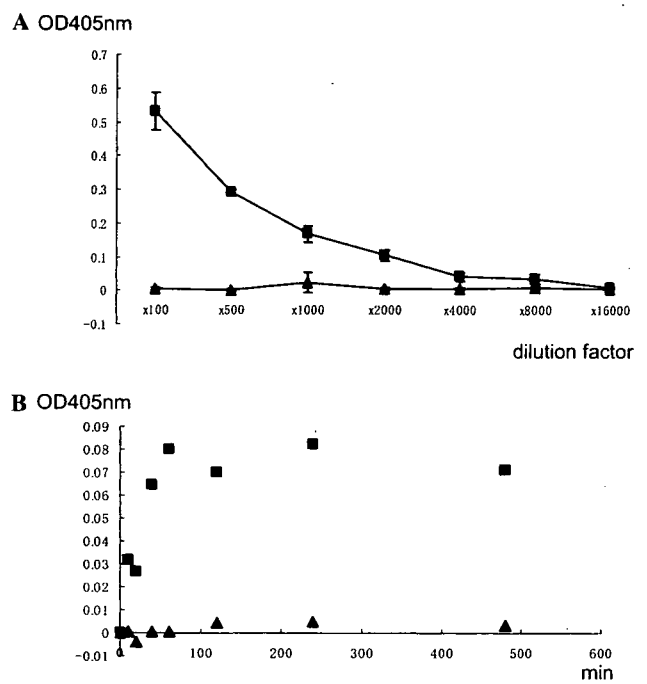

Fig. 2. Detection of citrullinated CI in sera of a RA patient and a normal control. (A) Citrullinated huCI modified by rPADI2 was detected by ELISA, using antisera from a RA patient (square) and a normal control (triangle). (B) Time course was confirmed using serum from the patient in (A).

Also, we measured the relative levels of anti-citrullinated collagen antibodies in RA sera, non-RA sera, and healthy control sera (Figs. 3C and D). The relative levels of anti-citrullinated huCI were significantly higher for RA patients than for normal controls or non-RA patients ($p = 0.000026$, $p = 0.0011$, Student's t test; Fig. 3C). Thirty-eight of 117 sera (32%) from RA patients, none of 37 sera from non-RA patients (0%), and 1 of 47 sera from normal controls (2%) were positive for anti-citrullinated huCI antibodies. In contrast, there was no significant difference in levels of anti-citrullinated huCII between RA patients and either normal controls or non-RA patients. Thirty-seven of 56 sera (66%) from RA patients, 7 of 13 sera from non-RA patients (54%), and none of 9 sera from normal controls (0%) were positive for anti-citrullinated huCII antibodies. In addition, no correlation between age and both of the level of anti-citrullinated CI ($R^2 = 0.0037$) and anti-citrullinated CII ($R^2 = 0.022$) was observed.

We investigated correlation between the relative levels of anti-citrullinated collagen antibodies and anti-CCP antibodies (Figs. 4B and D). Correlation between the level of anti-CCP and anti-citrullinated huCI antibodies was higher than the correlation between anti-CCP and anti-citrullinated huCII antibodies. We also compared the level of anti-CCP with that of anti-collagen antibody. Weak correlation or no correlation was observed between anti-CCP and both anti-huCII and anti-huCI (Figs. 4A and C). Furthermore, we found that anti-citrullinated huCI weakly correlated with anti-citrullinated huCII (Fig. 4E). We hypothesize that some of the anti-citrullinated huCI antibody cross-reacted with citrullinated huCII.

Discussion

Citrullinating enzymes [35] and autoantibodies that specifically recognize peptidylcitrulline, including anti-keratin antibody [26], anti-filaggrin autoantibodies [10], anti-Sa [19], and anti-CCP [11,14,29], are associated with RA. Citrullination of self-peptides is strongly suspected to be pathogenic in RA. However, self-peptides that are citrullinated have not been found to be pathologically linked to RA. Because anti-citrullinated antibodies are thought to be locally produced in RA synovium [17,22], we adopted RA synovial tissue as a source of pathologic citrullinated self-peptides. Among five PADI isoforms in humans, PADI2 and PADI4 are present in synovial fluid as well as synovial fluid mononuclear cells [36]. Citrullinated proteins have been detected not only in nuclear and intracellular areas, but also in amorphous deposits and extracellular matrix in RA synovial tissue [18]. Therefore, we widely targeted proteins expressed in synovium including intra- and extracellular proteins, regardless of their expression level. To identify substrates of PADIs as candidate autoantigens, we immunoscreened an expression cDNA phage library of RA synoviocytes. Our method allows

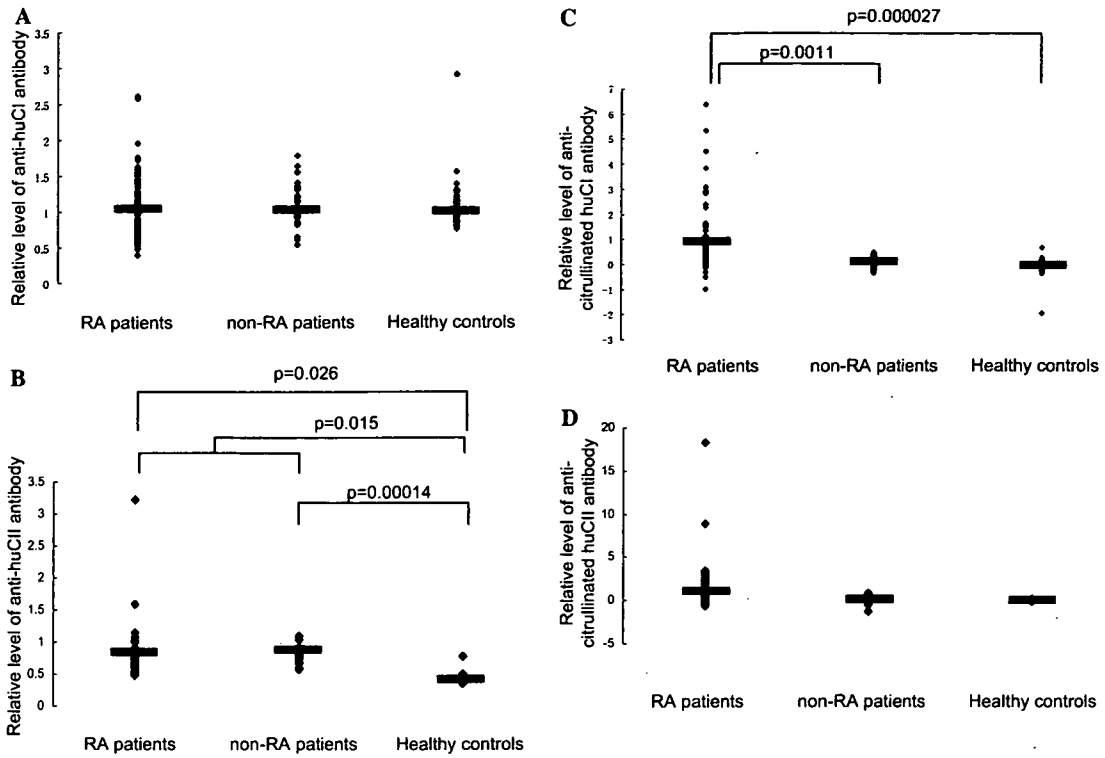

Fig. 3. Measurement of anti-collagen antibodies and anti-citrullinated collagen antibodies in RA patient sera and normal control sera. After coating, citrullination was produced by incubation with PADI2, followed by testing of the titers of anti-huCI antibodies and anti-citrullinated huCI antibodies (A,C, 117 RA sera, 37 non-RA patient sera, and 47 normal control sera). We also tested the titers of anti-huCII antibodies and anti-citrullinated huCII antibodies (B,D, 56 RA sera, 13 non-RA patient sera, and 9 normal control sera). The mean + 2 SD of healthy control values (A, >1.71; B, >0.72; C, >0.58; D, >0.12, respectively) was positive.

detection of insoluble proteins (including extracellular matrix proteins) as well as soluble proteins. This method also detects target proteins expressed at low levels, and is superior to other methods such as 2D-PAGE. One of several clones we identified was huCI peptides, which we examined in conjunction with other collagens known to be relevant to RA [9]. We confirmed citrullination of huCI by Western blotting, ELISA, and LC/MS/MS. We identified many citrullinated sites in huCI.

In the present study, we identified huCI peptide as a candidate substrate of citrullinated autoantigens by immunoscreening and found that anti-citrullinated huCI peptide antibody was specific to RA patients. huCI is one of the collagens that function as structural proteins, all of which have a characteristic triple helix structure with cyclic glycine and a high content of proline and hydroxyproline in their amino acid sequence. Among the collagens, CII has been the most studied, and there is evidence that it plays a pathologic role in RA. CII is major collagen in cartilage, and immunization with CII induces arthritis in mice and rats [6]. Bovine CII is also highly antigenic in transgenic mice that express HLA-DR1(*0101) and (*0401), which are associated with susceptibility to RA [5]. Anti-CII antibodies were observed in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. In the present study, anti-huCII antibody was also detected (41%) in RA patients. Compared to CII, there have been few reports indicating that CI plays a pathologic role in RA, although CI is widely expressed in bone, tendon, vascular tissues, synovial tissue, and various other tissues. In the present study, we observed no autoantibody recognizing non-citrullinated huCI in RA or control sera. In RA subjects, we observed that a marked increase in autoantibody positivity was associated with citrullination of huCI, but not with citrullination of huCII.

Although the present data indicate that anti-citrullinated huCI antibody is an RA-specific autoantibody, there are several issues that remain unresolved. First, collagen molecules form a triple helix with post-translational modification and their tertiary structure is believed to be a determinant of epitopes [13,15,20], although epitopes of anti-CCP antibodies are modified peptides. Second, it is not known how peptidylcitrullination alters antigenicity and breaks immunologic tolerance. The present findings, obtained by peptide-based immunoscreening and confirmation of recognition of citrullinated acid-extracted huCI molecules, provide a basis for further investigation to clarify the mechanisms of the roles of anti-citrullinated peptide antibodies in RA.

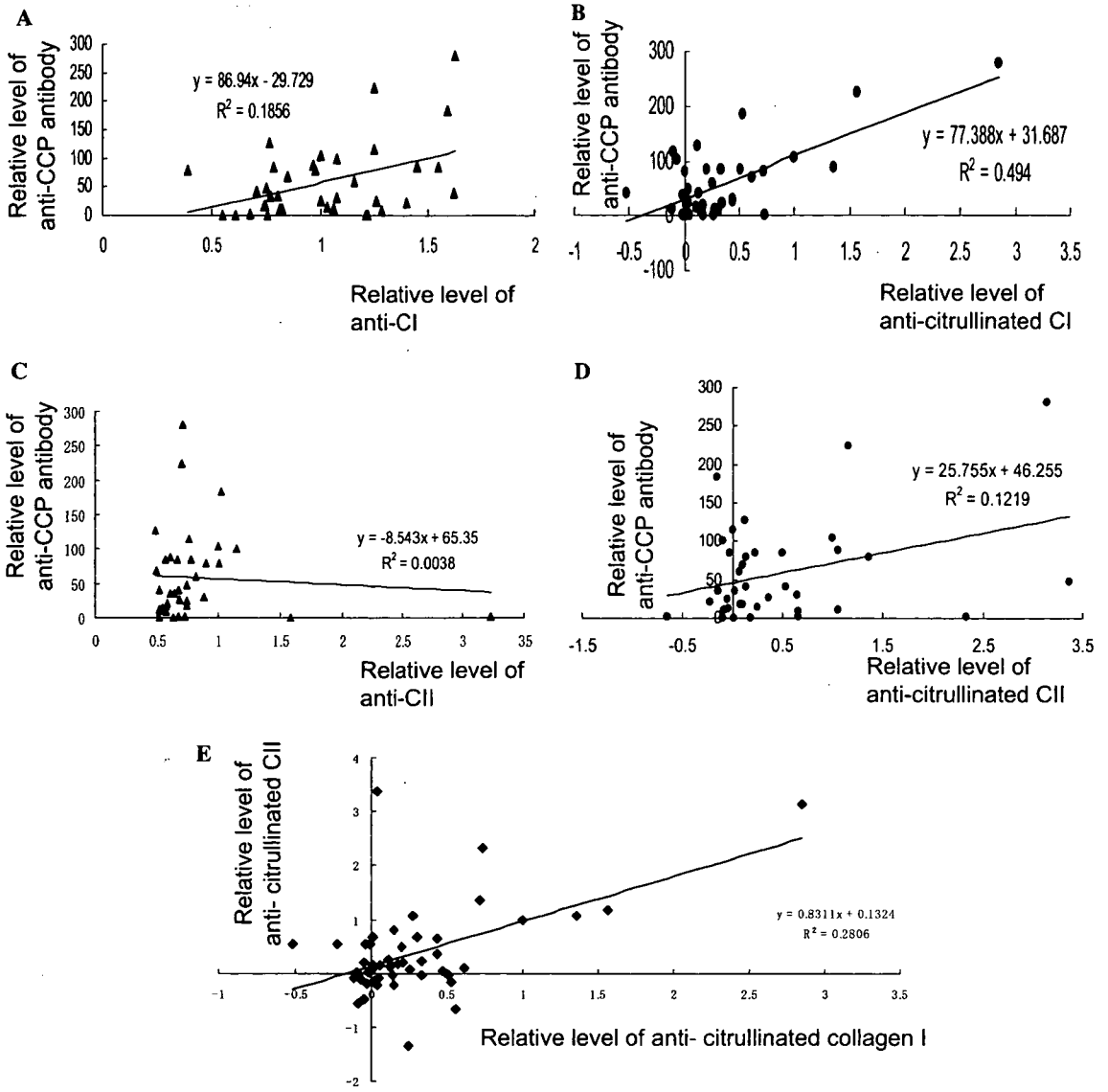

Fig. 4. Comparison of titers of anti-CCP and relative levels of anti-citrullinated collagen in RA sera. Comparison of (A) anti-huCI and (B) anti-citrullinated huCI levels with anti-CCP levels in RA sera. Comparison of (C) anti-huCII and (D) anti-citrullinated huCII levels with anti-CCP levels in RA sera. (E) Comparison of anti-citrullinated huCI levels with anti-citrullinated huCII levels in RA sera. There were no significant differences in any of these comparisons for any of the 37 RA samples. Regression line and correlation coefficient (R^2) are shown.

The present sensitivity and specificity of anti-citrullinated huCI antibody were 32% and 99%, respectively, and they correlated strongly with those of anti-CCP. The specificity of anti-citrullinated huCI antibodies was nearly equal to that of anti-CCP antibody, but the sensitivity of anti-citrullinated huCI antibodies was significantly less than that of anti-CCP antibody. Although almost all subjects who were positive for anti-CCP antibody were also positive for anti-citrullinated huCI antibody, a few were positive for anti-citrullinated huCI antibody but not for anti-CCP antibody. Because anti-CCP recognizes a mixture of synthetic peptides containing citrulline, and because huCI molecules contain multiple arginine residues that are citrullinated, it appears likely that epitopes of anti-CCP antibodies comprise the majority of those of anti-citrullinated huCI, but not all of them.

In conclusion, we found that huCI is a substrate of PADIs and that citrullinated huCI strongly correlates with RA. However, the present results indicate that CI can become an autoantigen via citrullination by PADIs, and citrullination as post-translational modification appears to be an important factor in RA. In addition, the present results suggest that anti-citrullinated collagen antibodies comprise a subclass of anti-CCP. To produce autoantigens in RA patients, PADIs must modify their substrates, but the mechanisms of this modification are unclear. However, PADIs are also clearly present in

the extracellular region [36]. Also, PADIs may be activated in the extracellular region, because the calcium ion concentration is sufficiently higher in the extracellular region than in the cytoplasm or intracellular region [21]. We speculate that autoantibodies for citrullinated collagens react or cross-react with other citrullinated proteins that are locally produced at the site of rheumatoid inflammation of synovial tissue. We believe that anti-citrullinated huCI plays important roles in the development of RA. More study of the mechanisms of citrullination in vivo may provide findings that are applicable to RA therapy.

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Enhanced Efficacy of Regulatory T Cell Transfer Against Increasing Resistance, by Elevated Foxp3 Expression Induced in Arthritic Murine Hosts

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Objective. **To investigate the efficacy of type II collagen–reactive Foxp3-expressing T cell transfer in suppressing collagen-induced arthritis (CIA) in relation to disease progression.**

Methods. **CD3-activated CD4 T cells were retrovirally transduced with the Foxp3 gene, and their in vitro suppressive activity on T cell proliferation was assessed for correlation with Foxp3 levels. To suppress CIA, Foxp3-transduced T cells generated with type II collagen– or ovalbumin (OVA)–pulsed dendritic cells (DCs), which were fractionated by Foxp3 levels, were adoptively transferred to mice at various time points.**

Results. **The in vitro suppressive activity of Foxp3-transduced cells correlated positively with Foxp3 levels. Type II collagen–reactive, but not OVA-reactive, Foxp3-transduced cells significantly suppressed CIA when they were transferred before immunization, and this suppression was accompanied by decreased anti–type II collagen antibody production. Larger cell numbers were required to suppress CIA when transfer occurred 20 days after immunization, indicating that hosts became resistant to suppression. Transfer of 1×10^5 $Foxp3^{low}$ cells had only a marginal effect on CIA suppression in immunized hosts, while transfer of $Foxp3^{high}$ cells at smaller doses significantly suppressed CIA. Transfer of 1×10^5 $Foxp3^{high}$ cells after establishment of arthritis attenuated disease progression but did not reverse joint swelling.**

Conclusion. **Resistance to Foxp3-transduced T cells proceeded as CIA progressed, suggesting that late-stage aggressive arthritis is more resistant to regulatory T cell transfer. An elevated expression level of Foxp3 in type II collagen–specific T cells improved their suppressive function in CIA. Thus, transfer of T cells expressing high levels of Foxp3 could be a strategy to overcome the induced resistance to regulatory T cell therapy.**

Recent studies have added to the accumulated evidence that CD4+,CD25+ naturally arising regulatory T cells (Treg) play a crucial role in the maintenance of peripheral self tolerance (1,2). Foxp3, a transcription regulator belonging to the forkhead/winged helix transcription factor family (3), is expressed exclusively in T cells with regulatory activities, including naturally arising CD4+,CD25+ Treg (4–6) and some adaptive Treg subsets (7–9). Foxp3 is considered as a master gene characterizing phenotypes and functions of Treg. Retrovirus-mediated ectopic Foxp3 expression confers CD4+,CD25− non-Treg characteristics similar to those of CD4+,CD25+ Treg, such as expression of CD25, CTLA-4, CD103, and glucocorticoid-induced tumor necrosis factor receptor (GITR) (4). The suppressive activities induced in these cells are independent of soluble factors such as interleukin-10 (IL-10) or transforming growth factor β (4,10).

The involvement of CD4+,CD25+ Treg in auto-

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immune diseases in humans and animals has been intensively investigated over the past several years. Analyses of clinical samples demonstrated that patients with multiple sclerosis (11), autoimmune polyglandular syndromes (12), juvenile idiopathic arthritis (13,14), or rheumatoid arthritis (RA) (15–17) had an abnormal number and/or abnormal regulatory function of CD4+,CD25+ T cells. These facts brought forth the idea that supplementation of functionally intact Treg may reverse the activation of autoreactive T cells in patients with these diseases. The development of autoimmunity induced by transfer of CD25-depleted CD4 T cells to athymic nude mice as well as by neonatal thymectomy of normal mice was successfully prevented by adoptive transfer of polyclonal CD4+,CD25+ T cells (18,19).

Similarly, adoptive transfer of naturally arising CD4+,CD25+ Treg as well as Foxp3-transduced T cells was also successful in suppressing autoimmunity in various murine models of lymphopenia, which offer a favorable environment in which the transferred lymphocytes have room to expand. These models included experimental autoimmune encephalomyelitis, autoimmune diabetes, and autoimmune colitis (4,20–23). In some studies, antigen-specific CD4+,CD25+ cells were transferred, while in other studies, nonspecific T cells were transferred. Generally, larger numbers of polyclonal T cells were required to suppress the same disease (i.e., autoimmune diabetes) (21,22). Thus, antigen specificity is a factor promoting the in vivo effects of Treg, probably because they are recruited and/or expand at the sites of local inflammation.

A previous study demonstrated that in vivo depletion of CD25+ cells prior to initiation of collagen-induced arthritis (CIA), a model of RA, worsened arthritis. This finding suggested that CD4+,CD25+ Treg have an inhibitory role in disease development (24). As shown in other animal models of lymphopenia, transfer of non–antigen-specific Treg CD4+,CD25+ T cells inhibited CIA development when host mice received a lethal dose of total body irradiation followed by rescue with syngeneic bone marrow transplantation (25). If larger numbers of CD4+,CD25+ T cells were transferred, a suppressive effect was also observed, without induction of lymphopenia. However, transferring a large number of non–antigen-specific Treg may lead to systemic immune suppression.

The aim of this study was to determine whether antigen-specific Treg transfer efficiently suppresses arthritis in immunocompetent hosts, and whether susceptibility to suppression by Treg changes during the disease course.

MATERIALS AND METHODS

Mice. Male DBA/1J mice were purchased from the Oriental Yeast Company (Tokyo, Japan). The mice were housed in the animal facility under specific pathogen–free conditions at the Research Center for Allergy and Immunology, RIKEN.

Monoclonal antibodies (mAb) and flow cytometry. Fluorescein isothiocyanate–conjugated anti-mouse CD3e (145-2C11 antibody), phycoerythrin (PE)–conjugated anti-mouse CD4 (L3T4 antibody), biotinylated anti-mouse CD25 (mAb 7D4; IgM), and allophycocyanin (APC)–conjugated streptavidin were purchased from BD PharMingen (San Diego, CA). APC-conjugated anti-mouse CD11c (N418) antibody was purchased from eBioscience (San Diego, CA). Single-cell suspensions were incubated with fluorescent or biotinylated mAb; incubation with biotinylated mAb was followed by incubation with PE-conjugated streptavidin. Data were acquired on a FACSCalibur and were analyzed using CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA).

Preparation of retroviral construct and supernatants. Two Foxp3-containing vectors were prepared for these experiments. The first vector consisted of an MIGR1 vector with a mouse Foxp3 complementary DNA insert (MIGR1-Foxp3) (4). The second vector contained the *Eco* RI–*Bgl* II fragment from MIGR1-Foxp3, which was ligated into an *Eco* RI–*Bam* HI–cleaved pMCs-IG vector (Foxp3-pMCs-IG) (26). MIGR1 and pMCs-IG encode green fluorescent protein (GFP) under the control of an internal ribosomal entry site. Retroviruses were prepared by introducing the empty vectors or the vectors with Foxp3 into the Plat-E packaging cell line (26,27).

Dendritic cell (DC) preparation. Bone marrow cells were collected from 6–7-week-old male DBA/1J mice. The bone marrow cells were cultured in RPMI 1640 medium containing 20 ng/ml granulocyte–macrophage colony-stimulating factor (PeproTech, London, UK), 10% fetal bovine serum, 2 μM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics for 7 days. The cells were then cultured in the presence of 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) with 50 μg/ml bovine type II collagen (Collagen Research Center, Tokyo, Japan), ovalbumin (OVA; Sigma-Aldrich), or LPS alone for a further 24 hours. These cells were used as mature DCs. A majority of the cells harvested by this method were CD11c positive.

Priming of CD4 T cells with antigen-pulsed DCs, and retroviral infection. DBA/1J mice were immunized twice (1-week interval) with 200 mg of either bovine type II collagen or OVA in Freund's complete adjuvant (CFA; Difco, Detroit, MI), in the hind footpads. CD4 T cells were isolated from the draining lymph nodes of immunized mice or from the splenocytes of naive mice, using magnetic-activated cell sorting microbead-coupled mAb and magnetic cell separation columns (Miltenyi Biotec, Auburn, CA). The cells were cultured with type II collagen–pulsed DCs, OVA-pulsed DCs, or DCs treated with anti-mouse CD3e mAb (145-2C11; BD PharMingen) in the presence of 100 units/ml recombinant human

IL-2 (Shionogi Pharmaceuticals, Osaka, Japan) for 24 hours. The activated CD4 T cells were mixed with an equal volume of retroviral supernatant and Polybrene (6 μg/ml; Sigma-Aldrich), centrifuged at 1,750*g* at 32°C for 1 hour, and incubated for a further 7 hours at 37°C in a 5% CO_2 atmosphere. The cells were then cultured in complete medium supplemented with 100 units/ml IL-2. After 2 or 3 days, live GFP-positive fractions of the infected cells were isolated using a BD FACSAria Cell Sorter (BD Biosciences Immunocytometry Systems). To assess antigen-specific priming of CD4 T cells, 1×10^5 CD4 T cells were cultured with antigen-pulsed DCs or unpulsed DCs at various ratios, in 96-well plates. ^{3}H-thymidine uptake was measured after 3 days. For immunoblot analyses and suppression assays, activated CD4+,CD25+ T cells were prepared by culturing CD4+,CD25+ T cells with DCs in the presence of anti-CD3 mAb (0.5 μg/ml) and IL-2 (100 units/ml) for 3 days.

Immunoblot analyses. Whole cell lysates were prepared using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and a fraction (10 μg) was loaded to each lane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblot analyses were performed using anti-Foxp3 rabbit antibody (28) as primary antibody and horseradish peroxidase–conjugated anti-rabbit antibody as secondary antibody (Santa Cruz Biotechnology), with detection accomplished using the enhanced chemiluminescence technique (Amersham Biosciences, Uppsala, Sweden). The measurement of band intensity was performed using ImageJ software (29).

Suppression assay. To assess the suppressive activities of regulatory T cells, CD4+,CD25− responder T cells (5×10^4/well) and irradiated antigen-presenting cells (whole splenocytes; 1×10^5/well) were cultured in a 96-well plate with Treg at various ratios, in the presence of anti-CD3 mAb (0.5 μg/ml). ^{3}H-thymidine (1 μCi/well) uptake was measured after 3 days.

Induction of CIA and clinical assessment of arthritis. Eight-week-old male DBA1/J mice were immunized intradermally at the tail base with 200 μg of bovine type II collagen in CFA (Difco). The mice received booster immunizations in the same manner, 21 days after the primary immunization. After the booster immunization (day 0), the disease severity in each limb was recorded using the following scoring system: 0 = normal, 1 = mild swelling in 1 joint, 2 = mild swelling in ≥2 joints, 3 = severe swelling in the paw or digits, and 4 = severe swelling in entire paw and digits.

Detection of bovine type II collagen–specific antibodies. Bovine type II collagen–specific antibodies in mouse serum were measured by enzyme-linked immunosorbent assay. Microtiter plates were coated with 2 μg/ml bovine type II collagen, which was denatured by boiling in phosphate buffered saline (PBS). The plates were washed with 0.05% Tween 20 in PBS (PBST), blocked with 2% bovine serum albumin (BSA) in PBS, and incubated with diluted mouse serum (1:1,000 ratio). Positive reactions were detected by incubation with rabbit anti-mouse IgG1, IgG2a, or IgG2b conjugated to horseradish peroxidase (Zymed, Burlingame, CA) in PBST with 2% BSA. The final reaction was visualized using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). Optical density values were measured at 450 nm. Bovine type II collagen–specific antibody units were determined using a reference serum created from pooled sera from arthritic or nonarthritic mice. A 1:40 dilution of serum from arthritic mice was assigned a value of 1,000 units/ml.

Histologic assessment of arthritis. Mice with CIA were killed 14 days after receiving the booster immunization. The knee joints were removed, fixed in formalin, and decalcified in 10% EDTA. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical analysis. Experimental groups were analyzed by fitting a repeated-measures model with a random intercept (30), using the linear mixed effects function in the "nlme" (31) package of R, version 2.4.1 (32). Full models were compared with models without the treatment term. *P* values less than 0.05 were considered significant.

RESULTS

Suppression of T cell proliferation in vitro by Foxp3-transduced cells. To investigate whether adoptive transfer of Foxp3-expressing T cells can suppress CIA in nonlymphopenic animals, we generated CD4 Treg by retroviral Foxp3 gene transfer. Splenic CD4 T cells from naive mice were mixed with mature DCs at a 10:1 ratio, in the presence of anti-CD3 mAb and IL-2. Stimulated T cells were infected with retroviral vectors including both GFP and Foxp3 genes (Foxp3-pMCs-IG) or GFP gene alone (pMCs-IG). The percentage of GFP-expressing cells was initially 30–50% (Figure 1A) but gradually decreased after 4 days in Foxp3-pMCs-IG–infected cells but not in pMCsIg–infected cells (data not shown), which indicates that Foxp3 expression repressed cell growth. The Foxp3-expressing cells up-regulated expression of CD25, GITR, and intracellular CTLA-4, which correlated with the GFP expression level (data not shown). Foxp3 was expressed at a higher level in Foxp3-transduced cells than in freshly isolated or anti-CD3–activated CD4+,CD25+ T cells from naive mice, based on Foxp3:β-actin intensity ratios of 1.2, 0.7, and 1.0, respectively (Figure 1B). In vitro suppressive activity was enhanced in activated cells compared with fresh cells (Figure 1C). CD3-primed Foxp3-transduced T cells significantly suppressed the proliferation of responder T cells, with efficiency that was remarkably higher than that of freshly isolated CD4+,CD25+ T cells and was comparable with that of activated CD4+,CD25+ T cells (Figure 1D).

Suppression of CIA by adoptive transfer of antigen-reactive Foxp3-transduced T cells. CD4 T cells isolated from the lymph nodes of type II collagen–immunized mice were activated in vitro with type II collagen–pulsed DCs and transfected with retroviruses. Because proliferating cells are susceptible to integration of retroviral genomes, reactivity to type II collagen of the transduced cells should be enriched. The enriched

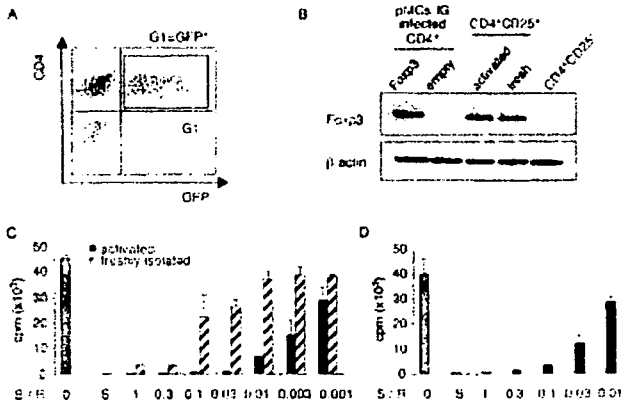

Figure 1. Foxp3 expression and suppressive activity of regulatory T cells. A, Splenic CD4 T cells from naive mice were stimulated with dendritic cells (DCs) and anti-CD3 monoclonal antibodies (mAb) and infected with Foxp3-pMCs-IG. The green fluorescent protein (GFP)–expressing fraction at 48 hours after infection is shown. B, CD4 T cells were activated and infected with Foxp3-pMCs-IG or pMCs-IG. After 48 hours, GFP-expressing fractions were isolated in order to prepare whole cell lysates for immunodetection of Foxp3 and β-actin. Whole cell lysates were also prepared from freshly isolated and activated CD4+,CD25+ T cells as well as CD4+,CD25− T cells. Activated CD4+,CD25+ T cells were prepared by culturing with DCs treated with anti-mouse CD3e mAb in the presence of 100 units/ml interleukin-1 for 72 hours. C, Freshly isolated and activated CD4+,CD25+ T cells (suppressors; S) were cultured with CD4+,CD25− responder T cells (responders; R) in the presence of irradiated splenocytes and anti-CD3 mAb. Proliferation of responder T cells at the indicated S:R ratios was assessed by measuring ^{3}H-thymidine uptake. Values are the mean and SD and are representative of 5 individual experiments. D, Foxp3-transduced CD4 T cells were cultured with responder T cells for assessment of suppressive activity, in the same manner. Values are the mean and SD and are representative of 3 individual experiments.

specificity was confirmed by antigen-specific proliferation of CD4 T cells from lymph nodes of type II collagen–immunized mice reacting with type II collagen–pulsed DCs compared with antigen-unpulsed DCs (Figure 2A).

To evaluate whether Foxp3-transduced T cells effectively suppressed CIA, either 1×10^5 or 5×10^4 cells were adoptively transferred to the mice 1 day prior to the primary type II collagen immunization for initiation of CIA. Although arthritis should eventually develop after a single immunization, the mice received booster immunizations after 21 days, to synchronize the onset of joint swelling. Both doses of adoptively transferred cells were equally effective for lowering the arthritis scores of treated animals (Figure 2B). This indicated that transfer using this timing was effective in achieving suppression of arthritis, but that the amounts of transferred cells reached optimal levels in this setting. CD4 T cells from lymph nodes of OVA-immunized mice were activated with OVA-pulsed DCs (Figure 2C) and transfected with Foxp3-expressing retroviruses. The generated OVA-reactive Foxp3-transduced T cells exhibited suppressive activity in vitro against CD3-activated T cell proliferation, which was approximately comparable with that of CD3-primed Foxp3-transduced T cells. Transfer of OVA-reactive Foxp3-transduced T cells prepared in

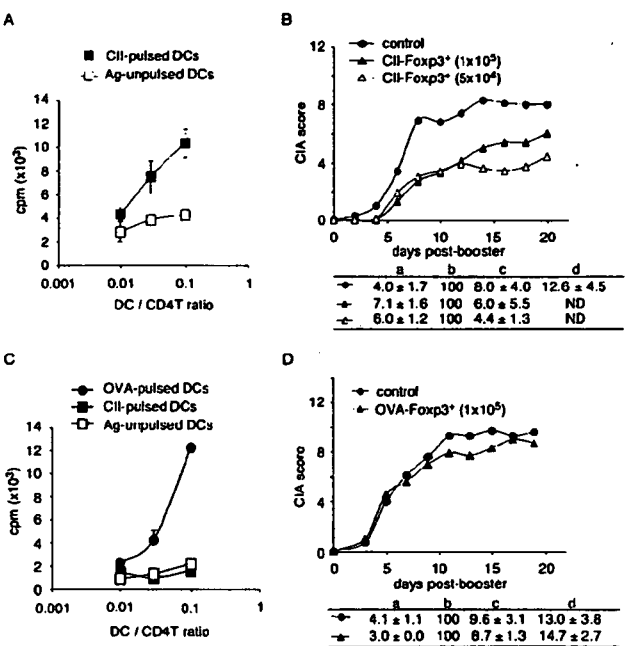

Figure 2. Suppression of collagen-induced arthritis (CIA) by type II collagen (CII)–primed Foxp3-transduced T cells transferred before the primary CII immunization. A and C, CD4 T cells derived from CII-immunized mice (A) or ovalbumin (OVA)–immunized mice (C) were mixed with either CII-pulsed dendritic cells (DCs), OVA-pulsed DCs, or antigen (Ag)–unpulsed DCs at the indicated ratios. Proliferation was measured by ^{3}H-thymidine uptake after 3 days. Values are the mean ± SD results from triplicate cultures. B and D, Naive DBA/1 mice received 1×10^5 (n = 7) or 5×10^4 (n = 7) Foxp3-transduced T cells primed by CII-pulsed DCs (CII-Foxp3+) (B) or 1×10^5 Foxp3-transduced T cells primed by OVA-pulsed DCs (OVA-Foxp3+; n = 7) (D) intravenously, 1 day prior to the primary immunization with CII. A group of mice received no T cell transfer (control; n = 15 in B, n = 7 in D). A booster immunization was administered 21 days after the primary immunization. B, Differences between the control group and the groups receiving CII-primed Foxp3+ (both doses) were significant ($P < 0.01$ by repeated-measures analysis of variance). Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. ND = not determined.

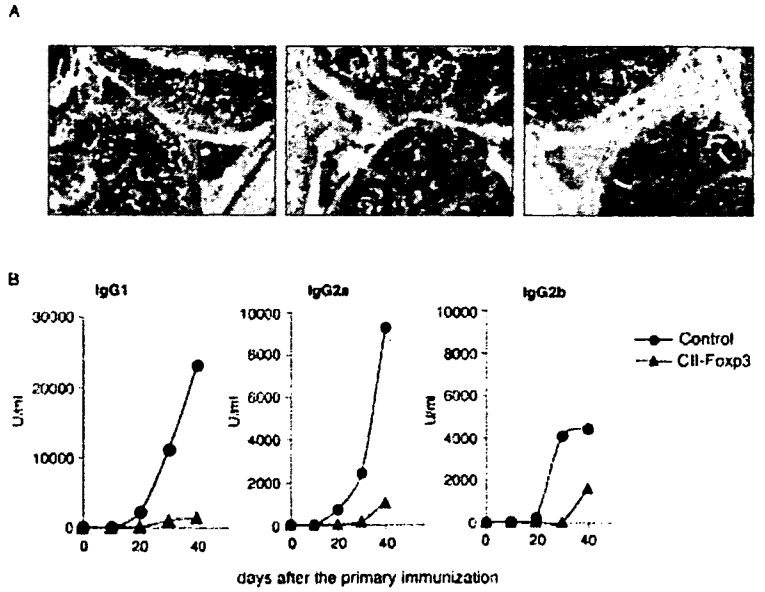

Figure 3. Suppression of joint inflammation and bovine type II collagen (CII)–specific antibody production by Foxp3-transduced T cell transfer. **A,** Histopathologic examination was performed 14 days after booster immunization to compare the knee joints of control mice with collagen-induced arthritis (CIA) (middle panel) and those of CIA mice that received transfer of 5×10^4 Foxp3-transduced T cells 1 day prior to the primary immunization (right panel) with the knee joints of normal naive mice (left panel). **B,** Mice received 1×10^5 Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3; n = 4) intravenously, 1 day prior to the primary immunization. A group of control mice (n = 4) received no T cell transfer. Serum was collected from each mouse on the day of the primary immunization and 10, 20, 30, and 40 days after the immunization. Unit values for each IgG subclass were measured using enzyme-linked immunosorbent assay. Plots represent the mean values for the 4 samples. Statistically significant differences, by repeated-measures analysis of variance, were observed between control and CII-Foxp3+ groups in the IgG1 and IgG2a subclasses ($P < 0.01$) and the IgG2b subclass ($P < 0.05$).

the same manner failed to suppress CIA (Figure 2D), suggesting that antigen specificity of Foxp3-transduced T cells is crucial.

Histopathologic assessment comparing the inflammation-related findings in control CIA mice, such as infiltration of mononuclear cells in the hypertrophic synovial tissue and destruction of joint cartilage (Figure 3A, middle panel), with those in normal mice (Figure 3A, left panel) demonstrated that these findings were suppressed in CIA mice that received transfer of type II collagen–reactive Foxp3-transduced T cells (Figure 3, right panel). Furthermore, the production of type II collagen–specific antibodies was suppressed for each of the IgG1, IgG2a, and IgG2b subclasses (Figure 3B).

We next examined whether adoptive transfer is effective when it is performed after immunization of the hosts. The same numbers of type II collagen–reactive Foxp3-transduced T cells as were used in the above-described experiments were transferred to the mice 20 days after the primary immunization, and booster immunization was performed on the following day. Transfer of 1×10^5 type II collagen–reactive Foxp3-transduced T cells significantly suppressed CIA, while transfer of 5×10^4 cells did not (Figure 4A). It should be noted that both approaches were effective in preventing arthritis in naive hosts (Figure 2B). Further, type II collagen–reactive Foxp3-transduced T cells were transferred intravenously 4 days after booster immunization, the time point at which joint swelling became apparent in most of the mice. At that time point, even 1×10^5 cells had no effect on CIA progression (Figure 4B). Thus, type II collagen–immunized mice became progressively more resistant to suppression by Treg.

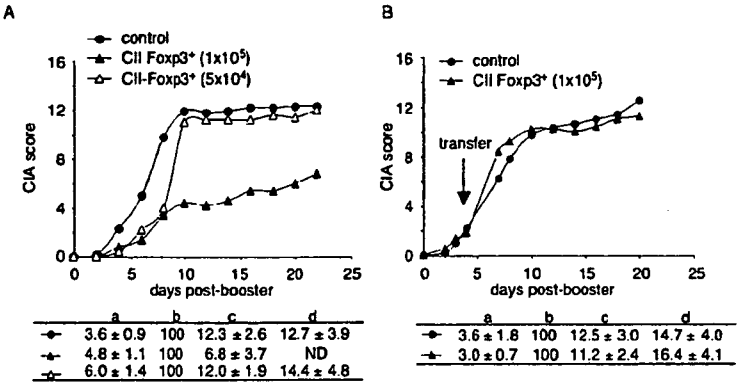

Figure 4. Induced resistance to Foxp3-transduced T cells after type II collagen (CII) immunization. Mice received transfer of 1×10^5 ($n = 5$) or 5×10^4 ($n = 5$) Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3+) intravenously, 20 days after the primary CII immunization (A) or 4 days after booster (B). A group of control mice received no T cell transfer ($n = 9$ in A; $n = 10$ in B). A, The difference between the control group and the group receiving Foxp3+ at a dose of 1×10^5 was significant ($P < 0.01$ by repeated-measures analysis of variance). Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. CIA = collagen-induced arthritis; ND = not determined.

Correlation of suppressive activity and expression levels of transduced Foxp3. As described previously, activated naturally arising Treg become more suppressive and express Foxp3 at higher levels than those expressed by freshly isolated cells. This fact led us to examine whether high-level Foxp3 expressers among Foxp3-transduced T cells exert stronger antiarthritic effects. Using a previously performed technique (4), we isolated $Foxp3^{high}$ and $Foxp3^{low}$ fractions based on their GFP expression level (Figure 5A, left). Immunoblot analyses of their cell lysates showed that Foxp3 expression in the fractionated cells correlated well with GFP expression. The low GFP fraction expressed Foxp3 at a level comparable with that expressed by naturally arising CD4+,CD25+ T cells (Figure 5A, right). Consistent with previous results (4), the $Foxp3^{high}$ fraction suppressed in vitro proliferation of CD4+,CD25− T cells more efficiently than did the $Foxp3^{low}$ fraction (Figure 5B).

The differential suppressive activity of the $Foxp3^{high}$ and $Foxp3^{low}$ cells may derive from differences in their activation levels. Theoretically, highly activated cells may enter the cell cycle more efficiently, thus making themselves more susceptible to retroviral integration. Actually, GFP expression levels of the T cells infected with empty vector (pMCs-IG) correlated positively with their surface CD69 levels (data not shown). To examine directly whether the Foxp3 expression level controls suppressive activity, we additionally used Foxp3-MIGR1, a retrovirus vector that was less efficient at protein expression, joined to the Foxp3 gene. The CD4 T cells activated with anti-CD3 mAb and DCs were divided for transfection with either Foxp3-pMCs-IG or Foxp3-MIGR1 retrovirus vector. Foxp3-transduced T cells generated by Foxp3-pMCs-IG expressed higher levels of Foxp3 (Figure 5C) and had higher suppressive activity (Figure 5B). Thus, the Foxp3 expression level regulated suppressive activity of Foxp3-transduced Treg.

Suppression of CIA by $Foxp3^{high}$ T cells versus $Foxp3^{low}$ T cells. For treatment of CIA, type II collagen–specific $Foxp3^{high}$ and $Foxp3^{low}$ T cell populations were generated from type II collagen–primed T cells. First, 1×10^5 cells from each population were transferred to mice 1 day prior to the primary type II collagen immunization. The 2 treatments suppressed arthritis equally, as shown by the arthritis score and measurement of joint swelling (Figure 6A). Transfer of the same number of OVA-reactive $Foxp3^{high}$ as well as $Foxp3^{low}$ T cells did not affect CIA development (Figure 6B). We next transferred $Foxp3^{high}$ or $Foxp3^{low}$ T cells to the hosts 20 days after the immunization (Figure 6C). When these mice were subjected to booster immunization for development of overt arthritis, $Foxp3^{low}$ T cells exerted only a marginal suppressive effect, demonstrating again that

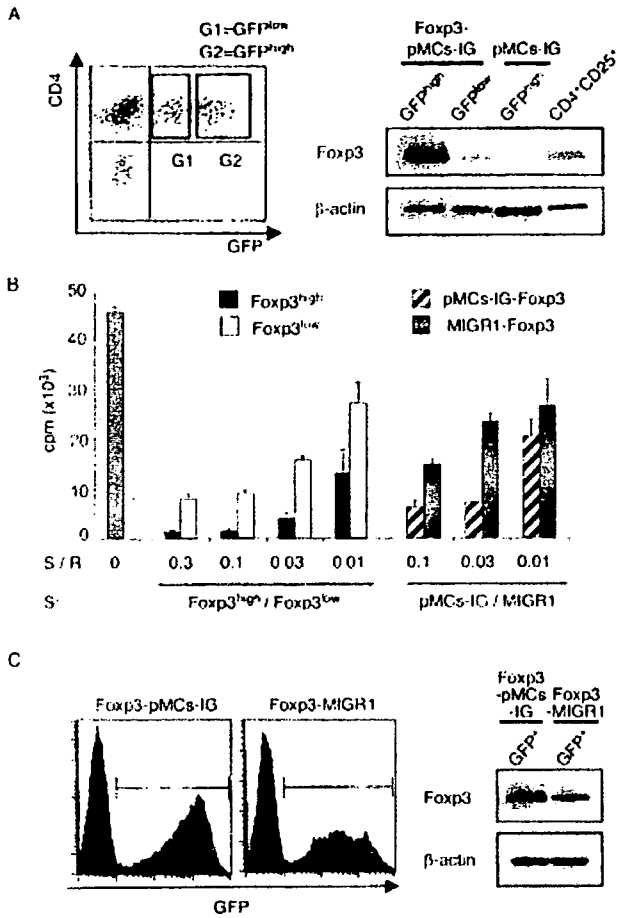

Figure 5. Dependence of Foxp3 expression level on suppressive activity of Foxp3-transduced T cells. **A**, CD4 T cells were activated and infected with Foxp3-pMCs-IG retroviruses as described in Figure 1. Panels represent the gates for isolating green fluorescent protein $(GFP)^{high}$ and GFP^{low} fractions (left), and immunoblots show Foxp3 or β-actin expression in GFP^{high} and GFP^{low} fractions of T cells infected with Foxp3-pMCs-IG, control pMCs-IG–infected cells, and naturally arising CD4+,CD25+ T cells (right). **B**, GFP^{high} or GFP^{low} fractions of T cells were infected with Foxp3-pMCs-IG viruses and isolated 48 hours after infection. Likewise, 2 T cell fractions were separately infected with Foxp3-pMCs-IG or Foxp3-MIGR1 retroviruses, and whole GFP-positive fractions were isolated. These suppressors (S) were cultured with CD4+,CD25− responder T cells (R) with irradiated splenocytes and anti-CD3 monoclonal antibody (0.5 μg/ml) for 72 hours. Proliferation of responders was assessed by ^{3}H-thymidine uptake. Bars show the mean and SD. **C**, CD4 T cells were activated as described in Figure 1 and infected with either Foxp3-pMCs-IG or Foxp3-MIGR1 retrovirus. Histograms (left) show GFP expression of the infected cells at 48 hours after infection. The gates on the histograms indicate Foxp3-expressing fractions, which were then sorted for detection by immunoblotting (right).

immunized hosts became resistant to suppression by Treg. In contrast, $Foxp3^{high}$ T cells overcame the resistance and suppressed CIA efficiently. A titration study demonstrated that as few as 1×10^4 $Foxp3^{high}$ T cells significantly suppressed CIA (Figure 6D). Because this number of unfractionated Foxp3-transduced cells had no effect, $Foxp3^{high}$ T cells had higher suppressive activity in vivo as well as in vitro.

Transfer of the same number of $Foxp3^{high}$ or $Foxp3^{low}$ fractions into knee joints at the same time did not suppress arthritis as efficiently as did systemic transfer. The effect was observed even in forelimbs (data not shown). This implies that the site of function for the Treg is not local synovial tissue. Finally, type II collagen–specific $Foxp3^{high}$ T cells (1×10^5 cells) were transferred intravenously 4 days after the booster immunization. The treatment attenuated progression of arthritis slightly but did not reverse the joint swelling (Figure 6E). Thus, arthritic mice gained further resistance to inhibition by Foxp3-expressing T cells.

DISCUSSION

The recent introduction of anticytokine therapies, including treatment with anti-TNFα agents, has improved the clinical outcome of RA that is refractory to conventional treatments (33). However, no current treatment targets pathogenic immune reactions to specific antigens. This often leads to the generalized immune suppression that is responsible for undesirable infections. We addressed this issue by generating antigen-specific Treg via transfer of the Foxp3 gene. Adoptive transfer of these cells to nonlymphopenic animals effectively suppressed CIA. In addition, we found that in vitro and in vivo suppressive activities of the genetically manipulated cells correlate well with the expression level of the Foxp3 gene. This allowed us to demonstrate that CIA becomes increasingly resistant to suppression by Treg during the disease course.

Activation of naturally arising CD4+,CD25+ T cells augmented their in vitro suppressive function. This is consistent with previous observations that stimulation with anti-CD3 and IL-2 (21,34) or antigen-pulsed DCs (22,35) remarkably enhanced the suppressive activity of CD4+,CD25+ Treg.

In vivo adoptive transfer experiments revealed that CD4+,CD25+ T cells specific to disease-relevant antigens were highly effective for disease suppression (21,22), which presumably was closely related to in vivo activation of the Treg. We observed that type II collagen–reactive Foxp3-transduced T cells suppressed CIA at much lower cell numbers compared with the number of non–antigen-specific CD4+,CD25+ Treg used in a previous study (25). The suppressed production of anti–bovine type II collagen antibodies (Figure 3B), which was not detected in the latter study, supports

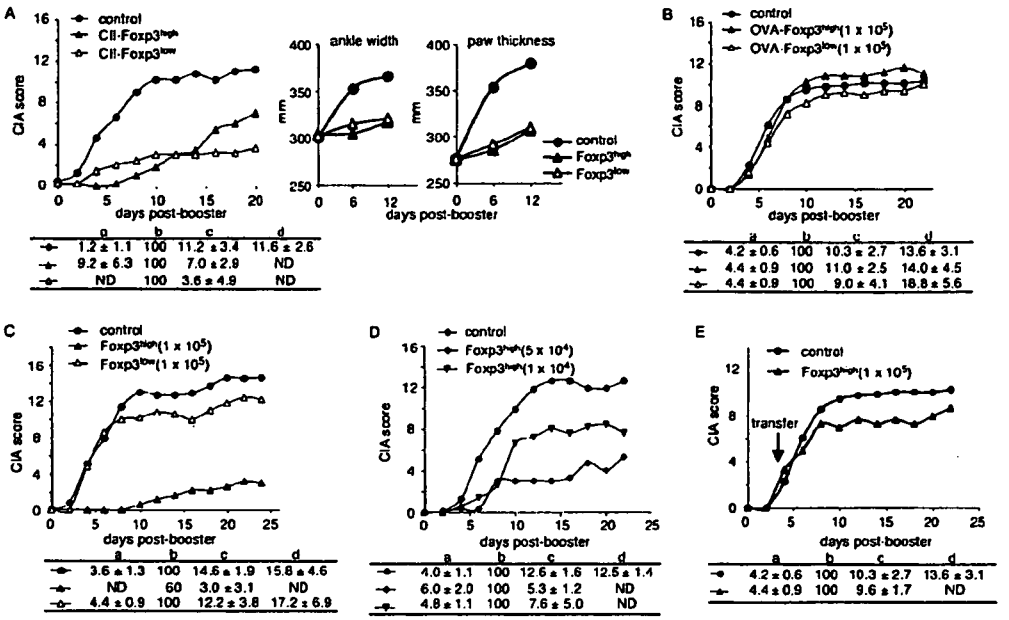

Figure 6. Collagen-induced arthritis (CIA) suppression by type II collagen (CII)–primed Foxp3^{high}/Foxp3^{low} fractions of Foxp3-transduced T cells. A and B, Naive mice received transfer of 1×10^5 Foxp3^{high}-transduced (n = 5) or Foxp3^{low}-transduced (n = 5) T cells primed with CII-pulsed dendritic cells (DCs) (A) or 1×10^5 Foxp3^{high}-transduced (n = 5) or Foxp3^{low}-transduced (n = 5) T cells primed with ovalbumin (OVA)–pulsed DCs (B) intravenously, 1 day prior to the primary immunization. Boosters were administered to the experimental mice as well as control mice, which did not receive Foxp3-transduced T cells (n = 10 in A and B). Joint swelling in the hind limbs was measured at the indicated days after booster immunization, and the mean values for the right and left sides of all mice in the experimental groups were plotted. Differences in CIA scores, ankle width, and paw thickness between the control group and the Foxp3^{high} and Foxp3^{low} groups were significant ($P < 0.01$ by repeated-measures analysis of variance [ANOVA]). C–E, Mice received transfer of 1×10^5 Foxp3^{high} (n = 5) or Foxp3^{low} (n = 5) T cells (C), 5×10^4 (n = 3) or 1×10^4 (n = 5) Foxp3^{high} T cells (D), or 1×10^5 Foxp3^{high} T cells (n = 3) (E) primed with CII-pulsed DCs, intravenously 20 days after the primary immunization (C and D) or 4 days after the booster immunization (E). A group of control mice did not receive T cells (n = 10 in C, n = 8 in D, and n = 10 in E). C, $P < 0.01$, control versus Foxp3^{high}, and $P < 0.05$, control versus Foxp3^{low}, by repeated-measures ANOVA. D, $P < 0.01$, control versus Foxp3^{high} (5×10^4) and Foxp3^{high} (1×10^4), by repeated-measures ANOVA. E, $P < 0.01$, control versus Foxp3^{high}, by repeated-measures ANOVA. Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. ND = not determined.

the fact that the antigen-specific Treg used in this study suppressed CIA more efficiently, because type II collagen–specific antibodies are known as the direct effectors giving rise to arthritis in the CIA model (36,37).

Treg can affect antibody production (38). Generating Foxp3-transduced T cells also circumvented the difficulty in preparing sufficient numbers of CD4+,CD25+ T cells, because this population occurs only as a small portion of peripheral T cells. Non–antigen-specific CD4+,CD25+ T cells (1×10^6) derived from 20 donors were required to treat 1 nonlymphopenic mouse with CIA (25). The Foxp3-transduced T cells needed to treat 1 CIA mouse (1×10^5 cells) in this study were generated from ~5×10^6 lymph node cells, which can be derived from 1 donor. At the moment, in vitro generation of Treg appears to be more practical for cell transfer therapy.

BDC2.5 T cell receptor–transgenic CD4+,CD25+ T cells, which presumably recognize an antigen derived from β cells in pancreatic islets, were shown to be capable of suppressing diabetes induced by diabetic NOD mouse splenocytes (21,22). Thus, Treg specific to a single antigen suppress immune responses induced by

polyclonal effectors. We assume that Foxp3-transduced T cells reactive to a single antigen specifically expressed in joint tissue could suppress T cell reactions against multiple autoantigens in the joints; the original target antigens of the arthritis may not need to be determined.

The Foxp3 expression level in Foxp3-transduced T cells directly correlated with their antiarthritic activity. A previous study showed that CD4 T cells from Foxp3-transgenic mice expressed 10–15-fold higher Foxp3 messenger RNA compared with those from wild-type mice. However, their in vitro suppressive activity was slightly reduced relative to that of wild-type CD4+,CD25+ T cells (6). Those investigators speculated that excessive Foxp3 expression interfered with the functional activation of Treg. In our experiments, the Foxp3 gene was retrovirally introduced after activating the T cells. This technique, combined with use of 2 different retrovirus vectors, allowed us to observe that the Foxp3 expression level dictated the suppressive activity of Foxp3-transduced Treg, both in vitro and in vivo.

It has been shown that stimulated CD4+,CD25− responder T cells were more resistant to in vitro suppression by Treg than were fresh CD4+,CD25− T cells (39,40). We observed that a larger number of Foxp3-transduced T cells was required to suppress CIA when transfer occurred after the primary immunization. Moreover, $Foxp3^{high}$ cells suppressed arthritis when the same dose of $Foxp3^{low}$ cells failed to have an effect. These findings demonstrate that in vivo resistance to Treg was induced after effector activation. In the CIA model, various other effectors besides T cells are involved in pathogenesis, including synovial fibroblasts. Insensitivity of these effectors to suppression by Treg may also contribute to the observed resistance. It may be possible that transfer of even larger numbers of $Foxp3^{high}$ cells can ameliorate full-blown arthritis. However, based on our results, current techniques would require multiple donors to treat a single host.

Our results suggest a possible problem in terms of the clinical application of Treg transfer therapy for patients with RA. Because advanced-stage disease would be refractory to such therapy, Treg transfer should be most effective for the induction of remission in the early stage of RA and for remission maintenance later. In contrast, $Foxp3^{high}$ T cells overcame, to some extent, the insensitivity of the immunized mice. If Treg are generated using Foxp3 gene transfer, transfer of T cells expressing high levels of Foxp3 would be desirable in Treg therapy for autoimmune diseases.

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AUTHOR CONTRIBUTIONS

Dr. Ohata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ohata, Ziegler, Kohsaka, Hori.
Acquisition of data. Ohata, Miura.
Analysis and interpretation of data. Ohata, Ziegler, Kohsaka.
Manuscript preparation. Ohata, Johnson, Kohsaka.
Statistical analysis. Johnson, Kohsaka.

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Lipopolysaccharide-Induced Up-Regulation of Triggering Receptor Expressed on Myeloid Cells-1 Expression on Macrophages Is Regulated by Endogenous Prostaglandin E_2[1]

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Triggering receptor expressed on myeloid cells-1 (TREM-1) is a recently identified cell surface molecule that is expressed by neutrophils and monocytes. TREM-1 expression is modulated by various ligands for TLRs in vitro and in vivo. However, the influence of PGE_2, a potential mediator of inflammation, on TREM-1 expression has not been elucidated. In this study, we examined the effects of PGE_2 on LPS-induced TREM-1 expression by resident murine peritoneal macrophages (RPM) and human PBMC. PGE_2 significantly induced murine TREM-1 (mTREM-1) expression by RPM. Up-regulation of TREM-1 expression was specific to PGE_2 among arachidonic acid metabolites, while ligands for chemoattractant receptor-homologous molecule expressed on Th2 cells and the thomboxane-like prostanoid receptor failed to induce mTREM-1 expression. PGE_2 also increased expression of the soluble form of TREM-1 by PBMC. LPS-induced TREM-1 expression was regulated by endogenous PGE_2 especially in late phase (>2 h after stimulation), because cyclooxygenase-1 and -2 inhibitors abolished this effect at that points. A synthetic EP4 agonist and 8-Br-cAMP also enhanced mTREM-1 expression by RPM. Furthermore, protein kinase A, PI3K, and p38 MAPK inhibitors prevented PGE_2-induced mTREM-1 expression by RPM. Activation of TREM-1 expressed on PGE_2-pretreated PBMC by an agonistic TREM-1 mAb significantly enhanced the production of IL-8 and TNF-α. These findings indicate that LPS-induced TREM-1 expression on macrophages is mediated, at least partly, by endogenous PGE_2 followed by EP4 and cAMP, protein kinase A, p38 MAPK, and PI3K-mediated signaling. Regulation of TREM-1 and the soluble form of TREM-1 expression by PGE_2 may modulate the inflammatory response to microbial pathogens. *The Journal of Immunology,* **2007, 178: 1144–1150.**

Triggering receptor expressed on myeloid cells-1 (TREM-1)[3] is a recently discovered cell surface molecule that has been identified on neutrophils and monocytes (1, 2). The soluble form of TREM-1 (sTREM-1) is detected in bronchoalveolar lavage fluid from patients with microbial infection and has been demonstrated to act as an inhibitor of TREM-1 (3–6). TREM-1 is a 30-kDa glycoprotein belonging to the Ig superfamily and its expression is up-regulated by various ligands for TLRs (7–9). Activation of TREM-1 expressed on neutrophils and monocytes by an agonistic mAb has been shown to stimulate the expression of various proinflammatory cytokines, chemokines, and cell surface molecules (1, 7–9). Furthermore, LPS causes synergistic enhancement of cytokine production by monocytes in response to the agonistic mAb, indicating that TREM-1 amplifies inflammatory responses initiated by TLRs (1, 7–9). Although the natural ligands for TREM-1 have not been identified, its essential role in acute inflammatory responses has been demonstrated in murine models of septic shock, because blocking of TREM-1 by a sTREM-1 improves the survival of mice with bacterial sepsis (6, 9). Thus, activation of TREM-1 may play a crucial role in the inflammatory response to microbes.

PGs are multipotent mediators that modulate a number of pathophysiological responses. PGs are produced by metabolism of arachidonic acid through activation of cyclooxygenase (COX). COX has two isoforms, which are COX-1 and COX-2 (10). COX-1 is constitutively expressed, whereas COX-2 is expressed at low level by most normal resting cells. COX-2 expression is induced by various TLR ligands (11, 12) and release of PGs is significantly increased in various animal models of endotoxemia or sepsis (13, 14). In particular, PGE_2 has been shown to function as a mediator of sepsis-induced immunosuppression, an inhibitor of proinflammatory cytokine production by macrophages, and an inducer of IL-10 production (15). In contrast, PGE_2 has several detrimental effects in sepsis, including vasodilation and increased vascular permeability (16). Several previous studies have shown that COX inhibitors can improve the survival of mice after burn infection or administration of a lethal dose of LPS (17–19). These findings indicate that PGs play an important role in microbial inflammation, including sepsis or endotoxemia. However, the precise mechanisms by which PGs (particularly PGE_2) have a regulatory effect on microbial inflammation have not been determined.

Although TREM-1 is clearly induced by LPS, little is known regarding the biological influence of PGE_2 on TREM-1 during

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[3] Abbreviations used in this paper: TREM, triggering receptor expressed on myeloid cells; mTREM, murine TREM; hTREM, human TREM; sTREM, soluble form of TREM; hsTREM, human sTREM; PKA, protein kinase A; COX, cyclooxygenase; RPM, resident peritoneal macrophage; I-BOP, 1*S*-[1α,2α(*Z*),3β(1*E*,3*S*),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TP, thromboxane-like prostanoid; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; EP, E-series of prostaglandin.