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Increased Arthritis Susceptibility in Cartilage Proteoglycan–Specific T Cell Receptor–Transgenic Mice

Suzanne E. Berlo,[1] Teun Guichelaar,[1] Corlinda B. ten Brink,[1] Peter J. van Kooten,[1] Femke Hauet-Broeren,[1] Katalin Ludanyi,[2] Willem van Eden,[1] Chris P. Broeren,[†] and Tibor T. Glant[2]

Objective. **To better understand the role of antigen (arthritogenic epitope)–specific T cells in the development of autoimmune arthritis.**

Methods. **A transgenic (Tg) mouse expressing the T cell receptor (TCR) $V_\alpha 1.1$ and $V_\beta 4$ chains specific for a dominant arthritogenic epitope (designated 5/4E8) of human cartilage proteoglycan (HuPG) aggrecan was generated. This TCR-Tg mouse strain was backcrossed into the PG-induced arthritis (PGIA)–susceptible BALB/c strain and tested for arthritis incidence and severity.**

Results. **CD4+ TCR-Tg T cells carried functionally active TCR specific for a dominant arthritogenic epitope of HuPG (5/4E8). T cells of naive TCR-Tg mice were in an activated stage, since the in vitro response to HuPG or to peptide stimulation induced interferon-γ and interleukin-4 production. TCR-Tg mice uniformly, without exception, developed severe and progressive polyarthritis, even without adjuvant. Inflamed joints showed extensive cartilage degradation and bone erosions, similar to that seen in the arthritic joints of wild-type BALB/c mice with PGIA. Spleen cells from both naive and HuPG-immunized arthritic TCR-Tg mice could adoptively transfer arthritis when injected into syngeneic BALB/c.SCID recipient mice.**

Conclusion. **TCR-Tg BALB/c mice display increased arthritis susceptibility and develop aggravated disease upon in vivo antigen stimulation. This model using TCR-Tg mice is a novel and valuable research tool for studying mechanisms of antigen (arthritogenic epitope)–driven regulation of arthritis and understanding how T cells recognize autoantigen in the joints. This type of mouse could also be used to develop new immunomodulatory strategies in T cell–mediated autoimmune diseases.**

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases, characterized by chronic inflammation of the synovium of diarthrodial joints. Although the etiology of RA is unknown, accumulating evidence indicates that it is a T cell–mediated and autoantibody-dependent disease in which both genetic and environmental factors play crucial roles (1–3). The RA synovium is infiltrated with CD4+ T cells of the Th1 phenotype (4,5), and antibodies are also involved in the pathologic mechanisms of joint inflammation and progression of the disease (6,7). The demonstrated therapeutic efficacy of agents that block T cell activation (8) or deplete B cells (7) in RA patients has confirmed the critical role of the adaptive immune system. Among the candidate autoantigens, the cartilage proteoglycan (PG) aggrecan is one of the target autoantigens in RA joints (9–14). PG is a complex macromolecule consisting of a large core protein (>2,200 amino acids) to which

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[1]Suzanne E. Berlo, PhD, Teun Guichelaar, MS, Corlinda B. ten Brink, BS, Peter J. van Kooten, MS, Femke Hauet-Broeren, PhD, Willem van Eden, MD, PhD: Utrecht University, Utrecht, The Netherlands; [2]Katalin Ludanyi, PhD, Tibor T. Glant, MD, PhD: Rush University Medical Center, Chicago, Illinois.

[†]Dr. Broeren is deceased.

Address correspondence and reprint requests to Tibor T. Glant, MD, PhD, Professor, Director, Section of Molecular Medicine, Department of Orthopedic Surgery, Rush University Medical Center, Cohn Research Building, Room 708, 1735 West Harrison Street, Chicago, IL 60612. E-mail: tglant@rush.edu, or Willem van Eden, MD, PhD, Department of Infectious Diseases and Immunology, Division of Immunology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, 3584 CL Utrecht, The Netherlands. E-mail: w.eden@vet.uu.nl.

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more than 100 glycosaminoglycan and oligosaccharide side chains are covalently attached (15,16). The core protein of aggrecan is heavily degraded by proteases released during either degenerative or inflammatory processes, resulting in the loss of normal function of articular cartilage (17).

Immunization of BALB/c mice with human cartilage PG (HuPG) induces chronic progressive polyarthritis (18). This PG-induced arthritis (PGIA) has many similarities to human RA, as indicated by clinical assessments, radiographic analyses, scintigraphic bone scans, laboratory tests, and histopathologic examination of the peripheral joints (18–21). The development of the disease is based on the development of T and B cell responses that are cross-reactive between the immunizing HuPG and self (mouse) cartilage PG (MuPG). This cross-reactivity, most likely achieved through epitope spreading, could explain why these T cells home to mouse joints to initiate arthritis (22,23).

Several lines of evidence indicate T cell involvement in the pathogenesis of PGIA. For example, CD4+ T cells selectively proliferate in response to HuPG (24,25), and arthritis can be prevented when CD4+ T cells are depleted either in vivo (26) or in vitro prior to adoptive transfer to naive mice (27,28). A PG-specific T cell hybridoma clone, 5/4E8, can induce arthritis in BALB/c mice (29). Furthermore, CD4+ T cells from arthritic animals are resistant to activation-induced cell death (30). Finally, susceptibility to PGIA is associated with the class II major histocompatibility complex (MHC) (H-2^d haplotype in BALB/c) (20). In addition, immunization of BALB/c mice with PG induces a dominant Th1 T cell response, and treatment of arthritic mice with interleukin-4 (IL-4) can prevent disease development by inducing a switch from the originally Th1-polarized response to a Th2-polarized response (20,31). The importance of CD4+ Th1 cells was further supported by the observation that IL-4–deficient BALB/c mice develop a significantly more severe form of the disease than that seen in wild-type BALB/c mice (32).

The arthritogenic 5/4E8 T cell hybridoma has a CD4+ Th1 phenotype and expresses the T cell receptor (TCR) α and β chains (29). These hybridoma cells secrete IL-2 and interferon-γ (IFNγ), but not IL-4, upon stimulation with HuPG, and the antigen-specific response is class II MHC (I-A^d) restricted (29). The epitope recognized by 5/4E8 cells is located in the G1 domain of HuPG and has been identified in previous mapping studies as an immunodominant, and possibly the most arthritogenic, T cell epitope of HuPG (16,29). The 5/4E8 hybridoma shows cross-reactivity with a homologous epitope of MuPG (16).

In this study, to gain more insight into the role of antigen-specific T cells in the development of autoimmune arthritis, we generated transgenic (Tg) mice expressing the TCR of the 5/4E8 hybridoma. We found that a single PG injection provoked a severe form of PGIA in TCR-5/4E8–Tg (hereafter referred to as TCR-Tg) BALB/c mice. Our experiments demonstrated that splenocytes from both naive TCR-Tg mice, after in vivo activation, and HuPG-immunized arthritic TCR-Tg mice could adoptively transfer arthritis into BALB/c.SCID recipient mice.

MATERIALS AND METHODS

Isolation, amplification, and cloning of the α- and β-chains of TCR-5/4E8. T cell hybridoma 5/4E8 (29), a CD4+ Th1 cell line, recognizes the most dominant arthritogenic peptide sequence, ^{70}ATEGRVRVNSAYQDK^{84} (referred to as peptide P70; underline indicates the core sequence), in the G1 domain of HuPG, and cross-reacts with the mouse homolog sequence, ATEG*Q*VRVNS*I*YQDK (MuPG P70; italics indicate the substitutions). T cell hybridoma 5/4E8 carries V_α1.1 chain and V_β4 chain of the TCR (GeneBank accession no. AY823583 and U19234, respectively).

Genomic DNA was isolated from 5/4E8 hybridoma cells to obtain full-length, rearranged TCRα and TCRβ DNA, including leader and intron sequences, and the corresponding regions were amplified by polymerase chain reaction (PCR). The 2 PCR fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), introduced in *Escherichia coli* DH5α cells (Invitrogen, Breda, The Netherlands), and sequenced. The *Xma* I– and *Not* I–released DNA fragment containing the TCR-5/4E8 α-chain was recloned into the pTα cassette, and the *Xho* I– and *Sac* II–digested and purified TCR-5/4E8 β-chain fragment was inserted into the pTβ cassette (33). Both of the in vivo expression plasmid constructs (pTα and pTβ) were generous gifts from C. Benoist and D. Mathis (Brigham and Women's Hospital, Boston, MA). Subsequently, XL10-Gold cells (Stratagene, La Jolla, CA) were transfected with the constructs, and the correct sequences were confirmed.

Generation of TCRα/β-Tg mice. Linearized pTα TCR-5/4E8 and pTβ TCR-5/4E8 DNA fragments were purified by electroelution, phenol extraction, and ethanol precipitation. Both TCR fragments were coinjected in equal amounts into the pronuclei of fertilized eggs of (CBA $\times$ C57BL/6)F_1 mice (Charles River Laboratories, Sulzfeld, Germany). TCR-Tg founders were identified by PCR analysis of tail genomic DNA. The TCR α1 chain was genotyped by PCR using forward primer 5′-TGCTCCAGGCTAATGGTACA-3′ and reverse primer 5′-CGCTCTCCTGACTAGGGATG-3′; the V_β4 chain was detected using forward primer 5′-CTCGAGCACTGCTATGGGCTCCAT-3′ and reverse primer 5′-CCCAATCCCGCGGAGAAC-3′. The expression of TCR-V_β4 was confirmed by flow cytometric analysis on blood lymphocytes. Unfortunately, flow cytometric analysis of

Table 1. Summary of adoptive transfer experiments using transfer of spleen cells from wild-type and TCR-Tg mice into syngeneic BALB/c.SCID mice*

Source of donor cells, no. of cells ($\times 10^6$) per transfer	Challenging antigen (peptide or HuPG)	No. of arthritic animals/ total no. of animals	Arthritis incidence, %	Arthritis onset, days		Maximum arthritis severity score, mean ± SD†
				Earliest	Mean ± SD	
Naive TCR-Tg						
Up to 4 × 30	None	0/8	0	NA	NA	NA
30 + 15	P70-5/4E8	2/10	20	24	28.0 ± 5.7	3.25 ± 1.77
30 + 15	HuPG	8/14	57	22	26.3 ± 4.0	5.43 ± 2.99
PGIA						
Arthritic TCR-Tg						
30 + 15	None	7/10	70	14	18.4 ± 4.6	4.14 ± 4.68
30	P70-5/4E8	8/9	89	16	18.6 ± 2.8	9.75 ± 4.98
30	HuPG	13/15	87	6	9.8 ± 2.9	8.85 ± 2.94
Arthritic wild-type (BALB/c)						
30 + 15	None	1/15	6.7	42	42	3
30 + 15	P70-5/4E8	1/9	11	36	36	4
30 + 15	HuPG	15/17	88	7	12.1 ± 3.3	9.53 ± 4.63

* Spleen cells (30×10^6) were injected intraperitoneally alone or with 100 μg human cartilage proteoglycan (HuPG) P70-5/4E8 peptide (peptide P70; ^{70}ATEGRVRVNSAYQDK) or 100 μg HuPG alone. A second spleen cell transfer (15×10^6 cells), if indicated, was administered 1 week later without peptide or HuPG The first group received 4 times 30×10^6 spleen cells from naive (nonimmunized) T cell receptor–transgenic (TCR-Tg) mice. NA = not applicable.

† Animals were scored daily for arthritis symptoms (maximum score of 16 per animal), and all were killed on days 49–52 after the first transfer. Adoptive transfer experiments were performed after the backcrossing process into the BALB/c background was completed.

the V_α1.1 chain was impossible due to a lack of V_α1-specific antibody.

Since PGIA was restricted to the susceptible BALB/c strain and the CD4+ hybridoma 5/4E8 was of H-2^d class II MHC (BALB/c origin), the TCR-Tg founders were backcrossed onto BALB/c (H-2^d). A marker-assisted genome screening process was used (20) until the pure BALB/c genomic background was achieved (backcross 8). This was confirmed by using 244 simple sequence-length polymorphic markers as described previously (20). Throughout the backcross processes with the TCR-Tg mice, the coexpression of V_α1.1 and V_β4 chains was always detected in 1 Tg mouse line by genotyping, and all heterozygous TCR-Tg mice were tested for PGIA susceptibility at each backcross level (see below). Finally, male and female heterozygous TCR-Tg BALB/c mice were intercrossed to select homozygous offspring.

Antigens, animals, immunization, and experimental groups. The use of human cartilage, obtained from joint replacement surgeries, for isolation of PG was approved by the Institutional Review Board of Rush University Medical Center (Chicago, IL). HuPG aggrecan was extracted with 4*M* guanidinium chloride as described in detail previously (21,34). For immunoassays (T cell proliferation or antibody measurements), HuPG and MuPG were further purified on cesium chloride gradient centrifugation prior to treatment with chondrotinase ABC and endo-β-galactosidase (18,21,35).

All animal experiments were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center and by the Animal Experiment Committee of Utrecht University (Utrecht, The Netherlands). HuPG was used for immunization of 16–26-week-old TCR-Tg mice or their wild-type littermates and age-matched female BALB/c mice (National Cancer Institute [NCI], Kingston Colony, NY), using a standard immunization protocol as described earlier (19,21). Briefly, the antigen injection (100 μg HuPG protein in 100 μl phosphate buffered saline [PBS], pH 7.2) was administered intraperitoneally, with or without 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammonium bromide (DDA), on days 0 and 21. HuPG-immunized TCR-Tg and wild-type littermate mice were killed within 3–9 days after the onset of primary PGIA. Spleen cells were isolated for transfer experiments and in vitro tests, and sera were collected for antibody and cytokine measurements. Female SCID mice of the BALB/c background (NCI/NCrC.B-17-scid/scid; herein designated BALB/c.SCID) were purchased from the NCI and maintained under germ-free conditions.

Transfer of arthritis using spleen cells from naive and HuPG-immunized wild-type or TCR-Tg BALB/c mice with arthritis. Approximately 60 BALB/c.SCID mice were used for adoptive transfer experiments (summarized in Table 1) to optimize the cell number, time interval between injections (if repeated), and dose of HuPG peptide P70 or HuPG. For the adoptive transfer experiments, BALB/c.SCID mice received unseparated spleen cells (30 or 15×10^6 intraperitoneally) from either naive or HuPG-immunized arthritic mice. Spleen cells were injected first with 100 μg peptide P70 or 100 μg HuPG, or were injected without antigen (see Table 1), as described previously (28).

Assessment of arthritis. The paws of mice were examined daily to record abnormalities due to arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of the paws (19,21). The degree of joint swelling for each paw (scored from 0 to 4) was assessed and expressed as the cumulative arthritis score of 4 paws, with a possible maximum severity index of 16 per animal. The first day of the clinical appearance of paw swelling was recorded as the onset of arthritis. At the end of the experiments, the limbs of arthritic and nonarthritic mice were dissected, fixed, decalcified, and

sectioned. The tissue sections were then stained with hematoxylin and eosin for histopathologic examination.

Flow cytometric analysis. Single-cell suspensions of the thymus and spleens of naive or HuPG-immunized arthritic wild-type and TCR-Tg mice were separated and washed with PBS containing 0.5% bovine serum albumin and 0.01% sodium azide (fluorescence-activated cell sorter [FACS] buffer). Cells were stained with phycoerythrin (PE)–conjugated anti-$V_\beta 4$, anti-CD8, or anti-CD25, fluorescein isothiocyanate (FITC)–conjugated anti-CD19, anti-CD44, or anti-$V_\beta 4$, allophycocyanin-conjugated anti-CD4 or peridinin chlorophyll protein–conjugated anti-CD3 monoclonal antibodies (mAb), or identically labeled relevant IgG isotypes as controls (BD Biosciences PharMingen, San Diego, CA) for 30 minutes on ice. After incubation, cells were washed twice with FACS buffer and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry, San Jose, CA).

Measurement of antigen-specific T cell responses. Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Bodinco, Alkmaar, The Netherlands), 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5}*M* 2-mercaptoethanol was used as culture medium. Single-cell suspensions of spleens were cultured in triplicate in 96-well flat-bottom plates (Corning Life Sciences, Schiphol-Rijk, The Netherlands) at 2×10^5 cells per well, in the presence or absence of peptide P70 (0.1 μg/ml), HuPG (10 μg protein/ml), or control mouse peptide P70 (50 μg/ml). Proliferation was determined by overnight incorporation of ^{3}H-thymidine (0.4 μCi per well; Amersham Biosciences Europe, Roosendaal, The Netherlands) and measured using a liquid scintillation counter (Microbeta; Perkin-Elmer, Boston, MA). The magnitude of the proliferative response was expressed as the change in counts per minute, calculated by subtracting the cpm in nonstimulated cultures from the cpm in stimulated cultures.

Cytokine analysis. Supernatants were collected for cytokine assays after 72 hours of culture and analyzed for IL-4 and IFNγ simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA) according to the method described by Carson and Vignali (36). Briefly, fluoresceinated microbeads coated with enzyme-linked immunosorbent assay (ELISA) capture antibodies (BD Biosciences PharMingen) were added to 50 μl of culture supernatant. Beads were centrifuged after a 45-minute incubation period at room temperature and then washed twice. Biotinylated detection antibodies (BD Biosciences PharMingen) were added, incubated for 15 minutes, and washed, and PE-labeled streptavidin was subsequently added. Cytokines were measured using a Luminex model 100 (Luminex, Austin, TX), and results were analyzed using LMAT software (Becton Dickinson).

Measurement of antigen (PG)–specific antibodies. PG-specific antibodies were measured by ELISA as described previously (19,28). The 96-well ELISA plates (Corning) were coated overnight with HuPG (0.1 μg protein/well) or MuPG (0.15 μg protein/well), and the free binding sites were blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG (Accurate Chemical & Scientific, Westbury, NY) or rat mAb to mouse IgG1 or IgG2a (BD Biosciences PharMingen) as secondary antibodies (28). Serum antibody levels were calculated relative to the corresponding mouse IgG isotype (IgG1 or IgG2a) standards (all from BD Biosciences PharMingen) or mouse serum Ig fractions (Sigma-Aldrich, St. Louis, MO) (19,28).

Statistical analysis. Data are expressed as the mean ± SEM or mean ± SD, as indicated. Statistical analyses were carried out with the nonparametric Mann-Whitney U test, using Prism software (version 3.0; Graphpad Software, San Diego, CA). The significance level was set at a P value less than 0.05.

RESULTS

In vivo expression of the transgenic TCR. The CD4+ cell ratio was higher in the thymus of TCR-Tg mice than in their wild-type littermates, and almost all thymocytes (mean ± SEM 91.8 ± 4.0%) from TCR-Tg mice displayed the transgenic TCR-$V_\beta 4$ chain (Figure 1A). In addition, spleen cells from naive TCR-Tg and

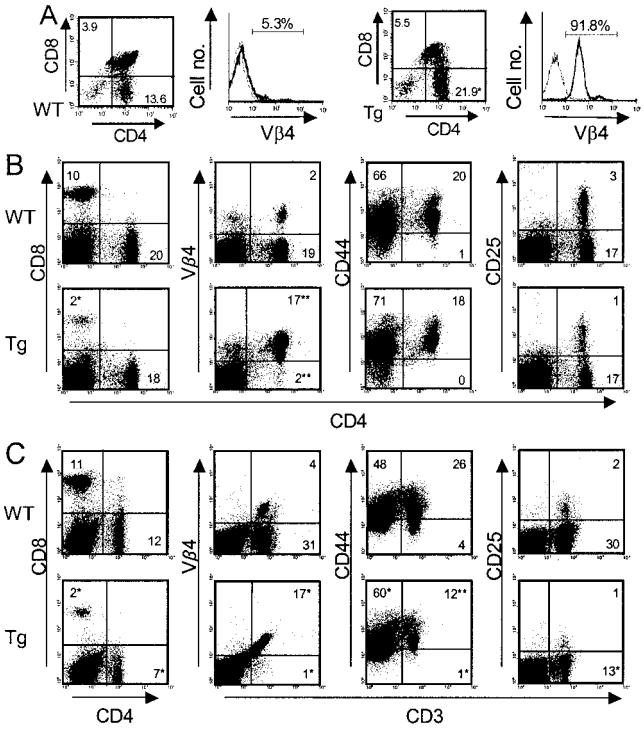

Figure 1. Flow cytometric analysis of the expression of cell surface molecules in T cell receptor–transgenic (TCR-Tg) mice. **A–C,** Cells were isolated from the thymus **(A)** and spleen **(B)** of naive (nonimmunized) mice or the spleen **(C)** of human cartilage proteoglycan–immunized arthritic TCR-Tg mice and their wild-type (WT) littermates. The percentages of single- or double-positive cells (the mean values of at least 5 independent assays in **A** and **B,** and summary of 8 flow cytometry results in **C**) are indicated in the quadrants of the scatter plots or in the histograms. $* = P < 0.05$; $** = P < 0.01$ versus WT mice, by nonparametric Mann-Whitney U test.

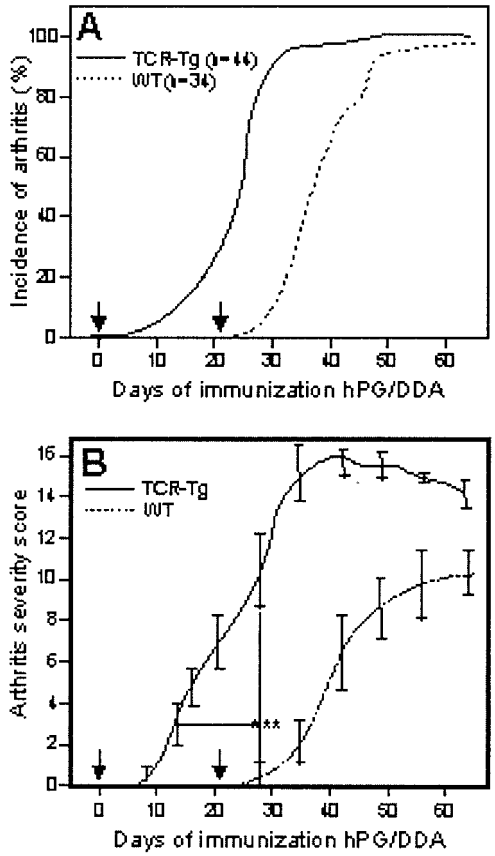

Figure 2. Incidence and severity of arthritis in T cell receptor–transgenic (TCR-Tg) and wild-type (WT) littermate BALB/c mice. Heterozygous TCR-Tg and WT mice (20–26 weeks old) were immunized with human cartilage proteoglycan in dimethyl-dioctadecyl-ammonium bromide (HuPG/DDA) on days 0 and 21 (arrows). **A,** Incidence of arthritis is expressed as the percentage of arthritic animals relative to the total number of TCR-Tg or WT mice. **B,** Disease severity is expressed as the mean ± SEM cumulative arthritis score in 2 independent experiments in arthritic animals only, using heterozygous TCR-Tg mice and their littermates after 8 backcrosses to BALB/c (representing the same groups as shown in **A**). The arthritis score was determined 3–4 times a week by 2 independent investigators, but only the weekly (cumulative) results are shown. *** = $P < 0.001$ between groups from day 27 until the end of the experiment (day 67).

wild-type (littermate) mice were analyzed for surface marker expression. The CD4:CD8 ratio in the spleen cells of TCR-Tg mice was significantly higher (9:1) than that in wild-type animals (2:1) (Figure 1B). Nearly all CD4+ T cells in the TCR-Tg mice expressed the $V_\beta 4$ chain, whereas the CD4+:$V_\beta 4$+ cell ratio was low (mean ± SEM 2.15 ± 0.93%) in the wild-type littermates (Figure 1B). The T cell:B cell ratio, measured as CD4+:CD19+ expression, was comparable in TCR-Tg and wild-type mice (data not shown). Moreover, the expression levels of CD25 and CD44 were comparable in both the spleen cells (Figure 1B) and popliteal lymph node cells (data not shown) of TCR-Tg and wild-type mice, and the $V_\beta 4$ chain was present in the majority of TCR-Tg CD3+ T cells (Figure 1C).

Arthritis in TCR-Tg mice. Since TCRα/β-5/4E8 was already present in heterozygous TCR-Tg mice, and because these CD4+ T cells were HuPG reactive (without priming) and also cross-reacted with MuPG, we were interested in determining whether the peripheral T cells in Tg mice were spontaneously activated by endogenous MuPG. Spontaneous activation of the self-reactive T cells might cause autoimmune arthritis in TCR-Tg mice. Indeed, during the backcrossing process to BALB/c (H-2^d), some TCR-Tg mice spontaneously developed arthritis (<15%). However, the majority of nonimmunized TCR-Tg mice, even on a BALB/c background, remained symptom-free.

We injected TCR-Tg and wild-type BALB/c mice with HuPG in DDA adjuvant intraperitoneally on day 0

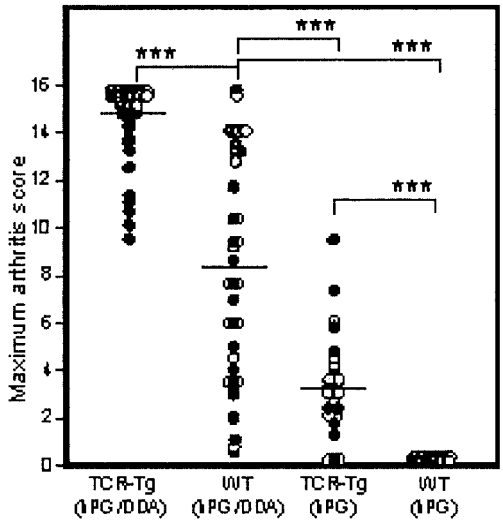

Figure 3. Immunization of T cell receptor–transgenic (TCR-Tg) mice with human cartilage proteoglycan (HuPG) with or without adjuvant dimethyl-dioctadecyl-ammonium bromide (DDA) (at backcross level 8) in relation to severity of arthritis, as compared with wild-type (WT) littermates. Each circle (either solid or open) represents 1 animal. The first 2 groups represent the same animals as in the longitudinal study shown in Figure 2. Mean values are indicated with horizontal lines. The mean ± SEM maximum arthritis scores were 14.6 ± 1.9 (n = 44), 8.6 ± 4.9 (n = 34), and 3.2 ± 2.1 (n = 24) in the the TCR-Tg and WT groups receiving HuPG/DDA and the TCR-Tg group receiving HuPG alone, respectively; this was negligible in the WT group receiving HuPG alone (n = 24). *** = $P < 0.001$ between groups, by nonparametric Mann-Whitney U test.

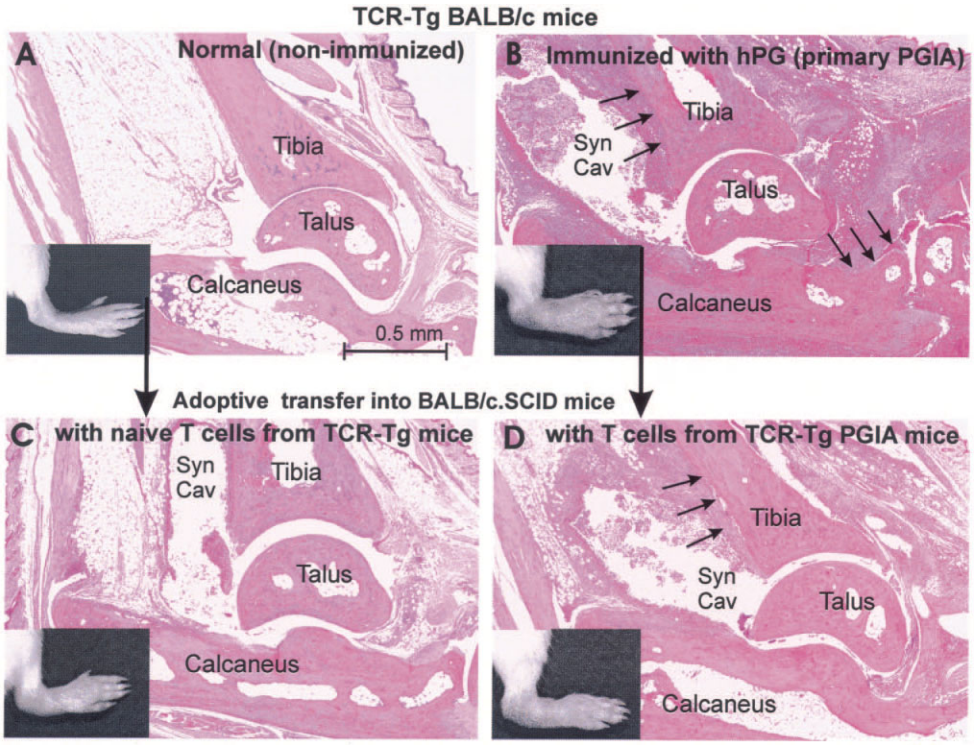

Figure 4. A–D, Histologic analysis of the ankle joints (insets) of naive (nonimmunized) mice **(A)** and human cartilage proteoglycan (HuPG)–immunized T cell receptor–transgenic (TCR-Tg) BALB/c mice **(B)**, and after adoptive transfer into BALB/c.SCID mice **(C** and **D)**. These BALB/c.SCID mice received spleen cells intraperitoneally (30×10^6 and 15×10^6 cells, 1 week apart) from naive TCR-Tg mice **(C)** or from HuPG-immunized arthritic TCR-Tg mice **(D)**. The first cell injection was administered along with 100 μg of HuPG, and spleen cells were harvested from acutely arthritic (having arthritis for <1 week) or naive TCR-Tg mice. Arthritic mice were killed 6–7 days after the onset of arthritis, when the joints showed massive cartilage and bone erosions **(arrows)**. SynCav indicates the expanded synovial cavity filled with a large volume of synovial fluid and inflammatory cells. Synovial hyperplasia accompanied by infiltrating cells and pannus formation is evident in all arthritic joints **(B–D)**. The overall histopathologic features of joint inflammation and tissue destruction in a BALB/c.SCID mouse receiving spleen cells from naive TCR-Tg mice **(C)** are similar, but the joint damage is less extensive, than in another BALB/c.SCID mouse that received spleen cells from arthritic TCR-Tg mice **(D)**. Sections are stained with hematoxylin and eosin. PGIA = proteoglycan-induced arthritis.

and, if necessary, on day 21, using a standard immunization protocol (19,21). Approximately 25–30% of the TCR-Tg mice developed arthritis after the first HuPG–DDA injection, whereas in the wild-type littermates, onset of arthritis occurred 8–10 days after the second HuPG–DDA injection (Figure 2A). When unaffected TCR-Tg mice and their wild-type littermates were boosted on day 21, the incidence of arthritis increased quickly and, in the TCR-Tg mice, reached 100% within a few days, with development of more severe disease than that in their wild-type littermates (Figures 2A and B). Nevertheless, 95–96% of age-matched wild-type littermates also developed arthritis, but with later onset (after the second HuPG–DDA injection) and milder clinical symptoms than in the TCR-Tg mice (Figures 2A and B).

We also tested whether HuPG alone (without adjuvant) could induce arthritis in TCR-Tg mice, especially in aging animals (older than 20–26 weeks). These experiments indicated that HuPG without adjuvant could induce arthritis in TCR-Tg mice, but not in wild-type animals (Figure 3). Although disease severity was lower in the TCR-Tg mice injected with HuPG alone as compared with the TCR-Tg mice that received HuPG in DDA adjuvant, our results suggest that constitutive (over)expression of TCRα/β-5/4E8 partially bypassed the requirement for adjuvant in the induction of arthritis.

The clinical appearance and histopathologic fea-

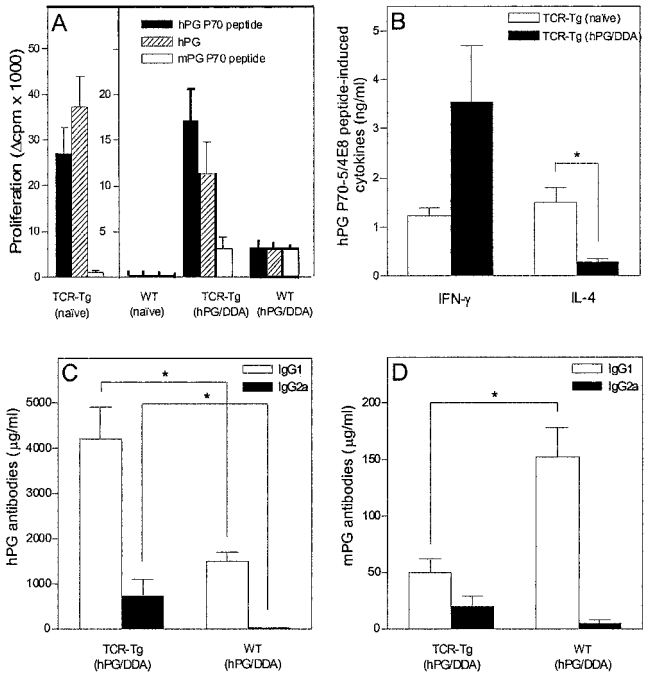

Figure 5. Analysis of antigen-specific T and B cell responses in naive and arthritic T cell receptor–transgenic (TCR-Tg) mice. The human cartilage proteoglycan–dimethyl-dioctadecyl-ammonium bromide (HuPG/DDA)–immunized arthritic TCR-Tg and wild-type (WT) mice were killed 9 days after arthritis onset (at ~31 days). Naive TCR-Tg and WT mice were used as controls. Bars show the mean and SEM of 4–10 mice per group. **A,** Proliferation assays involving spleen cells (2×10^5/well) cultured in the presence of peptide P70 (0.1 μg/ml), HuPG (10 μg/ml), or murine PG (MuPG) P70 peptide (50 μg/ml). **B,** Cytokine production in supernatants of peptide P70–stimulated spleen cell cultures of naive and HuPG-immunized (arthritic) TCR-Tg mice. Cells were harvested after 72 hours and assayed by Luminex analysis for interferon-γ (IFNγ) and interleukin-4 (IL-4). **C** and **D,** Production of serum PG-specific antibodies to immunizing HuPG (**C**) and MuPG (**D**) in the sera of arthritic TCR-Tg and WT mice. $* = P < 0.01$.

tures of arthritis in the TCR-Tg mice, as indicated by comparable arthritis scores and patterns of onset (Figure 4B), were very similar to those seen in the primary form of PGIA (18,20) in wild-type BALB/c mice. Joint inflammation resulted in pannus formation and extensive cartilage and bone erosions, leading to massive ankylosis and deformities of the peripheral joints in TCR-Tg mice (compare Figure 4A with Figure 4B), as has been reported for PGIA in wild-type BALB/c mice (18–20).

Recognition of peptide P70 and HuPG by peripheral T cells of TCR-Tg mice. Peripheral T cells of Tg mice were examined for in vitro responses to peptide P70 and to HuPG. Spleen cells from naive TCR-Tg mice proliferated vigorously when stimulated with peptide P70 or HuPG (Figure 5A), but did not respond to the control peptide (data not shown). An HuPG-specific response was not detected in spleen cell cultures from naive wild-type littermates (Figure 5A). Although T cell responses to HuPG or peptide P70 were higher in naive TCR-Tg mice than in HuPG-immunized arthritic TCR-Tg or wild-type mice, T cell proliferation in the presence of self (mouse) peptide P70 (5/4E8 epitope homolog) was detectable only in cell cultures of arthritic mice (Figure 5A).

IL-4 and IFNγ production was detected in naive TCR-Tg mice after in vitro stimulation with peptide P70, which then dramatically shifted toward the Th1 bias (IL-4 < IFNγ) at the time of onset of arthritis (Figure 5B). In contrast, the production of IFNγ after in vitro stimulation was much higher in HuPG–DDA–immunized arthritic TCR-Tg mice. These data indicate that T cells from naive Tg mice were not tolerized; instead, they appeared to be differentiated in vivo because they responded vigorously to peptide P70 by proliferation, as well as by production of IFNγ and IL-4.

PG-specific antibodies could not be detected in nonimmunized mice but were present at high levels in HuPG-immunized wild-type and TCR-Tg mice. Most of the antibodies were specific for the immunizing HuPG (Figure 5C), although there was a clear antibody response against self PG (MuPG) (Figure 5D). The amounts of HuPG-specific IgG1 and IgG2a antibodies were significantly higher in TCR-Tg mice than in wild-type mice ($P < 0.01$). While antibodies of the IgG1 isotype were predominantly against either HuPG (Figure 5C) or MuPG (Figure 5D) in both TCR-Tg and wild-type BALB/c mice, the IgG2a:IgG1 ratios of antibodies to either HuPG or MuPG were ~10 times higher in Tg mice than in wild-type control mice.

Adoptive transfer to BALB/c.SCID mice. To test whether spleen cells of TCR-Tg mice could transfer arthritis adoptively, we injected unseparated spleen cells from naive and arthritic TCR-Tg mice and from arthritic wild-type (BALB/c) mice into syngeneic BALB/c.SCID recipient mice. The results of these transfer experiments are summarized in Table 1. When SCID mice were injected with naive TCR-Tg cells alone (without activation), no arthritis was adopted. However, when these naive TCR-Tg cells were injected together with either peptide P70 or HuPG, some of the recipient animals developed arthritis (Table 1). The onset of arthritis was relatively late (~27 days after transfer), with a maximum incidence of 57% (Table 1). In contrast, spleen cells from arthritic TCR-Tg mice could induce arthritis upon a single transfer, which was even faster and more severe

when cells from arthritic mice were coinjected with either peptide P70 or HuPG. Remarkably, in this case, only 1 cell transfer was sufficient to induce arthritis in the BALB/c.SCID recipient mice (Table 1), which cannot be easily achieved using spleen cells from arthritic wild-type BALB/c mice (28).

The clinical appearance of the disease and the histopathologic characteristics of the acutely inflamed joints of TCR-Tg and wild-type mice with adoptively transferred arthritis were similar (Figures 4C and D) and indistinguishable from those described in primary PGIA (18,20). Synovial hyperplasia accompanied by infiltrating cells and pannus formation were evident in all arthritic joints. The overall histopathologic features of joint inflammation and tissue destruction in the BALB/c.SCID mice that received spleen cells from naive TCR-Tg mice were somewhat less extensive than in the BALB/c.SCID mouse that received spleen cells from arthritic TCR-Tg mice (Figures 4C and D).

DISCUSSION

PGIA is a T cell–dependent and antibody (B cell)–mediated autoimmune model of RA (20). We herein describe a novel and exaggerated model of PGIA, wherein TCR-5/4E8–Tg mouse T cells, mostly CD4+, respond only to a single arthritogenic epitope of HuPG. These TCR-Tg mice represent a unique source of naive antigen (arthritogenic epitope)–specific T cells that are capable of inducing progressive chronic arthritis. Histologic analysis of the inflamed joints showed extensive cartilage and bone erosions similar to those seen in arthritic joints of wild-type BALB/c mice, and these features were reminiscent of the histopathologic appearance of RA-affected joints. Arthritis onset, however, was much faster and the disease was more severe in the TCR-Tg mice compared with their wild-type littermates.

CD4+ T cells of the TCR-Tg mice carrying the $V_{\alpha}1.1$ and $V_{\beta}4$ chains of the TCR with class II MHC–restricted specificity for the consensus sequence of ^{73}GRVRVNSAY of HuPG (16) were positively selected in the thymus and exported to the periphery, where they constituted the vast majority of T cells (Figure 1). The dominant arthritogenic peptide P70 (the consensus sequence flanked with 3 amino acids at both sides) induced T cell proliferation (Figure 5A), indicating that a functional TCR was indeed generated in the TCR-Tg mice. In contrast to the classic (original) form of PGIA in wild-type BALB/c mice, which required multiple immunizations with HuPG in adjuvant (18,20,21), a single dose of HuPG, even in the absence of adjuvant, produced disease in TCR-Tg mice, whereas injection of the peptide P70 or adjuvant DDA alone did not induce arthritis. Altogether, this new model of PGIA is much more efficient, in the sense that it is epitope restricted. Moreover, since more than 90% of CD4+ T cells carry peptide P70–specific TCRs (Figure 1A), this transgenic model offers an excellent opportunity to test T cell activation events via a single epitope-specific TCR.

Flow cytometric analysis of cells from TCR-Tg mice showed a marked reduction in the CD8+ thymocyte population (Figure 1A). This reduction, however, was expected on the basis that the 5/4E8 epitope was class II MHC restricted. Shrinkage of the CD8+ T cell pool has also been observed in other class II MHC–restricted TCR-Tg mice expressing TCR specific for self antigens, such as type II collagen (37–39) or myelin basic protein (40). In addition to the reduced number of CD8+ cells and the expression of the TCR-$V_{\beta}4$ chain on almost all CD4+ T cells in TCR-Tg mice, the expression of all other surface markers tested on naive T cells was comparable between TCR-Tg and wild-type mice. In vitro studies (Figure 5), however, showed extensive proliferation of TCR-Tg T cells in response to either peptide P70 or HuPG, without clear evidence of activation-induced cell death (AICD).

Both IL-4 and IFNγ cytokine–producing cells were present, in either naive or HuPG-immunized Tg mice prior to the onset of inflammation (data not shown). However, the IL-4:IFNγ ratio shifted significantly toward a Th1 dominance at the time of onset of arthritis, illustrating a dynamic polarization during in vivo T cell activation. The presence of antibodies to both HuPG and MuPG in sera of arthritic TCR-Tg mice demonstrated that although CD4+ Tg T cells were capable of providing sufficient help in vivo for PG-specific B cells (41), and vice versa, B cells probably can transfer signals for T cell activation. Autoantibodies, such as those produced against MuPG, could play a role in initiating inflammation in the joints by binding to the cartilage surface (42), and by inducing chemokine and complement-dependent leukocyte recruitment (43).

A remarkable observation from this study was that we could transfer the disease into syngeneic BALB/c.SCID recipients using splenocytes from naive TCR-Tg mice, upon activation of these cells not only with HuPG, but also with peptide P70. Moreover, spleen cells of arthritic TCR-Tg donor mice were able to induce arthritis in recipient BALB/c.SCID mice without exogenous HuPG or specific peptide, which was not possible using splenocytes from arthritic wild-type donor BALB/c mice (20,27,28). In addition, adoptive transfer of PGIA

was achieved by the injection of cells from arthritic TCR-Tg mice (Table 1). It is conceivable that T cells from arthritic TCR-Tg mice migrate to the joints upon adoptive transfer and become reactivated by MuPG in the mouse joint, where self peptides are released during the normal turnover of the cartilage matrix. Production of MuPG peptides might be increased when PG degradation occurs in the inflamed joint, thus amplifying the inflammatory response.

T and B cell responses against cartilage matrix components are known to occur in RA, at least in a subset of patients, although the pathogenic role of these autoimmune responses is controversial (1,44,45). Among these matrix components, HuPG could be a candidate autoantigen in humans. Relevant to this, we previously demonstrated that several predicted epitopes within the core protein of HuPG were recognized by T cells in the context of human class II MHC in HLA–DR4– and HLA–DQ8–Tg mice (34). Collectively, the results of the present and earlier studies suggest that HuPG, especially the 5/4E8 epitope, not only plays a critical role in PGIA, but more importantly, may become a target of disease-associated T cell responses in patients with RA (12–14).

There are a number of fundamental questions regarding autoimmunity, including the processes seen in RA, which cannot be answered with the use of currently available technology. By using these TCR-Tg mice as a unique source of antigen (epitope)–specific arthritogenic CD4+ T cells, many of these questions, at least in part, can be answered. One of our immediate goals using these TCR-Tg mice is to understand the mechanisms of AICD, which, if defective, may lead to uncontrolled proliferation of antigen-specific peripheral T cells, creating an autoimmune homeostasis. Several lines of evidence indicate that a defect in the apoptotic process is a key component of autoimmunity (46,47). However, the mechanisms of T cell apoptosis in the breakdown of peripheral T cell tolerance in RA are poorly understood. Each lymphocyte produces only one type of TCR from billions of possibilities (48), and therefore the activation mechanisms of a relatively low number of self-reactive T cells cannot be studied. Consequently, AICD is studied in vitro by an indirect method using immobilized anti-CD3 and soluble anti-CD28 antibodies, which activate all T cells in a nonspecific manner. Although the activation of all T cells by anti-CD3/CD28 antibodies is an artificial approach, this has been the only, and widely used, method to monitor intracellular events of activated T cells.

We have detected AICD in our PGIA model (30), which is characteristic of T cells in RA patients (49), but the mechanisms could be studied only through this nonspecific and general T cell activation, i.e., via all TCRs of all T lymphocytes. The high expression of antigen (peptide)–specific TCR of the CD4+ T cells of these TCR-Tg mice allows us to directly study AICD via an arthritogenic epitope–specific mechanism. Recently, we generated HLA–DR4+/DQ8+ (and human CD4+), humanized BALB/c.SCID Tg knockout mice, which do not have their own MHC (H-2d). These humanized immunodeficient mice are being intercrossed with TCR-Tg animals to create a highly specific "autoimmune" environment, in which additional compounds (antibodies, B cells of different specificity, peptide P70 with altered sequence) can be used in a highly controlled environment (50).

In summary, based on the clinical and histopathologic features and the autoimmune aspects shared between PGIA and RA (20), we believe that these TCR-Tg mice are valuable tools for further analysis of the mechanisms associated with the initiation and pathogenesis of autoimmune arthritis, and more specifically, for the analysis of the role of antigen-specific T cells in disease development. Our TCR-Tg model also supports the hypothesis that antigen-specific T cells play a critical role in the initiation of arthritis, since disease could be induced in the absence of adjuvant. Given the paucity of relevant animal models for RA, this novel TCR-Tg mouse model is a valuable addition to the arthritis research armamentarium. Furthermore, these TCR-Tg mice may be useful for developing new immunomodulating agents, which might provide further insights into the immunopathogenic mechanisms of RA.

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