Chapter 5

Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor (TCR) transgenic mice

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Abstract

**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 T cell receptor (TCR) Vα1.1 and Vβ4 chains specific for a dominant arthritogenic epitope (designated 5/4E8) of human cartilage proteoglycan aggrecan (hPG) was generated. This TCR-5/4E8-Tg mouse was backcrossed into the PG-induced arthritis (PGIA)-susceptible BALB/c strain, and tested for arthritis incidence and severity.

**Results.** CD4+ TCR-5/4E8-Tg T cells carried functionally active TCR specific for a dominant arthritogenic epitope of hPG. T cells of naïve TCR-5/4E8-Tg mice were in an activated stage as they responded *in vitro* to hPG or peptide stimulation with IFNγ and IL-4 production. TCR-5/4E8-Tg mice, without exception, uniformly developed severe and progressive polyarthritis after a single hPG injection, even without adjuvant. Inflamed joints showed extensive cartilage degradation and bone erosions, similar to that seen in arthritic joints of wild-type BALB/c mice with PGIA. Spleen cells from either naïve or hPG-immunized arthritic TCR-5/4E8-Tg mice could adoptively transfer arthritis to syngeneic severe immunodeficient (BALB/c,SCID) recipients.

**Discussion/Conclusion.** TCR-5/4E8-Tg BALB/c mice display increased arthritis susceptibility and develop aggravated disease upon *in vivo* antigen stimulation. This TCR-5/4E8 transgenic BALB/c mouse 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. These mice could also be used to develop new immunomodulatory strategies in T cell-mediated autoimmune diseases.
Introduction

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 is unknown, accumulating evidences indicate that RA is a T cell-mediated and auto-antibody 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 pathological mechanisms of joint inflammation and progression of the disease (6,7). 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 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 degeneration or inflammatory processes, resulting in the loss of the normal function of articular cartilage (17).

Immunization of BALB/c mice with human cartilage PG (hPG) induces chronic progressive polyarthritis (18). This PG-induced arthritis (PGIA) shows many similarities to human RA, as indicated by clinical assessments, radiographic analyses, scintigraphic bone scans, laboratory tests and histopathology of the peripheral joints (18-21). The development of the disease is based upon the development of T and B cell responses cross-reactive between the immunizing human and self (mouse) cartilage PG (mPG). 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 are indicative of T cell involvement in the pathogenesis of PGIA. For example, as CD4+ T cells selectively proliferate in response to hPG (24,25), arthritis can be prevented when CD4+ T cells are depleted either in vivo (26) or in vitro prior to adoptive transfer to naïve mice (27,28) a PG-specific T cell hybridoma clone 5/4E8 can induce arthritis in BALB/c mice (29), CD4+ T cells from arthritic animals are resistant to activation induced cell death (30), and susceptibility to PGIA is associated with MHC class-II (H-2d 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 IL-4 can prevent disease development by inducing a switch from the originally Th1-polarized to a Th2-polarized response (20,31). The importance of CD4+ Th1-cells was further underlined by the observation that IL-4-deficient BALB/c mice developed a significantly more severe form of the disease than wild-type BALB/c mice (32).

The arthritogenic 5/4E8 T cell hybridoma has a CD4+ Th1 phenotype and expresses T cell receptor (TCR)-αβ* chains (29). These hybridoma cells secrete IL-2 and IFNγ, but not IL-4, upon hPG stimulation, and the antigen specific response is MHC class II (I-Ad) restricted (29). The epitope recognized by 5/4E8 cells is located in the G1 domain of hPG, and has been identified as an immunodominant, and possibly the most arthritogenic, T cell epitope of hPG in previous mapping studies (16,29). The 5/4E8 hybridoma shows cross-reactivity with a homologous epitope of mPG (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 BALB/c mice. Splenocytes from even naïve TCR-5/4E8-Tg mice, after in vivo activation, or cells from hPG-immunized arthritic TCR-5/4E8-Tg mice adoptively transferred arthritis into syngeneic severe combined immunodeficient (SCID) recipient mice.
Materials and Methods

Isolation, amplification, and cloning of the $\alpha\beta$ chains of TCR-5/4E8

T cell hybridoma 5/4E8 (29), a CD4$^+$ Th1 cell line, recognizes the most dominant arthritogenic peptide sequence $^{70}$ATEGRVRNSAYQDK$_{84}$ (henceforth: hPG P70-5/4E8 peptide; core sequence underlined) in the G1 domain of hPG, and cross-reacts with the mouse homologue sequence (ATEGQVRNSIYODK; mPG P70). T cell hybridoma 5/4E8 carries V$\alpha$1.1 and V$\beta$4 chains of the TCR (GeneBank accession numbers AY823583 and U19234, respectively).

Genomic DNA was isolated from 5/4E8 hybridoma cells to obtain full length rearranged TCR$\alpha$ and TCR$\beta$ DNA, including leader and intron sequences and the corresponding regions were amplified by polymerase chain reaction (PCR). The two PCR fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), introduced in E. coli DH5$\alpha$ cells (Invitrogen B.V., Breda, the Netherlands), and sequenced. The XmaI- and NotI-released DNA fragment containing the TCR-5/4E8$\alpha$ chain was recloned into the pT$\alpha$ cassette; and the XhoI- and SacII-digested and purified TCR-5/4E8$\beta$ fragment was inserted into the pT$\beta$ cassette (33). Both in vivo expression plasmid constructs (pT$\alpha$ and pT$\beta$) were generous gifts from C. Benoist and D. Mathis. Subsequently, XL10-Gold cells (Stratagene, La Jolla, CA) were transfected with the constructs, and the correct sequences confirmed.

Generation of TCR-5/4E8$\alpha$$\beta$-Tg (TCR-5/4E8-Tg) mice

Linearized pT$\alpha$ TCR-5/4E8 and pT$\beta$ TCR-5/4E8 DNA fragments were purified by electroelution, phenol extraction, and ethanol precipitation, respectively. Both TCR fragments were co-injected in equal amounts into the pronuclei of fertilized eggs of F1(CBAxC57BL/6) mice (Charles River Laboratories, Sulzfeld, Germany). TCR-5/4E8-Tg founders were identified by PCR analysis of tail genomic DNA. TCR$\alpha$1 chain was genotyped by PCR using forward primer 5'-TGCTCCAGGCTAA TGGTACA -3' and reverse primer 5'-CGCTCTCCTGACTAGGGATG-3'; the V$\beta$4-chain was detected by using forward primer 5'-CTCGAGCAGCCTCTGACTAGGGATG-3' and reverse primer 5'-CCCAATCCCCGGGAGAAC-3'). The expression of TCR-V$\beta$4 was confirmed by flow cytometric analysis on blood lymphocytes. Unfortunately, flow cytometric analysis of V$\alpha$1.1 chain was impossible due to lack of V$\alpha$1-specific antibody. Since the PGIA was restricted to the susceptible BALB/c strain and the CD4$^+$ hybridoma 5/4E8 was of H-2$d$ MHC class II (BALB/c origin), the TCR-5/4E8-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 (20). Throughout the backcross processes of TCR-5/4E8-Tg mice, the co-expression of V$\alpha$1.1 and V$\beta$4 chains was always detected in one transgenic line by genotyping, and all heterozygous TCR-5/4E8-Tg mice were tested for PGIA susceptibility at each backcross level (see below). Finally, heterozygous TCR-5/4E8-Tg BALB/c males and females were intercrossed to select homozygous offspring.
Antigens, animals, immunization, and experimental groups

The use of human cartilage from joint replacement surgeries for PG isolation was approved by the Institutional Review Board (Rush University Medical Center, Chicago, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee (Rush University Medical Center) and by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands). hPG was used for immunization of 16-26-week-old TCR-5/4E8-Tg or their wild-type littermates, and age matched female BALB/c (National Cancer Institute: NCI, Kingston colony, NY) mice using a standard immunization protocol described earlier (19,21). Briefly, the antigen injection (100 µg hPG protein in 100 µl phosphate-buffered saline; PBS, pH 7.2) was given intraperitoneally (IP) with or without 2 mg of the synthetic adjuvant dimethyldioctadecylammonium bromide (DDA) on days 0 and 21. hPG-immunized TCR-5/4E8-Tg and wild-type littermate mice were sacrificed 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. Severe combined immunodeficient (SCID) female mice of BALB/c background (NCI/NCr.C.B-17-scid/scid; henceforth designated BALB/c.SCID) were purchased from the NCI and maintained under germ-free conditions.

Transfer of arthritis using spleen cells from naïve, and from hPG-immunized wild-type or TCR-5/4E8-Tg BALB/c mice with arthritis

Prior to adoptive transfer experiments (summarized in Table 1), approximately 60 BALB/c.SCID mice were used to optimize cell number, time interval between injections (if repeated), and the dose of peptide (designated hPG P70-5/4E8) or hPG. For adoptive transfer experiments, BALB/c.SCID mice received unseparated spleen cells (30 or 15 x 10⁶) IP from either naïve or hPG-immunized arthritic mice. Spleen cells were injected first with 100 µg hPG P70-5/4E8 peptide or 100 µg hPG, or injected without antigen (see Table 1) as described earlier (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 used to express a 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 non-arthritic mice were dissected, fixed, decalcified, sectioned, and the tissue sections were stained with hematoxylin and eosin for histopathological examination.

Flow cytometric analysis

Single cell suspensions of thymus and spleens of naïve or hPG-immunized arthritic wild-type and TCR-5/4E8-Tg mice were separated and washed with PBS containing 0.5% bovine serum albumin and 0.01% sodium azide (FACS buffer). Cells were stained with phycoerythrin (PE)-conjugated anti-Vβ4, anti-CD8 or anti-CD25, fluorescein
isothiocyanate (FITC)-conjugated anti-CD19, anti-CD44, or anti-Vβ, allophycocyanin (APC)-conjugated anti-CD4 or peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 monoclonal antibodies (mAb), or identically-labeled relevant IgG isotypes as controls (BD Biosciences Pharmingen, San Diego, CA) for 30 min 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 B.V., Alkmaar, the Netherlands), 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10⁻⁵ M 2-mercaptoethanol was used as culture medium. Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates (Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands) at 2 x 10⁵ cells per well, in the presence or absence of hPG P70-5/4E8 peptide (0.1 µg/ml), hPG (10 µg protein/ml) or control mPG P70 peptide (50 µg/ml). Proliferation was determined by overnight incorporation of [³H]-thymidine (0.4 µCi per well; Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands) and measured using a liquid scintillation counter (Microbeta, Perkin-Elmer Inc., Boston, MA). The magnitude of the proliferative response was expressed as delta counts per minute (Δ cpm) calculated by subtracting the cpm of non-stimulated cultures from cpm of stimulated cultures.

Cytokine analysis
Supernatants were collected for cytokine assays after 72 h of culture and analyzed for IL-4 and IFNγ simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA) according to the method of Carson et al. (34). 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 min incubation period at room temperature, and then washed twice. Biotinylated detection antibodies (BD Biosciences Pharmingen) were added, incubated for 15 min, washed, and subsequently PE-labeled streptavidin was added. Cytokines were measured using a Luminex model 100 (Luminex Corporation, 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 (19,28). ELISA 96-well plate (Corning) was coated overnight with hPG (0.1 µg protein/well) or mPG (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 Corp., Westbury, NY) or rat mAbs 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 immunoglobulin fractions (Sigma-Aldrich, St. Louis, MO) (19,28).
Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the arthritis score at different time points and in vitro tests were carried out using the nonparametric Mann-Whitney U test using Prism software. Significance level was set at (p<0.05).
Results

In vivo expression of the transgenic TCR

As compared to wild-type littermates, TCR-5/4E8-Tg thymocytes showed an increase in CD4⁺CD8⁻ population (Fig. 1A), which was expected due to the 5/4E8 hybridoma being a CD4⁺ T cell clone. Importantly, almost all thymocytes (~86%) from TCR-5/4E8-Tg mice displayed the transgenic TCR Vβ4 chain (Fig. 1A). Spleen cells in naïve TCR-5/4E8-Tg and wild-type (littermate) mice were also analyzed for surface marker expression. The CD4/CD8 ratio in the spleen of TCR-5/4E8-Tg mice was significantly higher (9:1) than in wild-type animals (2:1) (Fig. 1B).

**Figure 1.** Flow cytometric analysis of the expression of cell surface molecules in TCR-5/4E8-Tg mice. Cells were isolated from the thymus (A) and spleen (B) of naïve (non-immunized) mice, or spleens (C) of hPG-immunized arthritic TCR-5/4E8-Tg mice and their wild-type (Wt) littermates. The percentages of single or double positive cells are indicated in the quadrants of the scatter plots or in the histograms.
Nearly all CD4+ T cells in the transgenic mice expressed the Vβ4 chain, whereas the CD4/Vβ4+ cell number was low in wild-type littermates (Fig. 1B). The T/B cell ratio measured by CD4/CD19 expression was comparable in TCR-5/4E8-Tg and wild-type mice (data not shown). Also, the expression levels of CD25 or CD44 were comparable in either spleen (Fig. 1B) or popliteal lymph node (data not shown) cells of TCR-5/4E8-Tg and wild-type mice, and the Vβ4 chain was present in the majority of TCR-5/4E8-Tg CD3+ T cells (Fig. 1C).

**Arthritis in TCR-5/4E8-Tg mice**

Since the TCR-αβ-5/4E8 was expressed already in heterozygous TCR-5/4E8-Tg mice, and because these CD4+ T cells were hPG-reactive (without priming) and also cross-reacted with mPG, we were interested in determining whether the peripheral T cells in transgenic mice were ‘spontaneously’ activated by endogenous mPG. Spontaneous activation of the self-reactive T cells might cause autoimmune arthritis in TCR-5/4E8-Tg mice. Indeed, during the backcrossing process to BALB/c (H-2d), some TCR-5/4E8-Tg mice spontaneously developed arthritis (<15%). However, the majority of TCR-5/4E8-Tg mice, even on a BALB/c background, remained symptom-free.

We injected TCR-5/4E8-Tg and wild-type BALB/c mice with hPG in DDA IP on day 0 and, if necessary, on day 21, using a standard immunization protocol (19,21). At the time of relatively early (5th-7th) backcross level into BALB/c mice, approximately 30% of the TCR-5/4E8-Tg mice developed arthritis after the first hPG injection, which occurred only 6-7 days after the second hPG/DDA injection in wild-type littermates (Fig. 2).

**Figure 2.** Incidence and severity of arthritis in TCR-5/4E8-Tg and wild-type (littermate) BALB/c mice. Mice were immunized with hPG in DDA on days 0 and 21 (arrows). The open squares represent the heterozygous TCR-5/4E8-Tg and the black circles represent the wild-type littermates (Wt). (A) Incidence of arthritis is expressed as the percentage of arthritic animals relative to the total number of TCR-5/4E8-Tg or wild-type mice (n = 10 in each group). (B) Disease severity is expressed as the mean of cumulative arthritis score in arthritic animals only. Results are the mean ± SEM of 3 independent experiments using heterozygous TCR-5/4E8-Tg mice and their littermates after 5-6 backcrosses to BALB/c. Severity of arthritis was significantly different between TCR-5/4E8-Tg and wild-type mice (p<0.0001) from day 27 till the end of the experiment (day 67).
Arthritis progressed and, by day 30-45 after the first hPG injection, all peripheral joints of all TCR-5/4E8-Tg mice became inflamed and/or deformed, and a second hPG injection was needed only to accelerate the onset of arthritis. When negative TCR-5/4E8-Tg mice and their wild-type littermates were boosted on day 21 (Fig. 2), the incidence of arthritis increased quickly and reached 100% within a few days in TCR-5/4E8-Tg mice with more severe arthritis than in wild-type littermates (Fig. 2A-B). Up to 70% of the wild-type littermates also developed arthritis after the second hPG/DDA injection, but with later onset and milder clinical symptoms than TCR-5/4E8-Tg mice (Fig. 2).

**Figure 3.** Immunization of TCR-5/4E8-Tg (TCR-Tg) mice with hPG (without adjuvant DDA) produced mild arthritis, but no inflammation was observed in wild-type (Wt) littermates. TCR-5/4E8-Tg and their wild-type littermates (at backcross level 8) were also immunized with hPG and adjuvant DDA (hPG/DDA). The maximum arthritis score, achieved at any time-point after the immunization, is shown. Significant differences (p<0.05) are indicated by the asterisks.

We also tested whether hPG alone (without adjuvant) could induce arthritis in TCR-5/4E8-Tg mice. These experiments indicated that hPG without adjuvant could induce arthritis in TCR-5/4E8-Tg mice, but not in wild-type animals (Fig. 3). Although disease severity was lower in adjuvant-free hPG-injected than in hPG/DDA-injected TCR-5/4E8-Tg mice, our results suggested that constitutive (over)expression of the TCR-αβ-5/4E8 partially bypassed the requirement for adjuvant in arthritis induction. The clinical appearance and histopathological features of arthritis in TCR-5/4E8-Tg mice with comparable scores and onset (Fig. 4B) were very similar to the primary form of PGIA in wild type BALB/c mice (18,20). Joint inflammation resulted in pannus formation, extensive cartilage and bone erosions, leading to massive ankylosis and deformities of the peripheral joints in TCR-5/4E8-Tg mice (Fig. 4B), as reported for PGIA in wild-type BALB/c mice (18-20).
Arthritis in cartilage PG-specific TCR transgenic mouse

Recognition of hPG P70-5/4E8 peptide and hPG by peripheral T cells of TCR-5/4E8-Tg mice

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

IL-4 and IFNγ production was detected in naïve TCR-5/4E8-Tg mice after in vitro stimulation with hPG P70-5/4E8 peptide, which then dramatically shifted toward the Th1 bias (IL-4 < IFNγ) at the time of onset of arthritis (Fig. 5B). In contrast, the production of IFNγ after in vitro stimulation was much higher in hPG/DDA-immunized arthritic TCR-
5/4E8-Tg mice. These data indicate that T cells from naïve transgenic mice were not tolerized, instead, they appeared to be differentiated in vivo because they responded vigorously to the hPG P70-5/4E8 peptide by proliferation, as well as by IFN\(\gamma\) and IL-4 production.

**Figure 5.** Analysis of antigen-specific T- and B-cell responses in naïve and arthritic TCR-5/4E8-Tg mice. hPG-immunized (hPG/DDA) arthritic TCR-5/4E8-Tg and wild-type mice were sacrificed 9 days after the onset (∼ day 31). Naïve TCR-5/4E8-Tg and wild-type (Wt) mice were used as controls. Values are mean ± SEM (n = 4-10). Levels of significance (*p<0.01) are indicated. (A) Proliferation assays: spleen cells (2 x 10^5/well) were cultured in the presence of P70-5/4E8 peptide (0.1 µg/ml; closed bars), hPG (10 µg/ml; dashed bars) or mPG P70 peptide (50 µg/ml; open bars). The results are expressed as ∆ cpm ± SEM. (B) Cytokine production: Supernatants of hPG P70-5/4E8 peptide-stimulated spleen cell cultures of naïve and hPG-immunized (arthritic) TCR-5/4E8-Tg were harvested after 72 h and assayed by Luminex analysis for IFN\(\gamma\) and IL-4. Values of naïve TCR-5/4E8-Tg mice are indicated by open bars and values of hPG/DDA immunized TCR-5/4E8-Tg mice by closed bars. (C-D) Serum anti-PG antibodies: PG-specific antibodies to immunizing hPG (C) and mPG (D) in the sera of arthritic TCR-5/4E8-Tg and wild-type mice (IgG1, open bars; IgG2a, closed bars) are shown.

PG-specific antibodies could not be detected in non-immunized mice but were present at high levels in hPG-immunized wild-type and TCR-5/4E8-Tg mice. Most of the antibodies were specific for the immunizing hPG (Fig. 5C), although there was a clear antibody response against self-PG (mPG) (Fig. 5D). The amount of hPG-specific IgG1
and IgG2a antibodies were significantly higher in TCR-5/4E8-Tg than in wild-type mice (p<0.01). While antibodies of the IgG1 isotype were predominant against either hPG (Fig. 5C) or mPG (Fig. 5D) in both TCR-5/4E8-Tg and wild-type BALB/c mice, the IgG2a/IgG1 ratios of antibodies to either hPG or mPG were about 10 times higher in transgenic mice than the ratios in wild-type control mice.

**Adoptive transfer to BALB/c.SCID mice**

To test whether spleen cells of TCR-5/4E8-Tg mice could transfer arthritis adoptively, we injected unseparated spleen cells from naïve and arthritic TCR-5/4E8-Tg, 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 naïve TCR-5/4E8-Tg cells alone (without activation), no arthritis was adopted. However, when these naïve TCR-5/4E8-Tg cells were injected together with either hPG P70-5/4E8 peptide or hPG, some of the recipient animals developed arthritis (Table 1). The onset of arthritis was relatively late (~ day 27), with a maximum incidence of 57% (Table 1). In contrast, spleen cells from arthritic TCR-5/4E8-Tg mice could induce arthritis upon a single transfer, which was even faster and more severe when cells from arthritic mice were co-injected with either hPG P70-5/4E8 peptide or hPG. Remarkably, in this case only one cell transfer was sufficient to induce arthritis in BALB/c.SCID recipient mice (Table 1), which can not be easily achieved using spleen cells from arthritic wild-type BALB/c mice (28). The clinical appearance of the disease and the histopathology of acutely inflamed joints of adoptively transferred arthritis from TCR-5/4E8-Tg and wild-type mice were similar (Figs. 4C-D), and indistinguishable from those described in primary PGIA (18,20). Synovial hyperplasia accompanied by infiltrating cells and pannus formation was evident in all arthritic joints. The overall histopathology of joint inflammation and tissue destruction in BALB/c.SCID mice that received spleen cells from naïve TCR-5/4E8-Tg mice was somewhat less extensive than in the BALB/c.SCID mouse that received spleen cells from arthritic TCR-5/4E8-Tg mice (Figs. 4C-D).
Table 1. Summary of adoptive transfer experiments using spleen cells from wild-type and TCR-5/4E8-Tg mice into syngeneic BALB/c.SCID mice

<table>
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<th>Source of donor spleen cells</th>
<th>Number of cells (x 10^6) per transfer*</th>
<th>Challenging antigen (peptide or hPG)*</th>
<th>Arthritic/total number of animals</th>
<th>Incidence (%)</th>
<th>Onset (day)</th>
<th>Maximum severity†</th>
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<td>None</td>
<td>1/15</td>
<td>6.7</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>30 + 15</td>
<td>P70-5/4E8</td>
<td>1/9</td>
<td>11</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>30 + 15</td>
<td>hPG</td>
<td>15/17</td>
<td>88</td>
<td>7</td>
<td>12.1 ± 3.3</td>
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</table>

*Spleen cells (30 x 10^6) were injected IP alone or with 100 µg hPG P70-5/4E8 peptide (ATEGRVRVNSAYQDK) or 100 µg hPG. A second spleen cell transfer (15 x 10^6 cells), if indicated, was given one week later without peptide or hPG as described in the Methods section. The first group received 4 times 30 x 10^6 spleen cells from naïve (non-immunized) TCR-5/4E8-Tg mice.

†Animals were scored daily for arthritis symptoms, and all were sacrificed on days 49-52 after the first transfer. Adoptive transfer experiments were performed after the backcrossing process into BALB/c background was completed. NA = not applicable, hPG = human proteoglycan.
**Discussion**

PGIA is a T cell-dependent and antibody (B cell)-mediated autoimmune model of RA (20). Here, we describe a novel and exaggerated model of PGIA, wherein TCR-5/4E8 transgenic T cells, mostly CD4+, respond only to a single arthritogenic epitope of human cartilage PG. These TCR-5/4E8-Tg mice represent a unique source of naïve antigen (arthritogenic epitope)-specific T cells that are capable of inducing progressive chronic arthritis. Histological analysis of inflamed joints showed extensive cartilage and bone erosions similar to those seen in arthritic joints of wild-type BALB/c mice, and was reminiscent of the histopathological appearance of RA-affected joints. Arthritis onset, however, is much faster and the disease is more severe in TCR-5/4E8-Tg mice compared to wild-type littermates. CD4+ T cells of the TCR-5/4E8-Tg mice carrying Vα1.1 and Vβ4 TCR chains with MHC-II restricted specificity for the consensus sequence of 73GRVRVNSAY of hPG (16) were positively selected in the thymus and exported to the periphery where they constituted the vast majority of T cells (Fig. 1). The dominant arthritogenic hPG P70-5/4E8 peptide (the consensus sequence flanked with 3 amino acids at both sides) induced T-cell proliferation (Fig. 5A) indicating that a functional TCR was indeed generated in the TCR-5/4E8-Tg mice. In contrast to the classical (original) form of PGIA in wild-type BALB/c mice that required multiple immunizations with hPG in adjuvant (18,20,21), a single dose of hPG, even in the absence of adjuvant, produced disease in TCR-5/4E8-Tg mice, whereas the injection of the hPG P70-5/4E8 peptide 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, as ~90% of CD4+ T cells carries hPG P70-5/4E8-specific TCRs (Fig. 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-5/4E8-Tg mice showed a marked reduction in the CD8+ thymocyte population (Fig. 1A). This reduction, however, was expected based on the fact that the 5/4E8 epitope was class II-restricted. Shrinkage of the CD8+ T cell pool has also been observed in other MHC class II-restricted TCR-Tg mice expressing TCR specific for self antigens such as type II collagen (35-37) or myelin basic protein (38). Besides the reduced number of CD8+ cells and the expression of TCR-Vβ4 on almost all CD4+ T cells in TCR-5/4E8-Tg mice, the expression of all other surface markers tested on naïve T cells was comparable between TCR-5/4E8-Tg and wild-type mice. In vitro studies (Fig. 5) however, showed extensive proliferation of TCR-5/4E8-Tg T cells in response to either hPG P70-5/4E8 peptide or hPG. Both IL-4 and IFNγ cytokine-producing cells were present, in either naïve or hPG-immunized transgenic 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 hPG and mPG in sera of arthritic TCR-5/4E8-Tg mice demonstrated that CD4+ transgenic T cells were capable of providing sufficient help in vivo for PG-specific B cells (39). Autoantibodies, such as those produced against mPG, could play a role in initiating inflammation in the joints by binding to the cartilage surface (40), and inducing chemokine and complement-dependent leukocyte recruitment (41).

A remarkable observation of this study was that we could transfer the disease into syngeneic BALB/c SCID recipients using splenocytes from naïve TCR-5/4E8-Tg mice, upon activation of these cells not only with hPG, but also with hPG P70-5/4E8 peptide. Moreover, spleen cells of arthritic TCR-5/4E8-Tg donor mice were able to induce arthritis in
recipient BALB/c.SCID mice without exogenous hPG 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-5/4E8-Tg mice (Table 1). It is conceivable that T cells from arthritic TCR-5/4E8-Tg mice migrate to the joints upon adoptive transfer and become reactivated by mPG in the mouse joint where self-peptides are released during the normal turnover of the cartilage matrix. Production of mPG peptides might be increased when PG degradation occurs in the inflamed joint, thus amplifying the inflammatory response.

Therapeutic efficacy of agents that can block T cell stimulation or deplete B cells has confirmed the pathogenic role of the adaptive immune system in RA (1,6-8). Adaptive immune responses seem to play an important role in PGIA as well (20). Although the critical function of CD4+ T cells was implicated in arthritis induction (29,39,40,42), both autoreactive T cells and B cells, and possibly autoantibodies, are required for the development of severe arthritis (20,43), indicating that B cell-mediated effector pathways contribute significantly to PGIA. Cooperation between antigen-primed CD4+ Th1 T cells and B cells appears to be essential for the development of a chronic progressive disease in this model (20,39); similar mechanisms may be involved in the pathogenesis of RA (3,44,45).

T and B cell responses against cartilage matrix components do occur in RA, at least in a subset of patients, although the pathogenic role of these autoimmune responses is controversial (1,46,47). Among these matrix components, hPG could be a candidate autoantigen in humans. Relevant to this, we showed earlier that several predicted epitopes within the core protein of hPG were recognized by T cells in context with human MHC class II in HLA-DR4 and HLA-DQ8 transgenic mice (48). Collectively, the results of the present and earlier studies suggest that hPG, especially the 5/4E8 epitope, plays a critical role not only in PGIA, but more importantly, this epitope may become a target of disease associated T cell responses in patients with RA (12-14).

Based upon the clinical and histopathological features and the autoimmune aspects shared between PGIA and RA (20), we believe that these TCR-5/4E8-Tg mice are a valuable tool for further analysis of the mechanisms associated with the initiation and pathogenesis of autoimmune arthritis; 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, as 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-5/4E8-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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References

Arthritis in cartilage PG-specific TCR transgenic mouse


(48) Szanto S, Bardos T, Szabo Z, David CS, Buzas EI, Mikesz K, Giant TT. Induction of arthritis in HLA-DR4-humanized and HLA-DQ8-humanized mice by human cartilage proteoglycan aggrecan but only in the presence of an appropriate (non-MHC) genetic background. Arthritis Rheum 2004;50:1984-95.