Research article

Susceptibility to collagen-induced arthritis is modulated by TGFβ responsiveness of T cells

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Abstract

The objective of our study was to determine the regulatory effects that endogenous transforming growth factor β (TGFβ) exerts on T cells in the pathogenesis of collagen-induced arthritis (CIA). CIA was induced in transgenic mice expressing a dominant negative TGFβ type II receptor in T cells under the control of the human CD2 promoter. Clinical and histological arthritis scores were determined and experiments on disease induction and the healing phase of disease were performed. The proliferation and cytokine production of draining lymph node cells *in vitro* were analyzed. Transgenic mice were more susceptible to induction of CIA. The overall incidence was higher in transgenic mice than in wild-type mice (57% vs 35%, $P < 0.05$). Affected transgenic animals displayed a significantly higher clinical (4.5 ± 0.6 vs 1.67 ± 0.19, $P = 0.001$) and histological arthritis score (8.01 ± 0.9 vs 4.06 ± 1.1, $P < 0.05$). Draining lymph node cells of transgenic mice secreted more tumor necrosis factor α and IFNγ and proliferated more vigorously in response to collagen type II and upon CD3/CD28 costimulation *in vitro*. Therefore, the regulation of T cells by endogenous TGFβ is important for the maintenance of joint integrity after arthritis induction. Defects in TGFβ-signalling as a susceptibility factor for rheumatoid arthritis may warrant further investigation.

Keywords: dominant negative TGFβ type II receptor, IFNγ, transgenic mice

Introduction

Collagen-induced arthritis (CIA) is an experimental model sharing several clinical and pathological features with rheumatoid arthritis (RA). CIA has been used to study the pathogenesis of RA [1]. The importance of T cells in the pathogenesis of CIA and RA has been established [2] and numerous studies have been performed to determine the cytokines and susceptibility factors involved in arthritis development [3]. However, little is known about the regulation of T cells that leads to the maintenance of immune homeostasis within the joint.

Transforming growth factor beta (TGFβ) family members are pleiotropic factors produced by a variety of cells and with actions depending on the context of their production [4]. Besides having effects on cell proliferation and differentiation and on matrix regulation and tissue repair, TGFβ1 is a major immunoregulatory factor [4]. TGFβ has been detected in RA synovial tissue, and suppressive effects of synovial fluid have been attributed to its actions [5]. In line with its site- and context-specific action, conflicting results have emerged from the use of exogenous TGFβ1 systemically or locally in joints and from the use of

CII = chicken collagen type II; CIA = collagen-induced arthritis; cpm = counts per minute; ELISA = enzyme-linked immunosorbent assay; IFNγ = interferon γ; IL = interleukin; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RPMI = Roswell Park Memorial Institute; SEM = standard error of the mean; TGFβ = transforming growth factor β; Th1 = T helper type 1; TNFα = tumor necrosis factor α.

anti-TGFβ antibodies. The systemic administration of TGFβ to mice ameliorated CIA [6], whereas its local administration to foot pads and joints in rats induced synovitis and aggravated their disease [4,7]. Similarly, blocking endogenous TGFβ by the systemic injection of anti-TGFβ antibody aggravated CIA in mice [6], whereas it ameliorated the ongoing inflammation when injected into the joints of rats [8]. TGFβ also has important functions in tissue repair and fibrosis and chondrocyte differentiation [9]. These conflicting results underline the need for a better understanding of the role of endogenous TGFβ in the maintenance of joint integrity.

The immunoregulatory effects of TGFβ have been clearly demonstrated in TGFβ-null mice, which die by four weeks of age because of multifocal inflammatory lesions, mainly in the lung and heart [10]. No joint lesions have been reported in these mice, but probably their life span was too short for the development of arthritis. In addition, it is difficult to delineate the effects of TGFβ to a specific cell type in this model. We have therefore used transgenic FVB/N mice with an impaired TGFβ-signalling pathway in T cells to delineate the regulatory effects of TGFβ on T cells in the maintenance of joint homeostasis in CIA [11]. The transgenic mice express a dominant negative TGFβ type II receptor under the control of the human CD2 promoter in T cells. This receptor lacks the intracellular kinase domain that is responsible for the phosphorylation of the type I receptor and the subsequent activation of the signalling cascade [12]. The truncated receptor competes with the endogenous type II receptor on the cell surface, thereby blocking TGFβ signal transduction.

We found a higher incidence of CIA in transgenic mice and a higher clinical and histological arthritis score with an increased production of Th1 cytokines by draining lymph node cells of transgenic mice. These findings indicate the importance of regulatory effects of endogenous TGFβ on T cells in the maintenance of joint integrity.

Materials and methods
Animals
The generation and characterization of transgenic hCD2-ΔkTβRII mice is described elsewhere [11]. In these mice, impaired TGFβ-signalling in T cells was shown to be similar to that in other models reported [13,14]. All transgenic lines were established and maintained as heterozygotes on an FVB/N background. FVB/N mice are naturally resistant to the induction of CIA [15]. Therefore, hCD2-ΔkTβRII mice were crossed with DBA/1 mice (Charles River, Sulzfeld, Germany). The male F_1 generation was genotyped using PCR as described elsewhere [11] and included in the experiments at 6 to 12 weeks of age. Nontransgenic male littermates were used as controls. In four separate experiments, 49 transgenic and 29 wild-type F_1 mice were included in the analysis of acute arthritis. An additional

14 transgenic and 17 wild-type mice were included in the analysis of the chronic phase of disease. Animal care was in accordance with governmental and institutional guidelines.

Induction of CIA
Chicken collagen type II (CII) (Sigma, Deisenhofen, Germany) was dissolved and stored in 0.01 M acetic acid at 4 mg/ml. Wild-type and transgenic F_1 mice were injected intradermally with 100 μg of CII emulsified in complete Freund's adjuvant (charge H37Ra) (Difco, Detroit, MI, USA) in both ears (25 μg each) and the base of the tail (50 μg). A booster injection of 100 μg CII in 100 μl PBS was given intraperitoneally 21 days later. Arthritis usually developed within the first week after the booster injection.

Clinical arthritis scoring
Mice were scored every two to three days in the acute phase and once a week in the chronic phase of arthritis, and grades ranging from 0 to 4 were allotted to each limb: grade 0, no visible abnormalities; grade 1, mild redness or swelling of wrist or up to three inflamed digits; grade 2, more than three inflamed digits or moderate redness and swelling of ankle or wrist; grade 3, severe ankle and wrist inflammation; grade 4, extensive ankle and wrist inflammation including all digits, or new bone formation with reduced motion. A maximum score of 16 could be achieved for each mouse.

Histological assessment
For the analysis of acute arthritis, anesthetized mice were killed by cervical dislocation when no further clinical deterioration occurred, which was within the first six weeks after the onset of arthritis. For the analysis of the healing phase of arthritis, mice were observed up to 24 weeks after arthritis induction. After removal of draining lymph nodes, all four limbs of mice with a clinical arthritis score of at least grade 1 were removed. Specimens were fixed in formalin and decalcified in 10% Tris-buffered EDTA (pH 7.3) for 24 to 72 hours using standard methods. Sections 5 μm thick were cut and stained with hematoxylin and eosin.

The histological arthritis score was determined in a blinded fashion for inflammatory and degenerative changes and graded from 0 and 3 for each limb as follows:
Synovial lining – grade 0, no changes; grade 1, localized monolayer cubical transformation; grade 2, localized multilayer cubical transformation; grade 3, multilayer synovial lining with extensive necrosis
Cellular infiltrate – grade 0, no changes; grade 1, few focal infiltrates; grade 2, extensive focal infiltrates; grade 3, extensive infiltrates invading the capsule with aggregate formation
Cartilage – grade 0, no changes; grade 1, superficial, localized cartilage degradation in more than one region;

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grade 2, localized deep cartilage degradation; grade 3, extensive deep cartilage degradation at several locations *Pannus* – grade 0, no changes; grade 1, pannus formation at up to two sites; grade 2, pannus formation at up to four sites, with infiltration or flat overgrowth of joint surface; grade 3, pannus formation at more than four sites or extensive pannus formation at two sites.

Of the four limbs analyzed per animal, the maximum score for each category was used. Therefore, a maximum score of 12 could be reached per animal.

Cell culture and cell proliferation assay

Popliteal and axillary draining lymph nodes were removed and ground through a 40-µm nylon mesh. Cells were cultivated in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 5% fetal calf serum supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies, Eggenstein, Germany). 2×10^6 cells/ml were plated and incubated with 50 µg/ml of CII or costimulated with anti-CD3/CD28 antibodies at 37°C in a water-saturated atmosphere with 5% CO_2 in air. For costimulation, plates were precoated with 10 µg/ml antimouse CD3 monoclonal antibodies (BD Pharmingen, Heidelberg, Germany) in 0.1 M sodium phosphate buffer, pH 8.5, overnight at 4°C, and 10 µg/ml antimouse CD28 monoclonal antibodies (BD Pharmingen) was then added to the medium. Supernatants were collected after 48 hours and frozen in liquid nitrogen. For proliferation assays, cells were seeded at 5×10^5 cells per well in 96-well flat-bottomed plates (Greiner Bio-One, Frickenhausen, Germany) in RPMI medium. Cells were incubated for 48 hours and pulsed with 0.25 µCi/well ³H-thymidine (37 MBq/ml) for the last 16 hours of culture. Samples were harvested and counted in a Betaplate liquid scintillation counter (Wallac, Freiburg, Germany).

ELISA

Cytokine levels of IL-2, IL-4, IL-5, IL-6, IL-10, tumour necrosis factor α (TNFα), and IFNγ in supernatants were measured using Mouse BD OptEIA ELISA Sets (BD Pharmingen) in accordance with the manufacturer's instructions.

Statistical analysis

Means ± SEM are given. For comparison of groups, the two-sided Mann–Whitney rank sum test was applied. A value of $P < 0.05$ was considered significant.

Results

Clinical and histological severity of arthritis

Mice with signs of inflammation at any time point during the observation period were included in the analysis of the severity of arthritis. In four separate experiments analysing the acute phase of arthritis, the overall arthritis incidence in transgenic mice was 57% (28/49), compared with only 35% (12/34) in wild-type littermates ($P < 0.05$). Arthritis

Figure 1
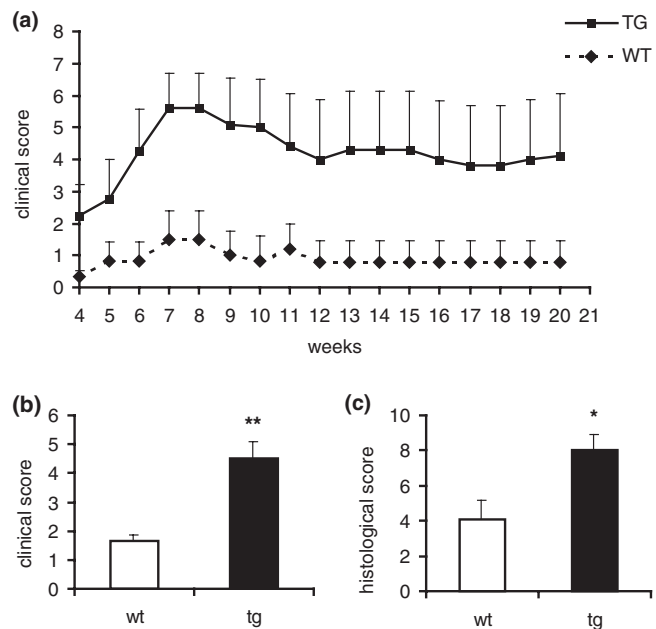
Increased clinical and histological severity of arthritis in transgenic mice with impaired TGFβ-signalling in T cells. **(a)** The time course of the severity of arthritis after the booster injection (day 0) of one long-term experiment is shown. This experiment involved six wild-type and eight transgenic mice. Means ± SEM are shown. **(b)** The maximum clinical arthritis score of mice with a clinical score of at least grade 1 during the observation period of four separate short-term experiments was significantly higher in transgenic mice than in wild-type mice (mean ± SEM; **$P = 0.001$). **(c)** Mice with a clinical score of at least grade 1 were analyzed histologically for inflammatory and degenerative changes. Transgenic mice had significantly higher histological scores than wild-type mice (mean ± SEM; *$P < 0.05$). TG, tg, transgenic; WT, wt, wild-type.

usually developed within the first 10 days after the booster injection of CII and lasted for at least four weeks, when a steady state was reached and the mice were killed. The clinical arthritis score was significantly higher in transgenic than in wild-type mice (4.5 ± 0.6 vs 1.67 ± 0.19, $P = 0.001$; Fig. 1b). No significant joint inflammation was observed in wild-type or transgenic FVB/N mice (data not shown). In long-term experiments analysing the healing phase of the disease, a plateau of disease activity in transgenic F_1 mice was reached after the initial flare had subsided after about 12 weeks. Thereafter chronic arthritis developed, which remained stable over the next 10–12 weeks without a tendency to heal. Only minor changes in disease activity were observed in wild-type mice. The time course of arthritis development for one representative long-term experiment out of two is shown in Fig. 1a.

The histological arthritis score was determined in all limbs of mice with a clinical score of at least grade 1 during the observation period. Inflammatory and degenerative changes were more severe in mice with impaired TGFβ-

Figure 2
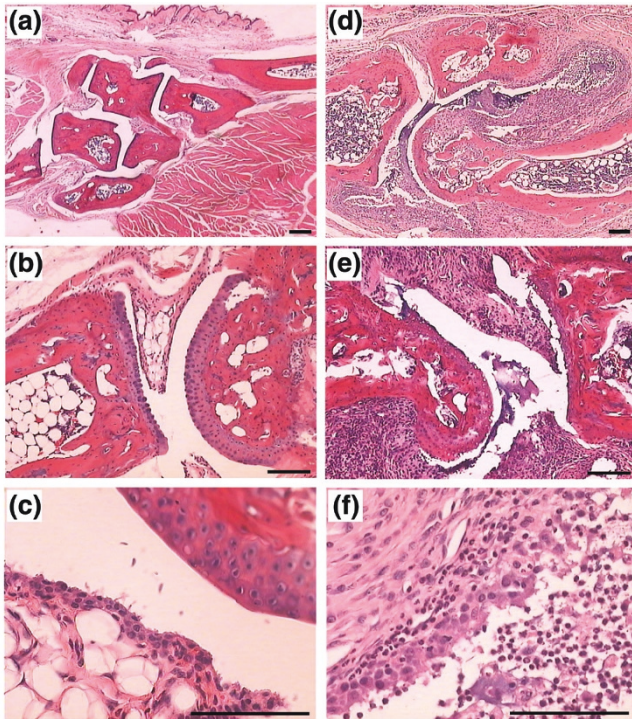
Increased inflammatory and degenerative changes in transgenic hCD2-ΔkTβRII mice after the induction of CIA. Representative sagittal histological sections stained with hematoxylin and eosin are shown. **(a–c)** A small joint of the extremities (a) of a wild-type mouse, and a larger joint (b), show a smooth cartilage surface without any cartilage or bone destruction. (c) The synovial lining layer is composed of flat synovial cells or is mildly hyperplastic. **(d–f)** Joints of transgenic mice with severe inflammatory changes also affecting the periarticular soft tissue are shown. (d) Destruction was seen in small joints, with fibroproliferative tissue (lower portion) and numerous neutrophils within the articular space (upper portion and f). Bone destruction has resulted in bone modulation. (e) In larger joints of the extremities, also, there is heavy proliferation of fibrocellular tissue (pannus formation) with joint destruction. Scale bars represent 100 μm.

signalling in T cells than in wild-type mice (histological score 8.01 ± 0.9 vs 4.06 ± 1.1, $P < 0.05$; Fig. 1c). In wild-type mice, only minor inflammatory changes and a smooth cartilage surface without significant cartilage or bone destruction were observed. In transgenic mice, however, severe inflammatory changes, also involving the periarticular soft tissue, and numerous neutrophils within the articular space were observed in small and large joints. Heavy proliferation of fibrocellular tissue leading to pannus formation with joint destruction was seen. Representative sections of small and large joints from wild-type and transgenic mice are shown in Fig. 2.

Increased cell proliferation and Th1 cytokine production in lymph node cells from transgenic mice

The draining axillary and popliteal lymph nodes of affected animals were removed and the cells cultured in the pres-

ence of CII. Increased cell proliferation was found in the lymph node cells of transgenic as compared with wild-type mice five weeks after arthritis induction (779 ± 85 vs 186 ± 27 cpm; Fig. 3a). In addition, a marked difference was noted in the production of TNFα and IFNγ in the supernatants of CII-stimulated cultures of draining lymph node cells (data not shown). Such cells from mice with long-standing arthritis were not significantly stimulated by CII *in vitro*, maybe because of an epitope spreading after the long period of joint inflammation (data not shown). However, after costimulation of these cells with CD3/CD28, a markedly increased proliferative capacity was observed in transgenic as compared with wild-type mice 20 weeks after arthritis induction ($23,603 \pm 2125$ vs 3554 ± 194 cpm; Fig. 3b). Th1 cytokines such as IFNγ and TNFα were highly up-regulated after costimulation as well as after stimulation with CII (Fig. 3c,d). In addition, it appeared that IL-5 was down-regulated in transgenic lymph node cells after stimulation with CII (Fig. 3d).

Discussion
Our results demonstrate the importance of endogenous TGFβ in regulating T cells in order to maintain joint integrity *in vivo*. Results of studies of the role of endogenous TGFβ in the development of joint lesions have been contradictory [6,8]. TGFβ is a pleiotropic cytokine, produced by a variety of cells and known to exert its effects depending on the effector cell and the context of production [4].

TGFβ has been detected in the synovium and effusions of arthritic joints, and an immunosuppressive role has been postulated from results of *in vitro* experiments [5]. The importance of TGFβ in maintaining immune homeostasis has been demonstrated in TGFβ knockout mice, which die within the first weeks of life as a result of multifocal inflammatory lesions, especially in the heart and lungs [10]. Because it is difficult to delineate the effects of the lack of TGFβ on a specific cell type in these mice, various methods have been used to impair TGFβ-signalling in specific cell types using cell-specific promoters. A dominant negative TGFβ type II receptor has been overexpressed in T cells using the CD4 and the CD2 promoter [13,14]. In addition, Smad7, an inhibitory Smad protein, has been expressed in T cells [16]. The phenotypes of these transgenic mice have turned out to be different from each other, probably because of strain differences and as yet unknown mechanisms.

Although T cells have been shown in several models to be important for the development of arthritis [2], in none of these mice has the spontaneous development of arthritis been described, indicating a tight regulation of immune homeostasis within the joint. The transgenic mice used in this study did not develop spontaneous arthritis even after an observation period of more than nine months [11]. Moreover, hCD2-ΔkTβRII mice developed only minimal

Figure 3
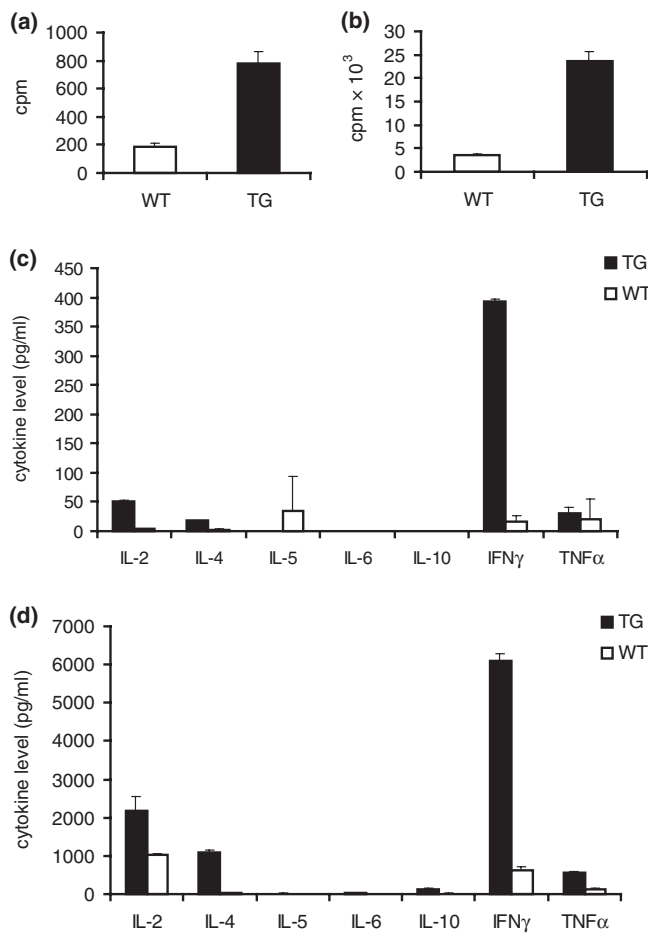
Increased proliferation and Th1 cytokine production in cultures of draining lymph node cells from transgenic mice. **(a)** Cell proliferation five weeks after arthritis induction. Cells were stimulated *in vitro* with 50 μg/ml CII and cultivated for 48 hours (mean ± SEM). This experiment involved 10 transgenic and 11 wild-type mice. **(b)** Draining lymph node cell proliferation 20 weeks after arthritis induction. Cells were stimulated with anti-CD3/CD28 antibody. Eight transgenic and six wild-type mice were included. **(c,d)** Cytokine levels were determined in the culture supernatants of draining lymph node cells after 20 weeks of arthritis using ELISA, in (c) cells stimulated with CII and (d) cells costimulated with anti-CD3/CD28 antibody. CII, chicken collagen type II; cpm, counts per minute; TG, transgenic; TNF, tumor necrosis factor; WT, wild-type.

inflammatory lesions on distal joints after immunization with CII. These mice were generated on an FVB/N background. FVB/N mice have been reported to be resistant to CIA, although they express the same MHC haplotype as DBA/1 mice, a major susceptibility factor for the development of CIA. Still, antigen recognition might be impaired in FVB/N mice, resulting in resistance to the induction of CIA due to deletions in the T-cell receptor $V\beta$ or mutations in the T-cell receptor $V\alpha$ genes [15]. Therefore, the F_1 generation of crossings with DBA/1 mice was used for the experiments. Wild-type F_1 mice still had a rather low inci-

dence and severity of CIA. In contrast, transgenic F_1 mice showed a marked increase in the incidence and severity of arthritis, demonstrating that the susceptibility of wild-type F_1 mice was greatly enhanced by the impairment of TGFβ-signalling in T cells. In addition, in long-term experiments, no resolution of arthritis was observed after an initial flare of disease. Clearly, these results indicate that impairment of TGFβ-signalling in T cells alone is not sufficient to overcome the resistance of FVB/N mice. However, in the setting of T-cell activation through efficient antigen presentation, impairment of TGFβ-signalling seems to be an additional susceptibility factor, a finding that underlines the importance of T cells in the regulation of joint homeostasis.

We also demonstrated an increased production of the Th1 cytokines TNFα and IFNγ in cultures of transgenic draining lymph node cells after arthritis induction and after long-standing arthritis. These cytokines are involved in the pathogenesis of arthritis and could be elevated either because of more severe inflammation in transgenic mice or because of the spontaneous differentiation and cytokine shift observed in T cells with impaired TGFβ-signalling [11,13].

In addition to its immunoregulatory effects, TGFβ has been shown to play an important role in matrix regulation and chondrocyte differentiation. As has been mentioned elsewhere, the injection of TGFβ1 into joints results in osteophyte formation and synovitis [7]. Moreover, the impairment of TGFβ-signalling in skeletal tissue of transgenic mice expressing a dominant negative TGFβ type II receptor under the control of a metallothionein-like promoter has resulted in degenerative changes and bone malformation, the changes in joints resembling those seen in osteoarthritis [9]. TGFβ therefore seems to have beneficial effects in the promotion of tissue repair and down-regulation of inflammation, but when these regulatory effects are not sufficient to control disease, negative effects such as fibrosis and bone remodelling could predominate in the long term.

Conclusion
A significantly higher incidence and severity of CIA were observed in transgenic mice with impaired TGFβ-signalling in T cells than in wild-type littermates. These results demonstrate that endogenous TGFβ acts on T cells to maintain joint integrity after the induction of arthritis and during the healing phase of disease. Several studies have been performed on the susceptibility factors contributing to the development of arthritis. Our data suggest assessment of the TGFβ-signalling cascade as an as yet unknown susceptibility factor.

Competing interests
None declared.

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