

Collagen-induced arthritis development requires $\alpha\beta$ T cells but not $\gamma\delta$ T cells: studies with T cell-deficient (TCR mutant) mice

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Abstract

Collagen type II (CII)-induced arthritis (CIA) in mice is a model for rheumatoid arthritis (RA) in which the role of T lymphocytes remains controversial. To clarify this, we have bred a targeted gene deletion of TCR β or δ loci into two mouse strains susceptible to CIA, the B10.Q and DBA/1 strains. The TCR $\beta^{-/-}$ mice lacked $\alpha\beta$ T cells, which was compensated by an expansion of B cells, $\gamma\delta$ T cells and NK cells. The $\beta^{-/-}$ mice, but not control $\beta^{+/+}$ littermates, were completely resistant to CIA. The production of anti-CII IgG antibodies was also abolished in $\beta^{-/-}$ mice, revealing a strict $\alpha\beta$ T cell dependency. In contrast, $\beta^{-/-}$ mice produced reduced, but significant, anti-CII IgM titers after immunization with either CII or ovalbumin, indicating a multispecificity for these $\alpha\beta$ T cell-independent IgM antibodies. The TCR $\delta^{-/-}$ mice lacked $\gamma\delta$ T cells but had no other significant changes in lymphocyte or monocyte subsets. The cytokine response (IL-2, IL-4, IL-10 and IFN- γ) in $\delta^{-/-}$ mice, quantified by flow cytometry staining of mitogen-stimulated lymphocytes, was indistinguishable from normal mice. Likewise, no statistically significant differences were observed in CIA between mice lacking $\gamma\delta$ T cells and control littermates, considering arthritis incidence, day of disease onset, maximum arthritic score, anti-CII IgG titers and disease course. We conclude that $\alpha\beta$ T cells are necessary for CIA development and for an IgG response towards CII, whereas $\gamma\delta$ T cells are neither necessary nor sufficient for development of CIA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease resulting in destruction of cartilage and bone in the joints. While the etiology and pathogenesis of RA remain unclear, a number of observations implicate the immune system. First, susceptibility to RA is associated with certain MHC alleles, particularly some HLA-DR4 and -DR1 subtypes. Most of the disease-associated HLA alleles share a sequence of amino acids (the so-called 'shared epitope') within the third hypervariable region (HVR3) of DRB1, a region involved in the binding of antigenic peptides and T cell recognition (1). Second, large numbers of mononuclear cells (T and B cells) infiltrate the synovium of arthritic joints. Third, sera and synovial fluids from RA patients often contain autoantibodies specific for the Fc portion of IgG (the so-called rheumatoid factors) and also autoantibodies to type II collagen (CII), which is a major component of cartilage (2–4). Although most

anti-CII antibodies belong to the IgG isotype, it has been unexpectedly difficult to isolate T cells specific for CII (5).

The presence of autoantibodies to CII, particularly in early disease, suggests that RA may be caused by an autoimmune response to this antigen. This hypothesis is supported by the fact that polyarthritis can be induced in rats, mice and apes by intradermal immunization with CII (6–8). This experimental disease, which is named 'collagen-induced arthritis' (CIA), resembles RA in many histological and clinical aspects and is also associated with certain MHC class II alleles (H-2^q and H-2^f in the mouse) (9). CIA has been intensively used as an animal model for RA, and provides the opportunity to address critical questions about the cell types and cell interactions involved in the disease.

Several strategies can be used to experimentally dissect the immunological events involved in CIA. A first approach is

to evaluate the effects of drugs and mAb on the disease course. Administration of anti-IL-2 receptor (10), anti-CD4 (11) or anti-TCR $\alpha\beta$ (12) mAb significantly reduced the incidence of CIA, thereby suggesting an important role for activated CD4⁺ $\alpha\beta$ T cells in the induction of CIA. A role for $\gamma\delta$ T cells has also been proposed in that injection of mice with mAb specific for TCR $\gamma\delta$ resulted in suppression of arthritis if the treatment was initiated before the immunization with CII, whereas CIA was exacerbated if the mAb were injected 40 days after immunization (13). Development of arthritis could also be suppressed by anti-IgM (14) or anti-C5 (15) antibody treatments, indicating a role for B cells and complement in CIA.

A second approach to study CIA is the adoptive transfer of arthritis to naive recipient mice. A transient arthritis is induced by transferring either concentrated anti-CII serum (16), purified anti-CII serum antibodies (17) or anti-CII mAb (18), indicating a critical role for anti-CII antibodies in CIA. Transfer of arthritis with T cells has generally been unsuccessful; however, we have shown that a mild arthritis could be transferred with CII-specific CD4⁺ T cell lines (19). A potent and long-lasting arthritis occurred after anti-CII antibodies had been transferred together with anti-CII CD4⁺ T cells to T cell-depleted mice (20) or to SCID mice (21), suggesting a synergy between B cells and CD4⁺ T cells in CIA.

In recent years, gene technology has provided a new approach to study CIA by targeted germline mutation of certain genes of immunological importance. Mice rendered deficient for B cells, due to a deletion of the IgM heavy chain gene, were resistant to CIA (22). Experiments with CD8-deficient mice suggested that CD8⁺ T cells may be both important in initiation of CIA and in providing resistance to reinduction of CIA (23). Unexpectedly, the role of CD4⁺ T cells in CIA has been questioned when CD4-deficient mice appeared to develop arthritis to the same extent as control CD4⁺ animals (23). The role of all lymphocytes in CIA has also been recently questioned when RAG-deficient mice, lacking mature T and B cells, were reported to develop arthritis after immunization with CII (24). In another model, the spontaneous development of arthritis observed in mice transgenic for human tumor necrosis factor (TNF) suggested that deregulation of TNF production may be involved in the pathogenesis of arthritis (25). Since TNF is mainly a product of activated macrophages, these data also question the importance of lymphocytes in arthritis. The current opinion remains, however, that T cells drive the production of TNF by macrophages.

In the present study, we used mice that selectively lack either $\alpha\beta$ or $\gamma\delta$ T cells as a result of targeted germline mutation in their TCR genes to investigate the role of both cell types in CIA. We show that $\alpha\beta$ T cells are required for CIA development and that the anti-CII B cell response is also $\alpha\beta$ T cell dependent. In contrast, no significant difference was observed in CIA development in mice lacking $\gamma\delta$ T cells compared to normal littermates. We conclude that $\gamma\delta$ T cells are neither necessary nor sufficient for development of CIA.

Methods

Mice

Two male mice, with a mixed (129 \times C57B^{L/6}) background, that selectively lacked either $\alpha\beta$ or $\gamma\delta$ T cells as a result of

targeted germline mutation in their TCR β or δ genes respectively (26,27), were purchased from the Jackson Laboratory (Bar Harbor, ME). The mutated TCR loci were backcrossed onto either B10.Q (originating from Dr Jan Klein, Tübingen, Germany) or DBA/1 (originating from the Jackson Laboratory) backgrounds for six generations. By subsequent intercrossing of mice that were either heterozygous or homozygous for a mutated TCR, we obtained both homozygous ($\alpha\beta$ or $\gamma\delta$ T cell-deficient) or heterozygous (with normal T cell phenotypes) offspring littermates. The different T cell phenotypes were determined by flow cytometry analysis of blood cells, using mAb specific for either TCR $\alpha\beta$ or TCR $\gamma\delta$ (see below).

Lymph node cell cultures

Single-cell suspensions of pooled lymph nodes (cervical, axillary, mesenteric, para-aortic and inguinal lymph nodes) were made by mechanically mashing the tissue. The cells were then cultured, at a concentration of 1.5×10^6 cells/ml, in Dulbecco's modified Eagle's medium supplemented with HEPES, 2-mercaptoethanol, penicillin, streptomycin, glutamine and 10% FCS, with either the superantigen *Staphylococcus aureus* enterotoxin A (SEA; 175 ng/ml, kindly provided by Annette Sundstedt at Pharmacia, Lund, Sweden) or with concanavalin A (Con A; 5 μ g/ml, Pharmacia) for 6 days in 5% CO₂ at 37°C. A culture duration of 6 days was chosen to obtain a high cytokine production. The cells were then restimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (1 μ g/ml) for 6 h in the presence of 3 μ M of the protein transport inhibitor monensin (all from ICN Pharmaceuticals, Costa Mesa, CA) before the staining procedure (see below).

Flow cytometry antibodies

Surface epitopes were stained with the following mAb, used at concentrations between 1 and 12 μ g/ml. H129.19-phycoerythrin (PE) (anti-CD4), GL3-PE (anti-TCR $\gamma\delta$), 145-2C11-PE (anti-CD3), DX5-FITC (anti-panNK cells) and 53.6.7-FITC (anti-CD8) (PharMingen, San Diego, CA). RA3-6B2-TriColor (anti-B220), CT-CD4-TriColor (anti-CD4) and CT-CD8-TriColor (anti-CD8) (Caltag, Burlingame, CA). H57-597-FITC (anti-TCR $\alpha\beta$), M170-FITC (anti-CD11b), H129.19-FITC (anti-CD4), 145-2C11-FITC (anti-CD3 ϵ) and 2.4.G2 (anti-FcRII ϵ), which were purified from culture supernatants by affinity chromatography on Protein G-Sepharose and conjugated. For intracellular cytokine detection, the following mAb were used. 11B11-PE (anti-IL-4) and JES5-16E3-PE (anti-IL-10) (PharMingen), and AN18-FITC (anti-IFN- γ), R4-6A2-FITC (anti-IFN- γ) and S4B6-FITC (anti-IL-2), which were purified from supernatants and conjugated. 145-2C11-FITC was used as a positive control for the staining procedure. FITC- and PE-conjugated antibodies with the same isotype as the anti-cytokine mAb were used as negative controls.

Flow cytometry analysis

For staining of fresh lymph node cells, single-cell suspensions of pooled lymph nodes (cervical, axillary, mesenteric, para-aortic and inguinal lymph nodes) were made by mechanically mashing the tissue. For peripheral blood lymphocyte staining (for mice screening), ammonium chloride (0.84%, pH 7.4) was first added to the blood, for 3 min, in order to lyse the

red blood cells. Cells (from lymph nodes or from the blood) were then washed and resuspended in PBS supplemented with 0.5% BSA (Sigma, St Louis, MO) and 0.01% NaN_3 (staining buffer). Cells (10^6) were stained for the various surface epitopes with mAb for 20 min at 4°C. The cells were washed with staining buffer and analyzed immediately or fixed in 1% formaldehyde/PBS and analyzed within 1 week.

For staining of lymph node cell cultures, cells were washed and resuspended in staining buffer. Then $1\text{--}2 \times 10^6$ cells were stained for various surface epitopes for 20 min at 4°C. An anti-Fc ϵ R1I mAb was used to inhibit antibody binding to the Fc γ receptor. The cells were washed with staining buffer and resuspended in 1% formaldehyde/PBS for fixation overnight at 4°C. For permeation and to inhibit non-specific antibody binding, the cells were incubated with 5% normal rat serum/1% saponin (Sigma) staining buffer for 30 min at 4°C. Cells were washed with 0.025% digitonin (ICN Pharmaceuticals)/1% saponin/2% BSA/0.01% NaN_3 /PBS (intracellular staining buffer) before intracellular staining. Intracellular staining was performed in intracellular staining buffer at 4°C for 20 min. The cells were then washed twice with intracellular staining buffer, once with staining buffer and resuspended finally in PBS for flow cytometry analysis. As a positive control for the intracellular staining procedure, intracellular CD3 ϵ was stained after blocking of surface-expressed CD3 ϵ . A negative control was also performed by blocking the binding to CD3 ϵ by a molar excess of pure anti-CD3 ϵ antibody before the conjugated antibody was added.

For flow cytometry analysis, a typical forward and side scatter gate for lymphocytes was set to exclude dead cells and aggregates, except for analysis of macrophages, for which the gate was extended to include all living cells. In total, 10^4 events in the gate were collected and analyzed using a FACSort (Becton Dickinson, San Jose, CA) and Becton Dickinson software. Quadrant and histogram statistics were placed on the basis of the staining of the negative controls. Less than 0.5% positively stained cells was not regarded as significant.

Induction of arthritis

Rat CII was prepared from the Swarm chondrosarcoma after pepsin digestion (28). CII and ovalbumin (Sigma) were dissolved at a concentration of 2 mg/ml in 0.1 M acetic acid and stored at 4°C. Arthritis was induced by intradermal injection at the base of the tail with 100 μ l of 100 μ g rat CII emulsified with an equal volume of complete Freund's adjuvant (CFA; containing *Mycobacterium butyricum*; Difco, Detroit, MI). In experiments 1, 3 and 4, mice were boosted intradermally at the base of the tail with 50 μ l of 50 μ g rat CII emulsified with an equal volume of incomplete Freund's adjuvant (IFA; Difco) 35–68 days after the first immunization (see Tables 2 and 3). Arthritis experiments were performed with B10.Q males or with DBA/1 females.

Clinical evaluation of arthritis

Arthritis was evaluated blindly using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness. In this scoring system, described in detail elsewhere (29), each inflamed toe or knuckle gives 1 point, whereas an inflamed wrist or ankle

gives 5 points, resulting in a score of 0–15 (5 toes + 5 knuckles + 1 wrist/ankle) for each paw and 0–60 points for each mouse. Healed joints that are deformed or swollen without redness are not considered in this system.

Quantification of CII-specific antibody titers in serum by ELISA

Mice were tail bled and the individual sera were stored at -20°C until assayed. For the ELISA, 96-well plates (Costar, Cambridge, MA) were coated overnight at 4°C with 50 μ l/well of PBS containing 10 μ g/ml of CII. All subsequent incubations were done in a volume of 50 μ l/well. Washings were performed using Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 (Tris/Tween). The sera were diluted in Tris/Tween 20 and tested in duplicate. The amounts of bound IgG and IgM antibodies were estimated after incubation with either a sheep anti-mouse IgG mAb or with a goat anti-mouse IgM mAb, both coupled to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA). Paranitrophenol was used as a chromogenic substrate and the absorbance determined in a Titertek multi-scan spectrophotometer. The amount of CII-specific antibodies in sera from immunized mice was determined by comparing the titration curve of the test serum with the titration curve of a standard consisting of affinity-purified CII-specific antibodies from DBA/1 mice (30) with known IgG and IgM concentrations.

Statistical analysis

Comparison of data between the groups were performed by the Student's unpaired *t*-test (for cell proportions, Tables 1 and 2), the Fisher's exact test (for arthritis incidence, Table 5, Figs 1 and 2) or the Mann–Whitney *U*-test (for mean day of onset and mean arthritic scores, Table 5, Figs 1 and 2). Antibody titers (Tables 4 and 5) were compared with the Student's paired or unpaired *t*-test, when appropriate. *P* < 0.05 was considered significant.

Results

Phenotypic analysis of mice rendered deficient for either $\alpha\beta$ or $\gamma\delta$ T cells

On both B10.Q and DBA/1 backgrounds, littermate mice that were either heterozygous ($\beta^{+/-}$) or homozygous ($\beta^{-/-}$) for a mutated TCR β , or littermates that were either heterozygous ($\delta^{+/-}$) or homozygous ($\delta^{-/-}$) for a mutated TCR δ were generated. It has been previously reported that homozygotes lack either $\alpha\beta$ ($\beta^{-/-}$) or $\gamma\delta$ ($\delta^{-/-}$) T cells, while heterozygotes have normal T cell subsets and are indistinguishable phenotypically from wild-type (+/+) mice (26,27,31). In order to evaluate the effects of the different mutations, the cell content in lymph nodes from adult mice of each strain were analyzed by flow cytometry.

As previously reported (31), the lack of $\alpha\beta$ T cells in $\alpha\beta$ T cell-deficient mice was mostly compensated by an increase of the B cell compartment (Table 1). This implies that the absolute number of B cells was increased in $\beta^{-/-}$ mice, since the total number of cells in lymph nodes is similar between adult $\beta^{+/-}$ and $\beta^{-/-}$ mice (31). The number of $\gamma\delta$ T cells was also significantly increased in $\beta^{-/-}$ mice, comprising 10–19% of lymph node cells (Table 1). NK cells (DX5 $^{+}$) and, in the

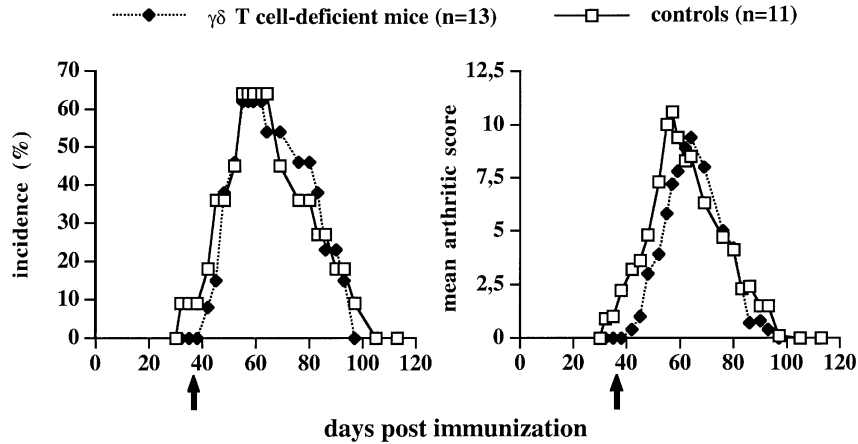


Fig. 1. CIA is similar in mice deficient for $\gamma\delta$ T cells as compared to normal littermates (B10.Q strain). The disease course is represented by arthritis incidence and mean arthritic score (for all mice in each group) over time. B10.Q males were immunized with 100 μg of rat CII in CFA at day 0 and boosted (indicated by an arrow) 36 days later with 50 μg of rat CII in IFA (see Table 5, experiment 3). No statistically significant difference was observed between the two groups at any time point.

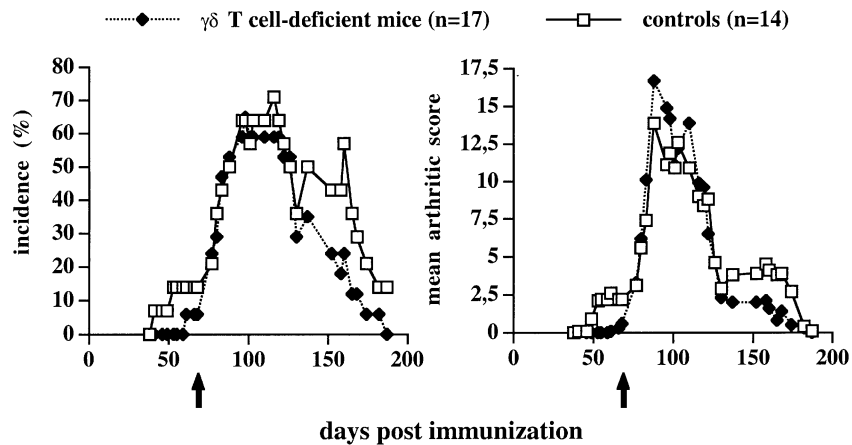


Fig. 2. CIA is similar in mice deficient for $\gamma\delta$ T cells as compared to normal littermates (DBA/1 strain). The disease course is represented by arthritis incidence and mean arthritic score (for all mice in each group) over time. DBA/1 females were immunized with 100 μg of rat CII in CFA at day 0 and boosted (indicated by an arrow) 68 days later with 50 μg of rat CII in IFA (see Table 5, experiment 4). No statistically significant difference was observed between the two groups at any time point.

DBA/1 strain, macrophages (CD11b⁺) also showed a slight, but significant, expansion in $\beta^{-/-}$ mice (Table 1). By contrast, the lymph node cell content of $\gamma\delta$ T cell-deficient mice ($\delta^{-/-}$) was indistinguishable from $\delta^{+/-}$ heterozygotes, except, of course, for the presence of $\gamma\delta$ T cells (Table 1).

Interestingly, the data shown in Table 1 reveal that the two strains used (B10.Q and DBA/1) significantly differ in the cell proportions in lymph nodes. These variations are not caused by the mutated TCR genes but are due to a polymorphism between the two strains (Å. Johansson *et al.*, manuscript in preparation). Although lymph node cell populations were apparently normal in $\gamma\delta$ T cell-deficient mice, we considered that the lack of $\gamma\delta$ T cells might affect the cytokine expression of CD4⁺ or CD8⁺ T cells. To check this possibility, lymph node cells from $\delta^{-/-}$ and $\delta^{+/-}$ littermates (from the B10.Q strain) were cultured together with two different mitogens (SEA or Con A) and the cytokine expression was analyzed

by flow cytometry. No significant differences were seen between the two groups (Table 2).

$\alpha\beta$ T cell-deficient mice are resistant to CIA

CIA development is known to be influenced by genes both within and outside the MHC (9,32). It is therefore valuable to use more than one genetic background (strain), when investigating general mechanisms of CIA. We selected two different CIA-susceptible inbred mouse strains (B10.Q and DBA/1) that share the same MHC haplotype (H-2^q). Arthritis incidence and severity, as well as the anti-CII antibody production is lower in B10.Q mice as compared to DBA/1 (30). In DBA/1 mice a single immunization with CII in CFA is sufficient to induce arthritis; however, an additional booster immunization (CII in IFA) is required in B10.Q mice (30). Another important difference between these strains is the spontaneous development of arthritis, which is the develop-

Table 1. Flow cytometry analysis of lymph node cells from mice with mutations in TCR β or TCR δ ^a

Strain ^b	n	Sex	B cells B220 ⁺	CD4 ⁺	CD8 ⁺	TCR $\gamma\delta$ ⁺		
						CD4 ⁺	CD8 ⁺	All
B10.Q TCR β ^{+/-}	3	M	44.5 ± 2.3	30.5 ± 1.2	20.9 ± 1.3	0.8 ± 0.2	0.5 ± 0.3	1.6 ± 0.2
B10.Q TCR β ^{-/-}	3	M	85.7 ± 3.2	0.7 ± 0.2	1.9 ± 0.4	0.7 ± 0.1	1.9 ± 0.4	10.5 ± 3.6
DBA/1 TCR β ^{+/-}	2	M	30.8 ± 0.7	43.6 ± 0.5	20.5 ± 0.5	0	0	1.5 ± 0
DBA/1 TCR β ^{-/-}	2	M	75.0 ± 4.3	1.9 ± 0	4.5 ± 0.1	1.9 ± 0	4.5 ± 0.1	19.1 ± 1.7
B10.Q TCR δ ^{+/-}	2	F	36.0 ± 0.4	36.1 ± 3.8	23.7 ± 0.7	1.0 ± 0.1	1.5 ± 1.2	1.4 ± 0.7
B10.Q TCR δ ^{-/-}	2	F	34.2 ± 4.2	36.7 ± 4.1	26.7 ± 1.8	0	0	0
DBA/1 TCR δ ^{+/-}	3	F	27.9 ± 1.0	49.3 ± 1.5	19.3 ± 1.4	0.5 ± 0.3	0.5 ± 0	1.1 ± 0.5
DBA/1 TCR δ ^{-/-}	3	F	29.2 ± 2.8	45.7 ± 3.0	19.6 ± 1.1	0	0	0

Strain ^b	n	Sex	NK cells DX5 ⁺	NK T cells DX5 ⁺ CD3 ⁺	Macrophages CD11b ⁺
B10.Q TCR β ^{-/-}	3	M	10.4 ± 3.0	8.2 ± 3.1	5.2 ± 1.0
DBA/1 TCR β ^{+/-}	2	M	1.2 ± 0.1	ND	5.0 ± 0.6
DBA/1 TCR β ^{-/-}	2	M	4.3 ± 0.1	ND	9.0 ± 1.0
B10.Q TCR δ ^{+/-}	2	F	3.6 ± 1.2	3.6 ± 1.2	6.6 ± 1.3
B10.Q TCR δ ^{-/-}	2	F	3.0 ± 0.6	2.7 ± 0.2	7.8 ± 3.5
DBA/1 TCR δ ^{+/-}	3	F	0.8 ± 0.2	ND	4.0 ± 2.0
DBA/1 TCR δ ^{-/-}	3	F	0.9 ± 0.2	ND	5.4 ± 0.2

^aThe figures represent the percentage of the total number of gated cells (mean ± SD). A typical gate for lymphocytes was used, except for analysis of macrophages, for which the gate was set to include all living cells. In bold are shown percentages that were statistically significantly different between -/- and +/- animals within each strain. ND, not determined.

^bThe background (B10.Q or DBA/1) and the TCR genotype (+, wild-type; -, mutated) are indicated.

Table 2. The cytokine expression of CD4⁺ and CD8⁺ T cells is not altered in mice deficient for $\gamma\delta$ T cells^a

Mitogen	$\gamma\delta$ T cells ^b	CD4 ⁺ IL-2	CD4 ⁺ IL-4	CD4 ⁺ IL-10	CD4 ⁺ IFN- γ	CD8 ⁺ IL-2	CD8 ⁺ IL-10	CD8 ⁺ IFN- γ	CD4 ⁺	CD8 ⁺
Con A	+	27 ± 13	2 ± 2	3 ± 3	23 ± 24	12 ± 9	0	18 ± 9	14 ± 10	83 ± 7
Con A	-	30 ± 13	2 ± 2	2 ± 2	14 ± 12	12 ± 7	0	20 ± 11	12 ± 9	86 ± 8
SEA	+	42 ± 19	0	4 ± 4	19 ± 11	19 ± 15	5 ± 5	49 ± 19	18 ± 4	78 ± 6
SEA	-	49 ± 18	0	4 ± 3	22 ± 19	21 ± 14	5 ± 4	55 ± 20	22 ± 6	75 ± 5

^aLymph node cells from B10.Q males were cultured *in vitro* for 6 days with either Con A or the superantigen SEA. Figures in the last two right columns represent the percentage of CD4⁺ and CD8⁺ cells recovered after culture (mean ± SD) as measured by flow cytometry. The other figures represent the percentages of cytokine producing cells within the CD4⁺ or CD8⁺ populations (mean ± SD). Each group contained lymph node cells from five mice, cultured individually. Statistical analysis showed no significant differences between mice deficient for $\gamma\delta$ T cells and controls for any of the measured parameters.

^b-, $\gamma\delta$ T cell-deficient mice (TCR δ ^{-/-}); +, heterozygous (TCR δ ^{+/-}) littermates with a wild-type T cell phenotype.

ment of arthritis without immunization. Spontaneous arthritis has been reported to occur with a high incidence in DBA/1 males (33,34), in contrast it has not been observed in DBA/1 females nor in B10.Q mice (33). Thus, we performed our CIA experiments with DBA/1 females (since spontaneous arthritis in DBA/1 males can be difficult to distinguish from CIA) and B10.Q males as B10.Q females have a low response to CIA.

In both B10.Q and DBA/1 strains, $\alpha\beta$ T cell-deficient mice (β ^{-/-}) were compared with heterozygous (β ^{+/-}) littermates for the development of CIA. In contrast to the controls, none of the $\alpha\beta$ T cell-deficient mice developed arthritis after immunization with CII (Table 3). Immunization with ovalbumin did not induce arthritis (Table 3). This indicates that recognition of CII by $\alpha\beta$ T cells is crucial for CIA development. These two experiments also demonstrate that $\gamma\delta$ T cells are unable to substitute for

$\alpha\beta$ T cells in CIA and we thus conclude that $\gamma\delta$ T cells are not sufficient for development of CIA.

The antibody response against CII is $\alpha\beta$ T cell dependent

The DBA/1 mice tested for development of arthritis in experiment 2 (Table 3) were bled before (day -6) and after the immunization with CII or ovalbumin (day 21 and 36). Ovalbumin was used as a non-arthritisogenic control antigen. The serum IgG and IgM antibodies specific for CII were measured by ELISA. As previously reported (35,36), the antibody response towards CII in CIA in normal mice was predominately IgG but a significant IgM response could also be detected at day 21 (Table 4). We demonstrate here that the CII-specific antibody response is $\alpha\beta$ T cell dependent, since it was absent (IgG) or strongly reduced (IgM) in $\alpha\beta$ T cell-deficient mice

Table 3. Mice deficient for $\alpha\beta$ T cells are resistant to CIA

Experiment ^a	Strain	$\alpha\beta$ T cells ^b	Immunization with	Incidence of arthritis ^c	Day of onset (mean \pm SD)	Arthritic score ^d (mean \pm SD)
1.	B10.Q	+	CII	6/11	60.5 \pm 12.9	19.2 \pm 9.4
1.	B10.Q	-	CII	0/11	-	-
2.	DBA/1	+	CII	5/7	37.2 \pm 18.1	26.0 \pm 6.5
2.	DBA/1	+	OVA	0/7	-	-
2.	DBA/1	-	CII	0/8	-	-
2.	DBA/1	-	OVA	0/8	-	-

^aExperiment 1: adult (7–8 weeks old) B10.Q males were immunized with 100 μ g of rat CII in CFA and boosted 37 days later with 50 μ g of rat CII in IFA. Experiment 2: adult (7–16 weeks old) DBA/1 females were immunized with 100 μ g of rat CII or ovalbumin (OVA) in CFA. Mice were scored for arthritis until day 80 (experiment 1) or day 68 (experiment 2).

^b-, $\alpha\beta$ T cell-deficient mice (TCR $\beta^{-/-}$); +, heterozygous littermates (TCR $\beta^{+/-}$) with a wild-type T cell phenotype.

^cNumber of arthritic mice/total number of mice in each group.

^dMean maximum arthritic score for arthritic mice only.

(Table 4). Interestingly, $\alpha\beta$ T cell-deficient mice showed low, but significant, titers of IgM antibodies specific for CII before immunization (day -6). These anti-CII IgM antibody titers increased significantly after immunization, even in the absence of $\alpha\beta$ T cells. This increase did not seem to depend on the immunogen since immunization with ovalbumin also increased the titers of anti-CII IgM antibodies (Table 4).

$\gamma\delta$ T cells are not required for development of CIA

In both B10.Q and DBA/1 strains, $\gamma\delta$ T cell-deficient mice ($\delta^{-/-}$) were compared with heterozygous ($\delta^{+/-}$) littermates for the development of CIA. In experiment 3, B10.Q males were boosted 36 days post-immunization. In experiment 4, immunization of DBA/1 female mice with a single injection of CII in CFA resulted in <20% of the mice developing arthritis. This unusually low CIA incidence revealed the importance of environmental factors in this model and consequently led us to boost the mice with CII in IFA at day 68. In both experiments 3 and 4, no statistically significant differences were observed between mice lacking $\gamma\delta$ T cells and controls, considering arthritis incidence, day of disease onset, maximum arthritic score and anti-CII IgG titers (Table 5). The disease course was also similar between the two groups of mice, both for the B10.Q (experiment 3, Fig. 1) and for the DBA/1 (experiment 4, Fig. 2) strain. The lack of $\gamma\delta$ T cells did not significantly influence either the IgG1 and IgG2a antibody titers towards CII (data not shown). We conclude that $\gamma\delta$ T cells are not required for development of CIA.

Discussion

A number of observations support an important role for anti-CII antibodies, in particular for anti-CII IgG, in the pathogenesis of CIA. CIA is associated with high antibody titers to CII (9,35,37) and a transient arthritis can be induced by transferring either anti-CII serum (16,17) or anti-CII IgG mAb (18). We report here that the CII-specific antibody response is $\alpha\beta$ T cell dependent, since it was absent (IgG) or strongly reduced (IgM) in $\alpha\beta$ T cell-deficient mice. It has previously been shown that the anti-CII IgG response could be significantly decreased by administration of antibodies to T cells, e.g. anti-CD4 (11), anti-TCR $\alpha\beta$ (38–40) or anti-gp39 (41). However, most of these

previous experiments failed to completely inhibit the anti-CII IgG response, presumably due to the limitations of these antibody treatments. The use of $\alpha\beta$ T cell-deficient mice allows us to demonstrate that the IgG anti-CII response is strictly $\alpha\beta$ T cell dependent. Our data support the idea that a critical role for $\alpha\beta$ T cells in CIA is to provide help to B cells for production of anti-CII antibodies. The role of $\alpha\beta$ T cells in RA may be similar, as suggested by reports showing that HLA-DR4⁺ RA patients had significantly greater IgG reactivity to CII compared with DR4⁻ RA patients (42,43).

An interesting observation was that anti-CII IgM antibodies could be produced in the absence of $\alpha\beta$ T cells in $\alpha\beta$ T cell-deficient mice. The physiological relevance of these antibodies could be questioned, since they were present in non-immunized $\beta^{-/-}$ mice and since abnormally high levels of autoantibodies have been reported in $\alpha\beta$ T cell-deficient mice (44). However, previous investigations have shown that CII-reactive multispecific IgM antibodies could be derived from normal mice after immunization with CII or ovalbumin (35,36). The anti-CII IgM antibodies that we observed in $\beta^{-/-}$ mice also appear to be multispecific since their titers increased after immunization with either CII or ovalbumin. We believe that these CII-reactive IgM antibodies belong to the natural autoantibody repertoire (reviewed in 45), that presumably expands in $\alpha\beta$ T cell-deficient mice. Characterization of the anti-CII IgM response may be a critical step for the understanding of CIA. IgM antibodies have been suggested to play an important role in CIA in primates and rats (46), and anti-CII IgM antibodies are also common in patients with RA (3,43,47).

Our data showing that the lack of $\gamma\delta$ T cells had no effect on the development of CIA are surprising. We had a number of reasons to suspect a role for $\gamma\delta$ T cells in the pathogenesis of arthritis. The presence of $\gamma\delta$ T cells has been reported in the blood and inflamed synovium of RA patients (48). Several cytokines of importance for inflammatory responses (e.g. IFN- γ , TNF, IL-4 and IL-10) are produced by $\gamma\delta$ T cells (49). In addition, IFN- γ production by $\gamma\delta$ T cells can prime macrophages to secrete large amounts of TNF (49). IgG production by B cells, as well as the development of T_h1 or T_h2 $\alpha\beta$ T cell subsets, may also be influenced by $\gamma\delta$ T cells (49). Some $\gamma\delta$ T cells have been shown to respond to mycobacterial antigens and may consequently, in CIA, be activated by the CFA

Table 4. Mice deficient for $\alpha\beta$ T cells produce no IgG and have reduced IgM antibody titers to CII^a

$\alpha\beta$ T cells ^b	Immunization with	IgG (mean $\mu\text{g/ml} \pm \text{SD}$)			IgM (mean $\mu\text{g/ml} \pm \text{SD}$)		
		Day -6	Day 21	Day 36	Day -6	Day 21	Day 36
+	CII	0	637 \pm 587	798 \pm 517	0	29.4 \pm 19.8	7.4 \pm 4.8
+	OVA	0	0.23 \pm 0.42	0.38 \pm 0.47	0	1.0 \pm 2.6	2.6 \pm 4.1
-	CII	0	0	0	2.3 \pm 2.5	7.4 \pm 3.8	5.4 \pm 4.4
-	OVA	0	0	0	3.0 \pm 3.9	6.3 \pm 4.9	3.4 \pm 3.6

^aAdult (7–16 weeks old) DBA/1 females were immunized with 100 μg of rat CII or ovalbumin (OVA) in CFA at day 0 (see Table 3, experiment 2). The antibody titers in serum were measured by ELISA. Statistical analysis: the titers significantly higher after immunization, compared to day -6, are in bold. At day 21, the anti-CII IgM titers were significantly higher in the $\alpha\beta$ T cell-positive mice immunized with CII compared to any other group. No significant difference in antibody titers was seen, at any time point, between the two $\alpha\beta$ T cell-deficient groups.

^b-, $\alpha\beta$ T cell-deficient mice ($\text{TCR}\beta^{-/-}$); +, heterozygous ($\text{TCR}\beta^{+/-}$) littermates with a wild-type T cell phenotype.

Table 5. CIA is similar in mice deficient for $\gamma\delta$ T cells as compared to normal controls

Experiment ^a	Strain	$\gamma\delta$ T cells ^b	Incidence of arthritis ^c	Day of onset (mean \pm SD)	Arthritic score ^d (mean \pm SD)	IgG anti-CII (mean $\mu\text{g/ml} \pm \text{SD}$) ^e		
						Arthritic	Healthy	All
3.	B10.Q	+	7/11 (64%)	46.6 \pm 8.3	11.5 \pm 16.1	151 \pm 229	90 \pm 71	129 \pm 184
3.	B10.Q	-	8/13 (62%)	49.1 \pm 4.6	10.8 \pm 13.3	163 \pm 113	85 \pm 66	133 \pm 102
4.	DBA/1	+	11/14 (79%)	84.3 \pm 23.8	20.9 \pm 13.5	511 \pm 330	150 \pm 141	434 \pm 332
4.	DBA/1	-	13/17 (76%)	86.4 \pm 13.9	26.2 \pm 18.6	320 \pm 250	49 \pm 65	256 \pm 249

^aExperiment 3: adult (7–12 weeks old) B10.Q males were immunized with 100 μg of rat CII in CFA and boosted 36 days later with 50 μg of rat CII in IFA. Experiment 4: adult (11–22 weeks old) DBA/1 females were immunized with 100 μg of rat CII in CFA and boosted 68 days later with 50 μg of rat CII in IFA. Mice were scored for arthritis until day 113 (experiment 3) or day 187 (experiment 4). Statistical analysis showed no significant difference between mice deficient for $\gamma\delta$ T cells and controls for any measured parameter.

^b-, $\gamma\delta$ T cell-deficient mice ($\text{TCR}\delta^{-/-}$); +, heterozygous ($\text{TCR}\delta^{+/-}$) littermates with a wild-type T cell phenotype.

^cNumber of arthritic mice/total number of mice in each group.

^dMean maximum arthritic score for arthritic mice only.

^eTiters of IgG antibodies specific for CII, in serum at day 35 (experiment 3) or day 38 (experiment 4) post-immunization, measured by ELISA, for arthritic, healthy or all animals in each group.

(containing *M. butyricum*) that is used, together with CII, to induce arthritis. As a matter of fact, $\gamma\delta$ T cells have been reported to expand in peripheral lymphoid tissues of mice with CIA and suggested to play a direct role in CIA (13,50). Injection of mice with 13D5, an anti- $\text{TCR}\gamma\delta$ mAb, was reported to delay the onset of CIA, if the treatment was initiated before immunization with CII, whereas CIA was exacerbated if the antibodies were injected 40 days after immunization (13). However, the effects of 13D5 administration on the immune system are not well understood. In the above-mentioned experiment, mice injected with intact 13D5 rapidly lost weight, suggesting that 13D5 may induce a cytokine-mediated syndrome similar to that observed in mice and humans after injection of anti-CD3 (13,51). Administration of large amounts of 13D5 may consequently result in stimulation rather than blocking of $\gamma\delta$ T cells, and this interestingly has a prophylactic effect in CIA.

A major concern when using gene-modified animals is that the immune system, due to its innate flexibility, may develop in an altered fashion in the absence of a particular gene product or cell subset. For instance, CD4-deficient DBA/1 mice have been reported to develop an abnormal population of CD4-CD8⁻ $\text{TCR}\alpha\beta^{+}$ cells that could explain the susceptibility of these mice to CIA (23). In this particular case, genetic

factors (mouse strain) seem to be very important, since we have found that the CD4 deletion on a C57Bl/10 background protects against CIA (M. Vestberg *et al.*, unpublished findings). Similarly, the immune system may somehow compensate for the lack of $\gamma\delta$ T cells in $\delta^{-/-}$ mice, thus the fact that $\delta^{-/-}$ mice are susceptible to CIA does not necessarily mean that $\gamma\delta$ T cells are not involved in disease of wild-type mice. However, since the absence of $\gamma\delta$ T cells, in two different mouse strains, failed to alter any of the CIA parameters we measured, we believe that $\gamma\delta$ T cells can only have a very limited, if any role in this disease.

The recently reported induction of arthritis in $\text{RAG}^{-/-}$ DBA/1 male mice, lacking both mature T and B lymphocytes (24), is clearly a surprising observation in the light of the current opinion on CIA. In particular, the data conflicts with the observations that B cell-deficient (22) or $\alpha\beta$ T cell-deficient mice (present report) are resistant to CIA. It is likely, however, that a mouse lacking all lymphocyte populations is biologically different from a more immune competent mouse. It should be also noted that a delay in clinical disease onset and a reduction in severity between RAG^{+} and RAG^{-} DBA/1 mice was seen, confirming that lymphocytes play an important role in CIA (24). This report suggests a lymphocyte-independent mechanism of disease induction in CIA. This is a challenging

view that requires further studies, in particular with another mouse strain.

A role for $\gamma\delta$ T cells has been suggested in other animal models for inflammatory diseases. In particular, in experimental allergic encephalomyelitis (EAE), a model for multiple sclerosis, the role of $\gamma\delta$ T cells has been analyzed by several groups but with diverging results. Administration of 13D5 in B10.PL mice led to an aggravation of EAE (52), whereas injection of another anti-TCR $\gamma\delta$ mAb, GL3, into SJL mice resulted into reduction in the severity of the disease (53). In the C57BL/6 strain, mice deficient for $\gamma\delta$ T cells were as susceptible as controls to passive transfer of EAE by encephalitogenic T cells, arguing against a regulatory role of the $\gamma\delta$ T cells (54). These contradictory reports about the role of $\gamma\delta$ T cells in EAE may be explained by differences in the genetic background of the mice and in the experimental set-up.

The role of $\gamma\delta$ T cells may also be difficult to identify in the complex events leading to EAE or CIA. The use of targeted germline mutation techniques may be particularly helpful to investigate the role of such small cell populations, as $\gamma\delta$ T cells, in a disease model. By using two strains of mice rendered deficient for $\gamma\delta$ T cells, we show in the present study that the absence of $\gamma\delta$ T cells does not affect CIA. This demonstrates that $\gamma\delta$ T cells are not required for CIA development and most probably, in contrast to $\alpha\beta$ T cells, do not play a major role in this disease.

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Abbreviations

CII	type II collagen
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
Con A	concanavalin A
EAE	experimental allergic encephalomyelitis
IFA	incomplete Freund's adjuvant
PE	phycoerythrin
RA	rheumatoid arthritis
SEA	<i>Staphylococcus aureus</i> enterotoxin A
TNF	tumor necrosis factor

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