Chondrocyte antigen expression, immune response and susceptibility to arthritis

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Abstract
The association of HLA-B27 with certain forms of arthritis implies a role for MHC class I-restricted T cells in the arthritic process. Our aim was to study CD8⁺ T cell responses towards specific antigens localized in joint tissue. Known determinants were introduced into chondrocytes of transgenic (TG) mice, under the control of the cis-regulatory sequences of the human type II collagen gene (COL2A1). Two Escherichia coli β-galactosidase (β-gal)-expressing lines were derived (CIIL73 and CIIL64) as well as two lines (CIINP) expressing influenza A virus nucleoprotein (NP). Expression of the antigens could be demonstrated in cartilaginous tissues. The TG lines showed variable degrees of responsiveness towards the transgene-introduced antigens; whilst 75% of CIIL73 mice had an impaired cytotoxic T lymphocyte (CTL) response towards β-gal, the response in CIIL64 mice was essentially normal. However, both lines displayed normal proliferative and antibody responses to β-gal. A reduced CTL response was seen to NP in the CIINP lines in ~65% of the animals. In spite of the persistence of T cell responses to the transgene antigens in these lines, induction of CTL responses alone has so far failed to induce clinical signs of arthritis. Interestingly, some animals expressing β-gal were susceptible to arthritis following challenge with type II collagen alone, whilst their non-TG littermates and TG mice from other lines remained unaffected. As β-gal is expressed by E. coli, a component of the normal gut flora, this suggests a possible role for gut-derived immune responses. We believe these lines could form the basis of a model for studying links between intestinal inflammation and arthritis.

Introduction
The human MHC allele, HLA-B27, is strongly associated with the seronegative spondyloarthropathies, including ankylosing spondylitis and reactive arthritis. One hypothesis to explain this association is that the MHC class I molecules present potentially arthritogenic peptides to cytotoxic T lymphocytes (CTL), which may contribute to the arthritic process when activated. However, the demonstration of CD8⁺ T cell responses to self-antigen has been hampered by the failure to identify potential arthritogenic antigens. Proteins expressed more specifically in joints, such as type II and type XI collagen, have been suggested as target antigens for autoreactive T cells (1). However, it has proved difficult to isolate collagen-specific CD8⁺ T cell clones from peripheral blood of patients suffering spondyloarthropathies (2).

Numerous transgenic (TG) models have been used to investigate the CD8⁺ T cell responses to ‘neo-self’ antigens in the periphery (3–5). In a few cases, the TG antigen itself can cause spontaneous development of disease. The over-expression of MHC antigens in the pancreas (6,7) or in the central nervous system (8) can lead to spontaneous diabetes or dysmyelination respectively. However, in most cases, autoimmune disease does not occur spontaneously, either because of T cell ‘ignorance’ (9,10) or tolerance (11,12). Challenging these TG animals with the relevant antigen can, however, yield very different results depending on the model. In the RIP-LCMV (9,10) and MBP-LCMV (13) models, LCMV infection can initiate an autoimmune response. On the other hand, in the RIP-HA system (11), influenza virus...
infection could not break the tolerance and pancreatic infiltration has not been observed. The human type II collagen gene (COL2A1) has been used to express an MHC class II-restricted T cell epitope involved in the development of collagen-induced arthritis (CIA) in TG mice (14, 15). However, there are no reports of this approach applied to MHC class I-restricted T cell responses in joint-related disease.

### Methods

#### Gene constructs and the generation of TG mice

The transgene construct, pKL80.3, has been described previously (16). It contains the 6.1 kb 5’ flanking DNA sequence of the COL2A1 gene, the 3.5 kb *Escherichia coli* β-gal gene (β-gal; LacZ) cassette including the SV-40 poly(A) tail and the 0.3 kb enhancer element essential for tissue-specific expression (CSE) cloned into the vector pPolyIII. The second construct, pANP, contained the 4.8 kb COL2A1 promoter fragment from a second plasmid pAA2 (17). cDNA encoding nucleoprotein (NP) from the influenza A virus, A/NT/60/68 (obtained from the plasmid NP28, a gift of A. Townsend), followed by a stop codon and the poly(A) tail, replaced the majority of the LacZ gene, being cloned into an *EcoR*I site at position 3016 (45 bases upstream of the LacZ stop codon). This construct also has the 0.3 kb CSE from pKL80.3. In arthritis induction experiments, a line, CIIB7.1, expressing human CD80 in place of NP was used.

Vector sequence was removed from the transgene constructs using the restriction enzyme NotI. TG mice were generated by pronuclear injection of DNA into (C57BL6× BALB/c) F2 oocytes. Lines of mice were produced from five independent TG founders, two of each of the constructs: LacZ (lines CIL64 and CIL73), NP (lines CIINP14 and CIINP45) and one for CD80 (line CIIB7.1). TG mice were genotyped by Southern hybridization of tail DNA using a 1.6 kb *EcoR*I fragment of the COL2A1 proximal sequence as a probe. Copy numbers were estimated by relative intensity of the transgene band to that produced by hybridization of the probe to the mouse COL2A1 promoter. Founders were backcrossed onto the BALB/c background and were kept in the specific pathogen-free unit at the Institute for Animal Health, UK.

#### X-gal staining of the neonatal joints

The X-gal staining procedure was modified from a previously described method (18). Briefly, ribs and limb joints were dissected out from 1- to 3-day-old neonatal mice, and were fixed in 0.2% glutaraldehyde, 2% formaldehyde, 5 mM EGTA, pH 7.3 and 2 mM MgCl₂ in PBS at room temperature for 30 min. This was followed by three 5 min washes with rinse solution (0.1% deoxycholic acid, 0.2% Nonidet P-40 and 2 mM MgCl₂ in PBS) at room temperature. The tissues were incubated at 30°C for 24 h in the dark in substrate solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in rinse solution. After colour development, the tissues were washed with rinse solution and processed for paraffin wax embedding. Sections (3 μm) were cut and counter-stained with eosin for microscopic examination.

#### Immunohistochemistry

Ribs and limb joints were carefully dissected out from neonatal mice and were fixed in 4% paraformaldehyde in PBS at 4°C for 20 min. After two PBS washes, the tissues were embedded in Tissue-Tek. The NP transgene expression was detected using the IMAGEgent influenza virus A and B detection kit (code K6105; Dako, High Wycombe, UK). Frozen sections (7 μm) were re-hydrated in PBS. Then 25 μl of reagent A (containing FITC-conjugated mAb against NP and matrix protein, and Evans Blue as a counter-stain) was added to each section and incubation was carried out in darkness in a humidified chamber at 37°C for 1 h. It was followed by two washes of PBS for 5 min. The sections were mounted and examined under a confocal fluorescence microscope (DM RBE; Leica, Heidelberg, Germany) and the images were captured using the True Confocal Scanner with TCS-NT software (Leica).

#### In vitro re-stimulation and ⁵¹Cr-release assay

Spleens were obtained 2 weeks after viral infection for in vitro re-stimulation. Autologous splenocytes were incubated with 1 μM β-gal876−884 (TPHPARIGL) or NP147−155 (TYQRTRALV) (Research Genetics, Huntsville, AL) peptide in RPMI at 37°C for 1 h and used as stimulators. In vitro re-stimulation cultures were set up with 1.5×10⁷ splenocytes and 0.3×10⁷ peptide-pulsed stimulators in 15 ml RPMI supplemented with 10% FCS, 50 μg/ml penicillin and streptomycin, 0.3 g/l l-glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, and 5 U/ml Lymphocult-T as a source of IL-2 (Biotech, Dreieich, Germany). The culture was maintained at 37°C, 5% CO₂ for 5 days, at which time a standard ⁵¹Cr-release assay was performed. P815 target cells were pulsed with 1 μM peptide or unpulsed as indicated.

#### Proliferation assay

Two weeks after immunization, draining lymph nodes were removed and disrupted to form single-cell suspensions. Lymph node cells (3–5×10⁷) were co-cultured, in triplicate wells, with E. coli β-gal at various concentrations in 200 μl RPMI supplemented with 10% FCS in a 96-well flat-bottomed plate. At least four control wells without the antigen were included to represent the background counts. The culture was incubated at 37°C, 5% CO₂ for 4 days and 1 μCi of [³H]thymidine (sp. act. = 25Ci/mmol; Amersham Life Science, Little Chalfont, UK) was added to each well for the last 18 h. [³H]Thymidine uptake was measured using the 1450 MicroBeta TRILUX β-plate counter (Wallac, Turku, Finland).
ELISA plates (Maxisorp; Nunc, Life Technologies Ltd, Paisley, UK) were coated with 50 µl β-gal solution at 5 µg/ml in PBS containing 0.05% sodium azide at 4°C overnight. The solution was flicked off and the plates were washed 5 times with PBS-T (0.1% Tween 20 in PBS). This washing procedure was repeated between the subsequent incubation steps. The plates were then pre-treated with 100 µl blocking buffer (1% casein in PBS-T) at room temperature for 30 min followed by 100 µl diluted serum samples at 37°C for 1 h and 50 µl of 1 µg/ml peroxidase-conjugated anti-mouse IgG (Vector, Laboratories, Peterborough, UK) for another 1 h. This was followed by incubation with 100 µl peroxidase substrate, ABTS (Vector), at room temperature for 20 min in darkness. Substrate colour change was measured as OD at 405 nm using the Biomek plate reader (Beckman Instruments, High Wycombe, UK).

Induction of arthritis with collagen

Bovine type II collagen (CII) (cat. no. C-1188; Sigma) was dissolved in 0.1 M acetic acid and emulsified with equal volume of complete Freund’s adjuvant (CFA; Difco, Detroit, MI) to a final concentration of 1 mg/ml. Mycobacterium tuberculosis H37 RA (Difco) was added to a final concentration of 5 mg/ml. Mice were immunized s.c. with 100 µg CII/CFA mixture. After 4 weeks, animals were boosted s.c. with 100 µg CII emulsified in incomplete Freund’s Adjuvant (IFA; Difco). When mice were also challenged with vaccinia, 10 days after CII priming, they were boosted with 10⁷ NPVV (vaccinia recombinant for NP and β-gal). Then, 40 days after the initial challenge, the mice were injected s.c. with 100 µg CII/IFA mixture. The genetic status of the mice was not known during the monitoring period of 3-4 months. Arthritis scoring: grade 1 = redness or slight swelling of the paw, grade 2 = obvious increase in paw thickness and grade 3 = visible joint distortion. Only those mice that had an arthritis score of ≥1 for 5 consecutive days were considered as showing clinical signs of arthritis. Onset was then defined as the first day the signs had appeared.

At the end of the monitoring period, joints were taken for histological assessment. Feet or knees were dissected out, fixed and decalcified in Decalciﬁer I solution (Surgipath Europe Ltd, Cambridge, UK). Normal samples were taken at the same time for comparison. Parafﬁn sections (3 µm) were cut along a longitudinal axis and stained with haematoxylin & eosin. In some experiments, only joints taken from animals having shown signs of arthritis were taken for histology. In experiments where all mice provided samples for histology, no abnormalities were detected in the absence of clinical signs.

Results

Tissue-specific expression of the transgene

The COL2A1 regulatory elements were used to direct expression of the β-gal, NP and CD80 to tissues that synthesize CII, principally chondrocytes in the developing animal (17,19). The two β-gal lines differed in the transgene copy number, CIIL73 having >100 copies while CIIL64 had ~20. Figure 1(B and C) shows X-gal-stained paraffin sections from the limb joints of β-gal TG 1-day-old neonates. Intracellular X-gal staining was observed in isolated patches of cells in cartilaginous tissues in the knee joint (Fig. 1B) and in the metatarsal joint (Fig. 1C) of TG animals. No detectable signal was seen in the corresponding area of non-TG animals (Fig. 1A). Although both β-gal-expressing lines showed similar patterns of expression in cartilage, there were differences in the non-chondrocyte expression patterns of the two TG lines. During embryonic development (day 15–16 gestation), CIIL73 was expressed strongly in the lungs, whilst CIIL64 had no lung expression but patchy expression in the heart (data not shown). Young adults, however, did not generally express the transgene antigen in tissues other than ribs, joints or, occasionally, brain (RT-PCR, data not shown).

NP expression in neonatal joints and ribs of the CIINP14 line was detected using immunofluorescence staining on frozen sections. Figure 1(E and F) shows positive staining for NP in neonatal ribs. Minimal background staining was observed in the corresponding area in non-TG mice (Fig. 1D). Positive staining for NP was also seen in scattered chondrocytes in the cartilaginous area in the knee joint of the TG animals (data not shown). Transgenes were not expressed in the thymus of CIINP14, CIIL64 or CIIL73 lines as assessed by RT-PCR assays or X-gal staining of 16.5-day embryos of the CIIL73 and CIIL64 lines (data not shown).
that could mount a β antigen, TG animals had an impaired CTL response to the transgene pulsed targets (Fig. 2 B). In contrast, most, but not all, of the diamonds, assay. Open diamonds, untreated P815 targets cells; closed circles, which also expresses lines were immunized i.p. with vaccinia recombinant for NP, then 2 weeks later spleen cells were re-stimulated with either β-gal876–884 (A) or NP147–155 (B) and then assayed in a standard 51Cr-release assay. Open diamonds, untreated P815 targets cells; closed diamonds, β-gal876–884-pulsed P815 target cells; closed squares, P815 cells transfected with β-gal.

CD80 expression in the CIIB7.1 line was demonstrated in the ribs and joints of TG animals by RT-PCR, and on the cell surface of a proportion of chondrocytes isolated from neonatal cartilage (data not shown).

Transgene-dependent reduction of CTL, but not proliferative, T cell responses

To determine the effect of transgene expression on CTL responses, TG and non-TG littermates from CIIL73 and CIIL64 lines were immunized i.p. with vaccinia recombinant for NP (which also expresses E. coli β-gal as a selection marker). Spleen cells were assayed for transgene-specific cytotoxicity and results for the CIIL73 line are represented in percent lysis of different targets at different E:T ratios (Fig. 2). Both the TG and non-TG mice mounted good CTL responses to the control antigen, as shown by the specific lysis of the NP147–155-pulsed targets (Fig. 2 B). In contrast, most, but not all, of the TG animals had an impaired CTL response to the transgene antigen, β-gal (Fig. 2 A). However, among those CIIL73 TG mice that could mount a β-gal CTL response, the magnitude of the response was comparable to that observed in the non-TG mice. Unlike the CIIL73 line, CIIL64 TG mice could mount a transgene-specific CTL response that was comparable to that of their non-TG littermates (Table 1 and data not shown). In order to assess the frequency of mice with an impaired β-gal CTL response, we counted those animals that showed <10% specific lysis at the maximum E:T ratio as non-responders. The results are summarized in Table 1, showing 72% of CIIL73 TG mice to be non-responders.

In order to examine CTL responses to the transgene antigen in the CIINP14 line (expressing NP), mice were challenged by intranasal infection with influenza A virus. Figure 3(A) shows results of individual mice from a representative experiment. All the TG animals retained the ability to respond to NP; however, the response from the majority of these titrated out more quickly, in terms of effector cell numbers, than their non-TG littermates. Interestingly, although the reduction in CTL response to the transgene was not complete, as in the CIIL73 line, a similar proportion of TG animals was affected. Mice were grouped as low responders if the specific lysis was <50% of the maximal killing after 1 log10 titration of the highest E:T, if >50%, as high responders. Figure 3(A) shows an example of one high- and one low-responder TG mouse. Figure 3(B) shows a box and whisker plot of responses from a total of 25 TG and 25 non-TG animals to NP147–155 at an intermediate E:T ratio of 8. The difference between the two groups is highly significant (P < 0.0001). Table 2 summarizes the results of five independent experiments, showing 64% of CIINP14 TG mice to be low responders. The difference in responder status between TG animals was unlikely to be due to a gross reduction of the affinity or avidity of the responding T cells, as a titration of peptide, at a constant E:T of 10, was equivalent for both TG and non-TG mice. Figure 4 shows a titration from six representative animals, including three low-responder and one high-responder TG mouse. Although the total lysis for the low-responder mice was lower than that for the others, peptide sensitivity remains the same for all the animals. In all, a total

![Fig. 2](image-url) Transgenic antigens in chondrocytes and arthritis

| Table 1. Summary of β-gal CTL response in the CIIL73 and CIIL64 lines |
|----------------|----------------|
| TG line         | Non-responderb |
| TG              | 5              | 12             |
| Non-TG          | 13             | 15             |
| CIIL64a         |                |                |
| TG              | 12             | 12             |
| Non-TG          | 3              | 3              |

aMice having <10% specific lysis of the βgal876–884-pulsed targets at the maximum E:T ratio were counted as responders.
bMice having <10% specific lysis of the βgal876–884-pulsed targets at the maximum E:T ratio were counted as non-responders.

cP-values were calculated using Fisher’s exact test (Arcus).
dData were pooled from four independent experiments.
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Fig. 3. Reduced CD8+ T cell response to the transgenic antigen in the CIINP14 line. (A) The responses of representative individual animals of the CIINP14 line are shown following in vivo immunization with influenza A virus followed by in vitro re-stimulation of spleen cells with NP147–155 peptide. Closed symbols, P815 cells pulsed with peptide NP147–155; open symbols, unpulsed P815 cells. Data are representative of results summarized in Table 2. Lo, low responder; Hi, high responder. (B) Box and whisker plot representing specifically lysis of NP147–155-pulsed P815 target cells at an E:T ratio of 8. Pooled data from five experiments with 25 TG and 25 non-TG animals are shown. The box represents ~50% of the values, the whiskers show the range, the filled circle is the mean response and the line bisecting the box is the median response; asterisk indicate outliers.

Table 2. Summary of NP CTL response in the CIINP14 line

<table>
<thead>
<tr>
<th>TG line</th>
<th>No. of mice</th>
<th>Respondera</th>
<th>Non-responderb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIINP14d</td>
<td>16</td>
<td>9</td>
<td>0.0003</td>
</tr>
<tr>
<td>TG</td>
<td>3</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Non-TG</td>
<td></td>
<td></td>
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</tbody>
</table>

aSpecific lysis of the NP147–155-pulsed targets cells <50% of the maximum lysis after 1 in 10 titration of the E:T.
bSpecific lysis of the NP147–155-pulsed targets cells <50% of the maximum lysis after 1 in 10 titration of the E:T.
cCalculated using Fisher’s exact test (Arcus).

dData were pooled from five independent assays.

of 12 mice tested, irrespective of whether they were high or low responders, had very similar dose–response curves.

CD4+ T cell response to the transgenic antigen in CIIL73 TG mice

To analyse whether CD4+ autoreactive T cells are still present in the TG animals, mice from the CIIL73 line were tested for proliferative and antibody responses towards the transgene antigen. Mice were immunized with β-gal in CFA s.c. Figure 5(A) shows proliferation of draining lymph node cells at various antigen concentrations. Both groups of mice responded equally well to the transgene antigen with no significant difference between the TG and non-TG mice. To measure the antibody response to the transgene antigen, blood samples were taken before immunization and at the time of sacrifice. Individual serum samples were assayed for anti-β-gal IgG by ELISA (Fig. 5B). Again, no significant
difference was seen between TG and non-TG animals. Thus, although there was often a profound effect on the CTL response to the transgene antigen in the CIIL73 line, the CD4⁺ T cell and antibody responses were unaffected.

**Arthritis induction in β-gal TG mice**

No mice of any of the TG lines has been observed to develop spontaneous arthritis or joint disease over and above that of normal or non-TG animals up to at least 18 months of age. Therefore expression of the transgenes per se does not appear to be detrimental to development or have any deleterious effects on the mice. In addition, some mice were left for several months following priming and therefore more susceptible to arthritis than of either of the TG, compared to non-TG, animals that remained clinically healthy and were not seen during a period of at least 3 months nor were any histological signs of arthritis seen in a selection of mice tested (data not shown). Therefore, it appeared that simple induction of immune responses against the transgene antigen was insufficient to induce a significant incidence of arthritis.

In other animal models of arthritis, such as CIA, transfer of disease required both autoantibodies and self-reactive T cells (20). In our system, the transgene antigens were expressed intracellularly, either in the cytoplasm or the nucleus. This cellular location could render antigen less available as a target for antibody recognition in healthy, intact cartilage. Thus, we set out to induce an antibody response to a cartilage matrix protein, which would be antibody accessible, prior to transgene antigen immunization. We chose CII because it could elicit an antibody response without inducing arthritis in BALB/c mice (21). In the first instance we were challenged with CII in CFA on day 0, infected with vaccinia on day 10 and boosted with CII in IFA on day 40. In two independent experiments two mice of the CIIL73 line developed arthritis, assessed by both clinical and histological analysis (Fig. 6 and Table 3: CII-Vaccinia-CII). However, surprisingly, when mice were challenged and boosted with CII alone (without specific induction of responses against β-gal) some animals from both β-gal-expressing lines developed arthritis (Table 3). Clinical and histological features were similar irrespective of induction protocol, although the two CIIL64 mice had less severe disease, histologically and of later onset. Arthritic signs have only been observed in the hind limbs, predominantly involving the knee, ankle and forefoot. Onset was relatively late and was asymmetrical (generally in only one limb), very much in contrast to classical CIA. Only one of the mice had overt clinical disease in both hind limbs, though with unequal severity. Although clinical signs were seen in a number of non-TG mice (Table 3), there was a statistically significant difference in the number of TG animals from either of the β-gal lines compared to their non-TG littermates (13 of 66 TG versus five of 63 non-TG $P = 0.046$). The histology, seen only in some of the TG mice, is particularly characterized by polymorph infiltration and synovial proliferation, with evidence of bone erosion and remodelling, cartilage damage, and chondrocyte loss (Fig. 6). There is a possibility that antigen expression using the COL2A1 promoter could render the chondrocytes, and thus the cartilage of TG animals, more ‘fragile’ and therefore more susceptible to arthritis than their non-TG littermates. A structural weakness of the cartilage is highly unlikely as the NP and β-gal transgenes are only expressed intracellularly and therefore would have no influence on the cartilage matrix. In addition, we did not see any signs of arthritis in a large number of animals expressing human CD80 (cell surface expression) under the same promoter nor did we see anything in the very high copy CIINP14 line (Table 3). Furthermore, histological analysis of TG, compared to non-TG, animals that remained clinically healthy did not show any deficit in cartilage (data not shown).

**Discussion**

The association of HLA-B27 with certain forms of arthritis implies a role for MHC class I-restricted T cells in their pathogenesis. However, this does not necessarily imply that these T cells could, by themselves, precipitate the disease. In this study, we have deliberately chosen antigens that have the potential to be efficiently presented in the MHC class I pathway of antigen presentation, being cytoplasmic or nuclear, but are less likely to be efficiently presented to MHC class II-restricted T cells (22,23). We have shown that intracellular expression of TG antigens in chondrocytes can have a variable, but at times profound, effect on the CTL response to that antigen, whilst leaving the CD4⁺ T cell and antibody responses intact. The lack of an effect on the CD4⁺ T cell response may be due partially to the cellular localization of the transgene protein. In addition, in chondrocytes can express MHC class II antigens on activation, they are normally class II⁺ in mouse and man (24,25). It is of note that the CD8⁺ T cells differed in the two lines expressing β-gal. Although they differed in expression in other tissues...
Table 3. Summary of arthritis induction experiments

<table>
<thead>
<tr>
<th>Induction protocol</th>
<th>No. of experiments</th>
<th>Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TG mice affected (total TG mice)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Non-TG mice affected (total non-TG mice)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. with clinical signs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>With abnormal histology</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day of onset&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Maximum severity&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>CII–Vaccinia–CII</td>
<td>2</td>
<td>CIIL73</td>
<td>6 (23)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CIIL64</td>
<td>0 (8)</td>
<td>0</td>
</tr>
<tr>
<td>CII–CII</td>
<td>2</td>
<td>CIIL73</td>
<td>3 (17)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CIIINP</td>
<td>0 (7)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CIIB7.1</td>
<td>0 (36)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 CIIB7.1</td>
<td>0 (36)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All mice had been back-crossed to BALB/c for at least seven generations.
<sup>b</sup>Combined numbers for each induction protocol.
<sup>c</sup>Only those mice with a clinical score ≥1 for at least 5 consecutive days were scored.
<sup>d</sup>Number of days following initial CII challenge of first appearance of signs, for individual mice.
<sup>e</sup>Maximum severity for each limb affected (see Methods) for individual mice.
<sup>f</sup>Both hind limbs affected on this animal.

during embryonic development, most notably with expression in the heart in the CIIL64 line and in the lung in the CIIL73 line, both lines had similar patterns of expression in cartilage. Nonetheless, a difference between the embryonic expression in the two lines may indicate other subtle differences in expression that could account for the differences seen in the CTL responses.

The reduction of the CTL response is unlikely to be the result of deletion or inactivation of the autoreactive T cells in the thymus, as the COL2A1 regulatory elements do not direct expression in lymphoid tissues (16,19). In addition, transgene expression was not detectable in the thymus by RT-PCR or X-gal staining. Failure to detect transgene expression does not completely rule out the possibility of thymic tolerance and if this were at a critical threshold, then it could explain some of the difference seen between littermates. These questions would be best addressed using TCR TG mice of the appropriate specificity.

Transgene dose is, however, a factor that has the potential to affect the CTL response. The CIIL73 line has ~5 times greater copy number than the non-tolerant CIIL64 line (data not shown) and, although this does not necessarily relate directly to expression level, it indicates a likely difference.

A more intriguing finding is the difference between littermates of the CIIINP and CIIL73 lines. Approximately 30% of TG animals are able to make a near-normal CTL response to their transgene antigen, whilst their TG littermates may be profoundly tolerant. The variation between littermates could be related to genetic differences in some of the earlier backcrosses, or to chimeric or variable expression of the transgene. However, this pattern of response appears to be maintained (data not shown) and is seen in three lines of TG mice with independent insertions; therefore, we feel this is unlikely as an explanation. TG TCR animals will be required to precisely define the mechanisms of tolerance. However, the variable nature of the reduction of CTL response between littermates does imply that presentation leading to inactivation or deletion of potentially autoreactive T cells is at or near a threshold, either in level of expression or timing. Both these elements have been found to be important in the development of peripheral tolerance in models with co-expression of antigen and a specific TCR transgene (26–28).

No evidence of arthritis was observed in any of the TG lines tested, even in instances when an immune response was efficiently induced against the transgene. This result is in contrast to many other TG models of autoimmunity, including diabetes and central nervous system disease (reviewed in 3,29). Indeed, expression of β-gal under a retinal promoter did not lead to β-gal-specific tolerance, but an autoimmune retinitis was precipitated by induction of β-gal-specific CTL responses in these animals (30). There are a number of potential reasons for the lack disease in our mice expressing similar antigens, but under the COL2A1 promoter. Access of potentially autoreactive T cells to the TG chondrocytes is likely to be restricted in normal healthy animals due to the relatively impermeable matrix of the cartilage, together with a lack of vascularization. Even if CTL had access to the chondrocytes in the absence of any inflammatory signals, the low level of MHC class I on cells would make them essentially invisible to the T cells (E. Cohen et al., manuscript in preparation). Access might be improved by inflammation following damage or stress of the cartilage or enthesis. Although there is speculation that this mechanism could be important in humans, it may be a relatively rare event in a specific pathogen-free mouse colony and therefore not detected in our experiments.

Other induced models of arthritis, in particular CIA, generally require both a specific T cell as well as an antibody response. Transferring either arthritic serum or spleen cells from arthritic mice alone cannot induce complete CIA, which probably requires the synergetic effect from both types of response (20,31). Autoantibodies against CII can be generated in many different strains of mice, although there is a qualitative difference in terms of epitope specificity and IgG...
subclass from the arthritic serum (32). BALB/c mice are, however, highly resistant to CIA, although there have been reports of a low incidence of disease in some studies (21,33). In our experiments we have not seen histological evidence of arthritis in either BALB/c mouse controls (data not shown) or in non-TG littermates (Table 3). Nevertheless, two mice from the CII.L73 line developed a severe inflammatory arthritis as a result of challenge with bovine CII and induction of an anti-β-gal response by infection with recombinant vaccinia virus.

Surprisingly, a few mice from both lines expressing β-gal also developed arthritis in the absence of any other antigen-specific challenge. That this was not seen in seven CIINP14 mice or in 36 mice expressing human CD80 under the same COL2A1 promoter indicates that the arthritis may be linked to the transgene antigen, rather than be caused by potential toxic effects of transgene expression on the chondrocytes. Although we have, as yet, no definitive explanation for the low level of susceptibility of the CII.L64 and CII.L73 animals to arthritis induced by CII, the clinical picture seen is distinct from classical CIA. In addition, the nature of the antigen could be important. As β-gal is an E. coli protein, animals are potentially exposed to this antigen in the resident bacteria of the intestine. We could not see spontaneous β-gal responses in the spleens of these mice; however, normal mice do show a low level of spontaneous β-gal-specific response detectable in the mesenteric lymph nodes, but not the spleen (unpublished results). There is a strong link between some forms of arthritis and gastrointestinal or genitourinary infection or inflammation (34,35). Indeed, a number of animal models also depend on the presence of gut bacteria (36,37). Therefore the TG lines described in this report could provide a useful tool for the investigation of an antigen-specific link between gut immune responses and arthritis susceptibility.

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Abbreviations

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<thead>
<tr>
<th>β-gal</th>
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<tr>
<td>CFA</td>
<td>Freund’s complete adjuvant</td>
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<tr>
<td>CIA</td>
<td>collagen-induced arthritis</td>
</tr>
<tr>
<td>CII</td>
<td>type II collagen</td>
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<tr>
<td>COL2A1</td>
<td>human type II collagen gene</td>
</tr>
<tr>
<td>CSE</td>
<td>cartilage-specific element</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>IFA</td>
<td>Freund’s incomplete adjuvant</td>
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<tr>
<td>LacZ</td>
<td>β-galactosidase gene</td>
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<tr>
<td>NP</td>
<td>nucleoprotein</td>
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<tr>
<td>TG</td>
<td>transgenic</td>
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References


