EXTENDED REPORT

Tumour necrosis factor receptor gene therapy affects cellular immune responses in collagen induced arthritis in mice

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Background: Collagen induced arthritis (CIA) is an animal model of rheumatoid arthritis (RA) amenable to immunotherapy directed against tumour necrosis factor α (TNFα).
Objective: To evaluate whether local TNF receptor (TNF-R) gene therapy in DBA/1 mice exerts an influence beyond anti-inflammatory effects. Two measures of CIA pathogenesis were investigated—namely, immunity to collagen II (CII) 245–270 peptide (the major immunodominant epitope within bovine CII) and the preferential activation of T cell Vβ8.2 variable region receptors in arthritic DBA/1 mice.
Methods: DBA/1 mice received single periarticular injections of media or retroviral vectors containing LacZ or human TNF-R into affected arthritic paws at disease onset. Disease severity was monitored, immune responses towards the immunodominant bovine CII 245–270 and subdominant CII 334–360 peptide epitopes were assessed by ELISA, and T cell Vβ usage was analysed by real time polymerase chain reaction for the LacZ transduced, TNF-R, and viral-free media treated control animals. The therapeutic influence of TNF-R gene transduction was compared with other groups at different times after treatment.
Results: Reduced disease severity was seen 15–35 days after treatment, with a concomitant increase in immunity towards the subdominant CII 334–360 peptide epitope rather than the immunodominant CII 245–270 peptide in TNF-R treated animals. Early in the disease, TNF-R treated animals demonstrated a reduction of bias towards the otherwise predominant Vβ8.2 T cell subset.
Conclusions: TNF-R gene therapy influences cellular immunity in CIA, leading to overall disease amelioration, thus suggesting that TNF inhibition may have therapeutic potential beyond the control of inflammation in RA.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterised by altered cellular and humoral immune response.[1 2] Collagen induced arthritis (CIA) is elicited by the immunisation of disease susceptible H-2^q and H-2^r mice with heterologous type II collagen (CII),[3–5] and is widely used to study the immunological mechanisms relevant to RA. CII reactive CD4+ T cells and complement fixing anti-CII IgG2a autoantibodies have been implicated as major contributors to the immunopathogenesis of chronic arthritis.[5] Disease suppression has been observed after administration of monoclonal antibodies to CD4[6] and the T cell receptor (TCR) variable region β (Vβ) chain,[7] supporting a critical role for T cells in CIA. Susceptibility to CIA relies on the ability of major histocompatibility complex (MHC) class II molecules to present specific CII peptides, which lead to the activation of arthritis related CII reactive T cells.[5 8] A major H-2^q restricted T cell epitope present on bovine, chick, human, and rat CII between amino acids 245 and 270 has been identified as immunodominant and generates the highest immune responses and regulates development of arthritis in disease susceptible DBA/1 mice.[9–11]

Beyond MHC restriction, non-MHC factors such as TCR Vβ genes have a crucial role in modulating the susceptibility and progression of CIA, with a preferential use of TCR Vβ6 and Vβ8,[7 12] similar to experimental autoimmune encephalomyelitis.[13]

Tumour necrosis factor α (TNFα) is an inflammatory cytokine that contributes to the cytokine cascade in CIA.[14 15] Immunotherapy against TNFα has been shown to have marked anti-inflammatory effects.[1 16] Our objective in this study was to evaluate whether local retrovirus mediated TNF receptor (TNF-R: MOIN-sTNF-Rc-Ig) gene therapy could exert effects beyond its anti-inflammatory activity, and influence the T cell responses in CIA.

MATERIALS AND METHODS

Retroviral vector production

Retroviral vector MOIN-sTNF-Rc-Ig encodes a fusion protein consisting of the extracellular domain of human 55 kDa TNFα receptor covalently linked to the mouse IgG1 heavy chain. Soluble human TNF receptor (sTNF-Rc-Ig) was amplified from sTNFRc.Ig plasmid[17 18] using TNF-sR5 and Ig3 oligomers: TNF-sR5, 5′-ATCGATGCCATGGGCCTCTCCACCGTG-3′; Ig3, 5′-ATCGATTCATTTACCAGGAGA-GTG-3′, and inserted into the *Bam*HI site of the retroviral construct MOIN[19] resulting in MOIN-sTNF-Rc-Ig. The MFG-LacZ retroviral vectors were made by insertion of LacZ cDNA into 5′ *Nco*l and 3′ *Bam*Hl cloning sites of a simplified vector that contains a region of the gag gene (helf-gag) to increase viral titre and the env splice acceptor. High titre virus was produced by transfecting the vector into Phoenix cells, a 293 based amphotropic packaging cell line, and harvesting the supernatant 48 hours after transfection.

Abbreviations: CIA, collagen induced arthritis; CII, collagen type II; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; INFγ, interferon γ; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RA, rheumatoid arthritis; TCR, T cell receptor; TNFα, tumour necrosis factor α; TNF-R, TNF receptor; Vβ, variable region β

Induction and assessment of CIA

Native bovine CII (kindly provided by Dr Marie Griffiths, University of Utah) was solubilised at 2 mg/ml in 0.01 M acetic acid at 4℃ overnight and emulsified with equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Arthritis was induced by intradermal injections of 100 µl of emulsion containing 50 µg CII at the base of the tails of 6–8 week old female DBA/1 LacJ mice (Jackson Laboratory, Bar Harbor, ME). Mice were monitored daily for onset of disease. Mice developing CIA between 20 and 55 days after immunisation were divided on the day of arthritis onset into different treatment groups: (1) MOIN-sTNF-Rc-Ig treated (n = 23), (2) MFG-LacZ treated (n = 13), and (3) virus-free media treated controls (n = 20). Mice were divided alternately to normalise the onset date.

The first paw (front or hind limb) developing arthritis was given a single periarticular injection of either 1.6×10^7 pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of Dulbecco's modified Eagle's medium (DMEM; group 1) or a similar titre of MFG-LacZ in 100 µl of DMEM (group 2) at disease onset. Mice in virus-free medium control group received 100 µl of DMEM periarticular injection at disease onset. Care was taken to prevent any disruption of skin while administering the injections. We found previously that this dose of MOIN-sTNF-Rc-Ig improved CIA.[20]

Arthritic animals were clinically assessed daily and paw measurements were recorded by a constant tension caliper (Dyer, Lancaster, PA) five times a week for 7 weeks after disease onset and start of treatment. An established arthritis scoring system[21] was used to evaluate disease: 0 = normal appearance and flexion; 1 = erythema and oedema; 2 = visible joint distortion; 3 = ankylosis detectable on flexion. Based on this, each animal could have a maximum score of 12.

Detection of transgene production

An enzyme linked immunosorbent assay (ELISA) was performed to evaluate the expression of human TNF-R (hTNF-R) in serum samples collected at days 0, 3, 7, 14, 21, 35, and 49 after the vector injection. The level of the transgene product was calculated by regression analysis from a standard curve included with each assay. X-gal staining (β-galactosidase assay) was performed on the mouse paws and major organs such as spleen, liver, lungs, heart, and brain collected from the mice receiving MFG-LacZ injections to evaluate LacZ gene expression systemically. Paws and frozen sectioned major organ tissues were fixed in 2% formaldehyde/0.2% glutaraldehyde for 30 minutes before incubation in 1 mg/ml X-gal solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP_{40}, and 0.1% sodium deoxycholate) overnight at 37℃. Positive LacZ transgene expression was determined by blue tissue staining.

Table 1 Primer sequences of Vβ genes used in this study

Target	Primer sequences
Vβ5.1	5′- TGAGTGCCTTGGAGCTAGAG-3′ (upstream)
	5′- GCCTGTCCCGGGAGA-3′ (downstream)
Vβ6	5′- GGGGGTGTCAACTATGCTG-3′ (upstream)
	5′-GAGACCTTGGGTGGAGTCA-3′ (downstream)
Vβ8.1	5′-GTTGGCTTCCCTTTCTCAG-3′ (upstream)
	5′-TGTGTTTGAACCCCTGTCA-3′ (downstream)
Vβ8.2	5′-GCAGCACTGAGAAAGGAGAT-3′ (upstream)
	5′-TTGGTCTGGAGGCCTTG-3′ (downstream)
Vβ8.3	5′-GTGTAGGGGCTGGAAATAC-3′ (upstream)
	5′-CAACAATGAGCCGGCTT-3′ (downstream)
Vβ16	5′-TTACGGGGGGACTATGCT-3′ (upstream)
	5′-GGTGAGTCGTGTCCCTGG-3′ (downstream)

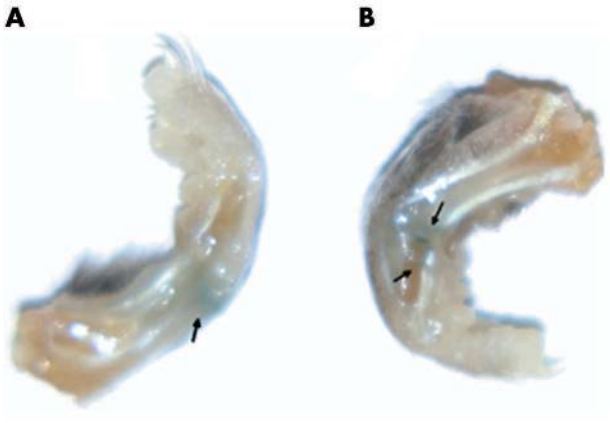

Figure 1 X-gal stain to show the expression of LacZ in the mouse paws given MFG-LacZ. (A) A paw at 3 days; and (B) 21 days after viral infection.

Measurement of interferon γ (IFNγ) responses to CII peptide epitopes

This assay was conducted as previously described.[9] Lymph nodes obtained at 3, 7, 14, 21, and 49 days after killing the arthritic mice were disrupted to yield single cell suspensions, counted, and assayed for viability. Cells were cultured in duplicate at a final concentration of 5×10^6 cells/ml with 100 µl of media containing either 100 µg/ml of immunodominant CII 245–270 peptide, 100 µg/ml of subdominant CII 334–360 peptide, 10 µg/ml concanavalin A, 10 µg/ml lipopolysaccharide, or media alone for 3 days at 37℃. Responses to the two CII peptides were assessed by measuring the IFNγ levels in culture supernatants by the standardised sandwich ELISA technique as previously described,[22] using purified rat antimouse IFNγ antibody and biotin conjugated secondary rat antimouse IFNγ antibody (Pharmingen, San Diego, CA).

Analysis of TCR Vβ gene expression by real time polymerase chain reaction (PCR)

Joints were harvested from arthritic mice post mortem at 3, 7, 21, and 49 days after disease onset. Total RNA was extracted using the manufacturer's instructions (Tel-Test, Friendswood, TX, USA), and possible DNA contaminants were removed by RNase-free DNase (RQ1, Promega, Madison, WI, USA) treatment. RNA samples with a ratio >1.7 at 260 nm and 280 nm, measured by a spectrophotometer (Beckman Instruments, Fullerton, CA, USA), were accepted for TCR Vβ analysis.

Firstly, a semiquantitative conventional PCR was used to screen for the most commonly expressed Vβ genes among a battery of 22 Vβ genes in the paw cDNA samples, generated from control and MOIN-sTNF-Rc-Ig treated mice. Real time reverse transcriptase PCR was performed to assess quantitatively the influence of MOIN-sTNF-Rc-Ig gene transfer on TCR Vβ expression in the paws of the arthritic animals. cDNA was reverse transcribed from 0.5 µg total RNA in a 40 µl reaction mixture containing 1× PCR buffer, 500 µM of each dNTP, 0.5 U/µl RNase inhibitor, 2.5 µM random hexamers, 5.5 mM $MgCl_2$, and 1.25 U/µl MuLV reverse transcriptase (Gibco-BRL, Gaithesburg, MD, USA) in a thermal cycler (Perkin-Elmer Cetus) at 25℃/10 min, 48℃/25 min, and 95℃/5 min. Real time PCR reaction mixtures (25 µl) containing 12.5 µl 2× SYBR Green Master Mix (PE Biosystems, Foster City, CA, USA), 2 µl cDNA, and 400 nM target Vβ (5.1, 6, 8.1, 8.2, 8.3, and 16) gene primer pairs (table 1) were run

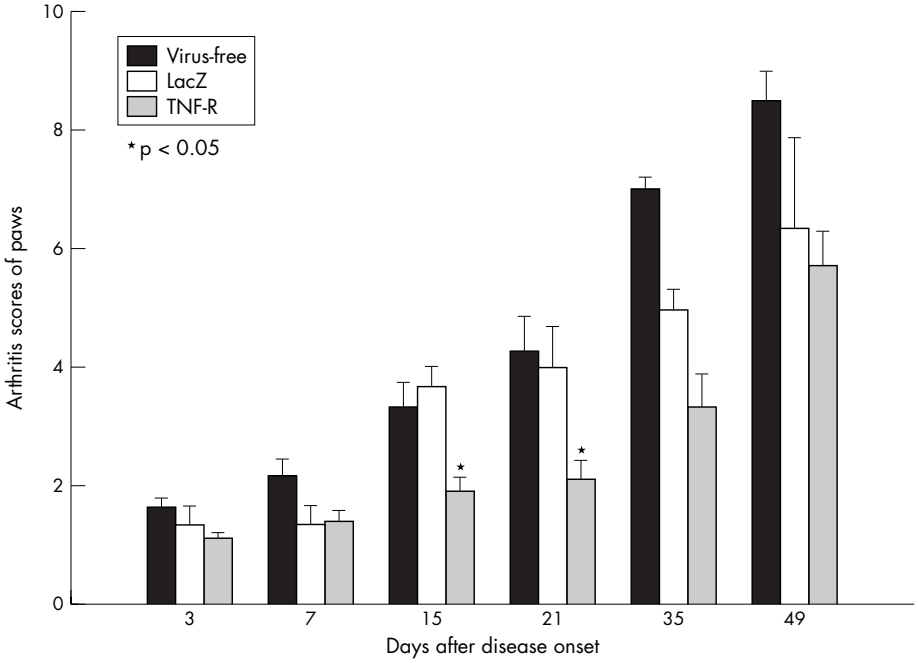

Figure 2 Influence of a single periarticular administration of retrovirus mediated MOIN-sTNF-Rc-Ig on the clinical progression of CIA: disease activity represented by the mean paw score was visually scored up to 49 days after disease onset. Mean paw score is the overall average of the paw scores of all animals in each group. Data are expressed as mean (SEM) for each group for each time point. Significant differences among the groups are indicated ($p<0.05$). The figure shows normalised mean data from three different individual experiments with similar results.

separately in the ABI Prism 7700 Sequence Detector (Applied Biosystems, CA) at 50℃/2 min, 95℃/10 min and followed by 40 cycles of 95℃/15 s; 60℃/min. The machine's built-in software recorded values of the threshold cycle (Ct) that showed a statistically significant increase in reporter dye signals (ΔRn) which were analysed by the v1.7 software (PE Biosystems). Target gene levels were standardised against the housekeeping gene 18S rRNA used as an endogenous control (Invitrogen, Carlsbad, CA, USA). Real time PCR products were run on 1.8% agarose gels to verify the correct amplification of the target gene.

Statistical analysis

Group comparisons between TNF-R treated and control mice were performed by the two tailed independent *t* test using SPSS-PC statistical software (SPSS, Chicago, IL). Comparisons of more than two means were conducted using one way analysis of variance, and p values <0.05 were considered to be significant.

RESULTS

Transgene expression after in vivo gene transfer

Retrovirus coding for the LacZ gene (β-galactosidase) was used as a reporter control. X-gal staining showed positive blue coloured LacZ expressing cells in the MFG-LacZ injected paws at 3 days (fig 1A) and at 21 days (fig 1B) after gene transfer. However, there was no evidence of X-gal positive staining in uninjected paws or remote organs (not shown). The protein level of sTNF-R in the circulation was measured by ELISA at different time points. Three days after disease onset and MOIN-sTNF-Rc-Ig administration, the human sTNF-R level in the circulation was 16.7 pg/ml. This value reduced to 12.7 pg/ml at 7 days after gene transfer and thereafter was undetectable at 14, 21, and 49 days.

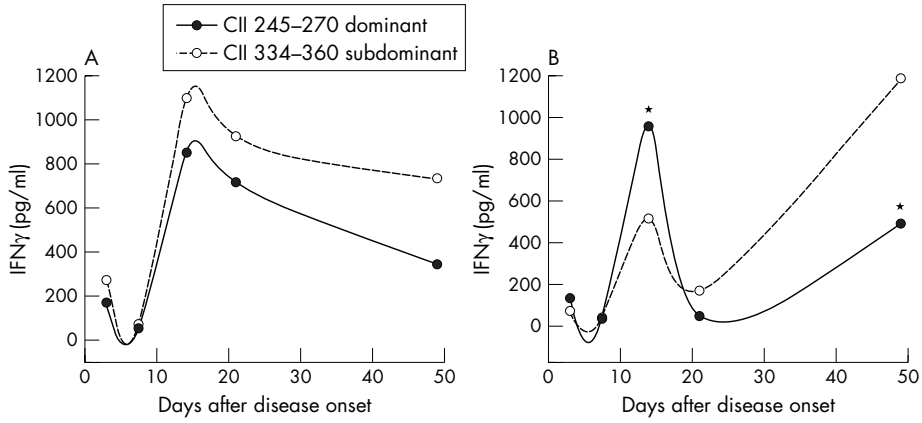

Figure 3 IFNγ responses to CII 245–270 and CII 330–360 peptides after MOIN-sTNF-Rc-Ig gene therapy: mononuclear cells from lymph nodes of (A) MOIN-sTNF-Rc-Ig or (B) media (control) treated mice were cultured with either 100 μg/μl of immunodominant CII 245–270 peptide or with subdominant CII 330–360 peptide for 72 hours. Cells cultured with media and concanavalin A were used as negative and positive controls respectively (not shown). IFNγ levels in resulting culture supernatants were measured by ELISA to evaluate CII peptide epitope usage. TNF-R treated animals showed an enhanced IFNγ response towards the subdominant epitopes rather than the immunodominant epitopes (A), while the normal response to the dominant epitope was observed in the controls (B). Data are expressed as mean for each group at each time point. n = 5 for each time point for both control and TNF-R treated groups. $p<0.05$.

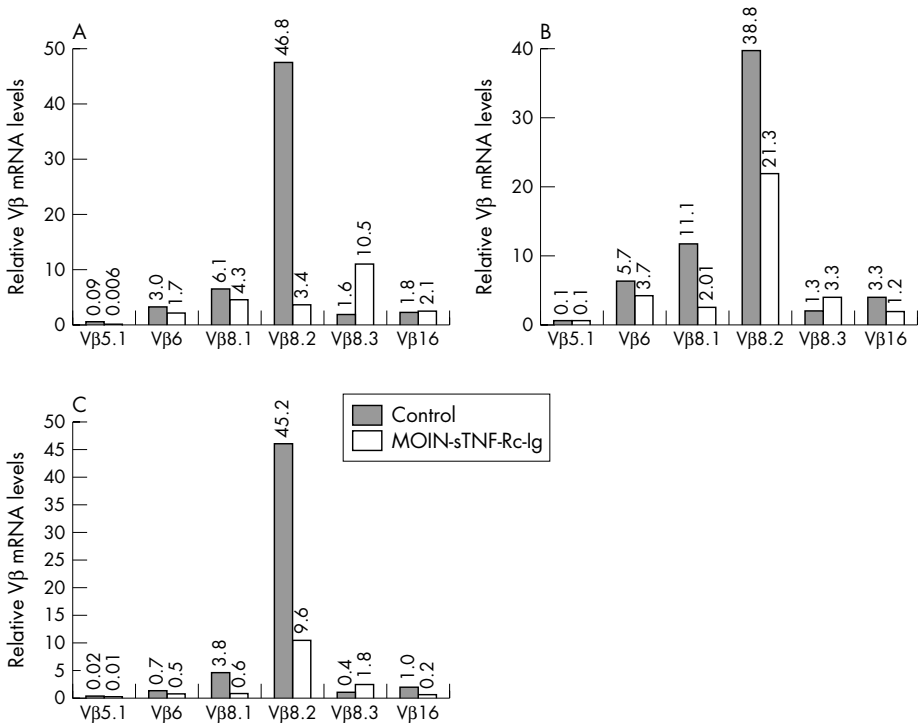

Figure 4 Comparison of Vβ expression levels in joints of arthritic animals: cDNA made from RNA preparations from (A) injected (B) ipsilateral (C) contralateral joints of media or MOIN-sTNF-Rc-Ig injected joints were amplified with different Vβ primer pairs using real time PCR. The figure shows the relative mRNA expression levels of the six Vβ genes quantitatively analysed normalised against the housekeeping gene 18S rRNA used as an internal control. n = 5 for both control and TNF-R treated groups.

Retroviral vector mediated periarticular delivery of TNF-R reduces clinical severity of CIA

On the day of arthritis onset (day 1), CII immunised mice received a single periarticular injection of 1.6×10^7 pfu/ml of either MOIN-sTNF-Rc-Ig or LacZ in 100 µl of media, or an equal volume of media (virus-free control) into the index affected arthritic paw. Thereafter, all arthritic paws (injected and uninjected contralateral and ipsilateral paws) were scored. A significantly lower mean paw score during 15–35 days after disease onset was seen in MOIN-sTNF-Rc-Ig treated DBA/1 mice than in virus-free or LacZ control groups (fig 2, $p<0.05$). Histology showed that there was significantly less severe synovitis; bone/cartilage damage, and overall joint destruction in the injected and uninjected arthritic limbs in the TNF-R treated animals relative to controls (data not shown), which was consistent with our previously published results.[20] These results demonstrated that local periarticular MOIN-sTNF-Rc-Ig delivery had significant effects in ameliorating the overall continuing arthritic disease process up to 35 days after treatment. These experiments were repeated three times for data reproducibility.

TNF-R gene therapy skews the immune response from the immunodominant CII 245–270 peptide to the subdominant CII 334–360 peptide epitope

Culture supernatants of lymph node cells from TNF-R treated mice cultured for 3 days with the subdominant CII 334–360 peptide demonstrated significantly higher levels of IFNγ than lymph node cells cultured with the immunodominant CII 245–270 peptide (fig 3A). This enhanced immune response to the subdominant CII 334–360 peptide in the TNF-R treated animals appeared 3 days after disease onset and gene therapy and was sustained for up to 49 days after disease onset (fig 3A). In the control group, the IFNγ levels in the culture supernatants obtained from lymph node cells cultured with dominant CII 245–270 peptide were higher than levels observed in lymph node cells cultured with the subdominant CII 334–360 peptide at 3–14 days after disease onset (fig 3B). From 21 days after disease onset, these control animals developed an increased IFNγ response towards the subdominant CII 334–360 peptide rather than the immunodominant CII 245–270 peptide. This finding suggests that the TNF-R treated animals showed a diversification of the T cell responses from the immunodominant CII peptide epitopes towards the subdominant CII epitopes, while in control mice the early immune response to the dominant CII peptide was stronger than to the subdominant peptide. However, an increased T cell response to the subdominant CII 334–360 peptide epitopes occurred later in the disease. This response pattern, together with the clinical findings, indicates that an early response to the immunodominant CII epitope may be more critical for determining the severity and progression of disease in CIA.

TNF-R gene therapy suppresses Vβ8.2 use in injected, ipsilateral and contralateral joints

cDNA obtained from paws of arthritic animals was amplified with a battery of 22 Vβ primers by semiquantitative PCR as an initial step to select the predominantly expressed Vβ genes in these samples. Expression of Vβ5.1, Vβ6, Vβ8.1, Vβ8.2, Vβ8.3, and Vβ16 was found to be dominant in the DBA/1 arthritic mice (not shown). Subsequently, real time RT-PCR was used to measure quantitatively potential changes in the TCR Vβ repertoire with respect to these genes. Vβ gene expression measured by real time PCR in the arthritic control and TNF-R treated animals was normalised against housekeeping gene 18S rRNA used as an endogenous control.

Real time PCR analysis demonstrated that Vβ5.1, Vβ6, Vβ8.1, and Vβ8.2 were decreased in the TNF-R injected joints compared with the control joints (fig 4A). Reduction in Vβ8.2 (known to be preferentially used in CIA[12]) was more marked than the changes seen in other Vβ subsets. Interestingly,

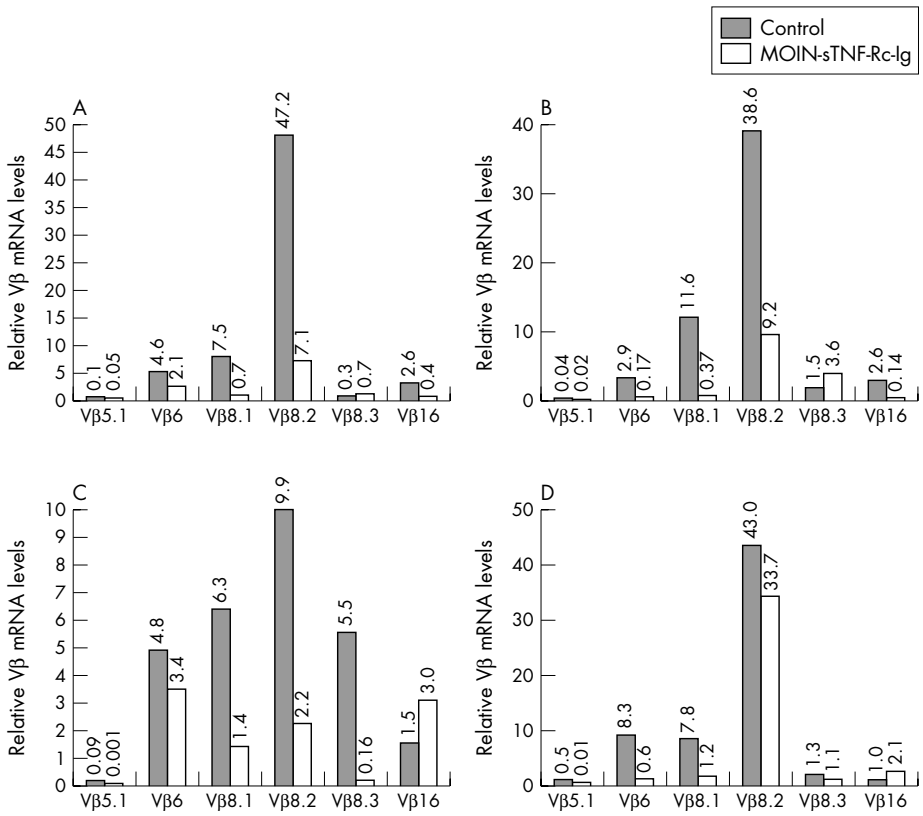

Figure 5 Local TNF-R treatment affects the TCR Vβ repertoire in CIA over time: Vβ mRNA levels in the joints of control and MOIN-sTNF-Rc-Ig treated groups were compared by real time RT-PCR at (A) 3; (B) 7; (C) 21; and (D) 49 days after disease onset. cDNA was obtained from RNA preparations from joints of the two groups of mice. Relative Vβ gene expression in both groups, normalised against the housekeeping gene 18S rRNA used as an internal control, is represented. n = 5 for both control and TNF-R treated groups.

there was also an increase in the use of Vβ8.3 in joints injected with TNF-R, compared with the controls (fig 4A). Similarly to injected joints, the uninjected ipsilateral (fig 4B) and contralateral (fig 4C) paws of the TNF-R treated animals also showed a marked suppression of the Vβ8.2 mRNA levels relative to all the Vβ genes tested in the appropriate control joints. However, skewing towards increased Vβ8.3 usage was more prominent in the injected joints than in the uninjected joints of the treated arthritic mice. These data suggest that local TNF-R treatment may suppress the Vβ8.2 gene that is preferentially activated in CIA.

Additionally, the above data suggest that local periarticular TNF-R administration influenced the Vβ8.2 usage in the uninjected arthritic paws of the TNF-R treated animals. This systemic effect after local TNF-R gene therapy is consistent with our histological data (not shown) and our previously published results,[20] in which periarticular TNF-R administration led to reduced synovitis and decreased pathological bone damage in the arthritic injected and arthritic uninjected joints of treated animals, compared with controls

Temporal effects of periarticular TNF-R gene therapy on the TCR Vβ repertoire

Vβ8.2 expression was markedly lower in the joints of the TNF-R treated animals than in those of media treated controls up to 21 days after disease onset (fig 5). At 49 days after disease onset, Vβ8.2 mRNA expression was still lower in the paws of the treated animals than in the controls (fig 5D), although the differences at this time were not as marked as those in the earlier stages of the disease (figs 5A, B, and C). TNF-R treated animals showed an increased trend for Vβ8.3 use early in the disease (fig 5B). These results indicate that periarticular TNF-R gene therapy led to a trend towards down regulation of the Vβ8.2 gene in the early stages of disease. Later in the disease, the extent of Vβ8.2 suppression diminished, and the increased use of Vβ8.3 seen in treated animals relative to controls was not sustained. The changes in the Vβ repertoire were reversed in the late stages of the disease, and the positive clinical effects were also diminished. This suggests that the reduction of Vβ8.2 seen in early disease may have a role in the amelioration of CIA.

DISCUSSION

Progression of CIA depends predominantly upon a Th1 response.[23 24] TNFα has a crucial role in the immune regulatory network of CIA by up regulating interleukin 1, interleukin 6, and granulocyte monocyte-colony stimulating factor levels, leading to synovial inflammation and joint erosions.[21 25–27] Soluble TNF-R fusion proteins[21 28] and anti-TNFα monoclonal antibody treatment[29–31] have strong anti-inflammatory effects in RA and CIA.[16] The potential of gene therapy as a therapeutic strategy for CIA and RA is gaining considerable importance in order to circumvent the disadvantages of protein based treatments.[32 33] We have focused on the effects of TNF-R gene therapy beyond its anti-inflammatory properties. Retrovirus mediated periarticular delivery of TNF-R at arthritis onset down regulated Th1 driven anti-CII IgG2a antibody levels.[20] Because interplay between both autoantibody and cellular immune responses is crucial to disease pathogenesis,[34] we examined the effects of local TNF-R treatment on cellular immune responses in CIA.

The immunodominant epitope CII 245–270[9 11] can suppress arthritis when used as a tolerogen, and does not stimulate T cells in disease resistant strains. In contrast, the subdominant

epitope CII 334–360 peptide does not generate T cell responses in susceptible DBA/1 mice.[9] We observed a stronger IFNγ immune response in TNF-R treated animals to the subdominant CII 334–360 peptide than to the immunodominant CII 245–270 peptide. Moudgil *et al* demonstrated that diversification of the T cell response from the initial focused dominant epitope to new epitopes, after priming with the whole antigen, led to protection in adjuvant induced arthritis.[35] Because we used the native whole CII molecule to induce CIA, the observed increased IFNγ responses towards subdominant CII peptide epitopes in TNF-R treated mice might possibly be due to a diversification of the T cell response. The differences in the responses towards the subdominant epitopes between the treated and control groups suggest that diversification of the immune response away from the immunodominant epitopes early in arthritis could influence the severity of disease. This differs from other autoimmune disease models, where spreading of T cell responses to new epitopes is associated with aggravation of the continuing disease[35 36] We recently showed that local TNF-R therapy also down regulates the complement binding anti-CII IgG2a antibody levels in treated animals.[20]

Resistance to arthritis observed in the SWR (H-2^q) and RIIIs/J (H-2^r) mice that possess CIA susceptible MHC haplotypes has been attributed to deletions in their Vβ genome.[37–40] Previous studies demonstrate the preferential selection of Vβ6 and Vβ8.2 in the arthritic joints of B10.Q (H-2^q) and DBA/1 (H-2^q) mice with CIA.[41] Vβ5.1, Vβ6, Vβ8.1, Vβ8.2, Vβ8.3, and Vβ16 mRNA were dominantly expressed in the joints of our arthritic animals. Early in the disease, treatment caused suppression of the otherwise predominant Vβ8.2 gene subset in the TNF-R treated animals. Down regulation of Vβ8.2 in both injected and uninjected (contralateral and ipsilateral) joints of arthritic animals suggests that local TNF-R gene therapy exerts systemic effects, consistent with our observations of reduced bone damage in the injected and uninjected arthritic joints of treated mice compared with the controls.[20] Retroviral vectors encoding a marker LacZ gene, administered periarticularly into arthritic joints at CIA onset, had no influence on the clinical disease. The results suggest that increased T cell responses towards the subdominant CII peptide epitope, and away from the immunodominant CII epitopes, together with down regulation of the Vβ8.2 bias associated with CIA, lead to improvement in the clinical arthritis seen in TNF-R treated animals.

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