

# Rheumatoid arthritis synovial T cells regulate transcription of several genes associated with antigen-induced anergy

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Rheumatoid arthritis (RA) is a chronic, inflammatory synovitis whose pathogenesis may involve autoimmune mechanisms. Anergy is a state of T-cell nonresponsiveness characterized by downregulated IL-2 production. Paradoxically, RA T cells are hyporesponsive and proliferate poorly to antigens and mitogens, thus sharing some characteristics with anergic T cells. We analyzed the molecular basis of anergy in cloned human CD4<sup>+</sup> T cells using differential display RT-PCR and subsequently examined the levels of differentially expressed transcripts in RA and, as control, reactive arthritis (ReA) synovium. Several transcriptional events were common to anergic T cells and RA synovium. These included downregulation of *Calmodulin*, which is critical to T-cell activation, and of *cellular apoptosis susceptibility protein*, which may mediate resistance to apoptosis in RA. Transcription of *Calmodulin* in RA synovium was less than 1% of that in ReA and was lower in RA synovial fluid mononuclear cells than in paired PBMCs. Following anti-TNF- $\alpha$  therapy in vivo, RA PBMC *Calmodulin* transcripts increased five- to tenfold. Pharmacological calmodulin blockade in vitro impaired antigen-specific proliferation. These data provide a link between reduced *Calmodulin* transcription and impaired T-cell responsiveness in RA. The identification of transcriptional changes common to anergic and RA synovial T cells should help interpret some of the characteristic RA cellular defects.

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## Introduction

Rheumatoid arthritis (RA) is a chronic disorder of the joints in which the synovium becomes hyperplastic, secondary to proliferation of the lining layer and infiltration by inflammatory cells (1). Its pathogenesis is unknown but linkage to the class II region of the MHC, the presence of circulating autoantibodies, and response to some T-cell targeted therapies, suggest an autoimmune contribution (2). Additionally, RA T cells have an activated phenotype, characterized by upregulated expression of HLA-DR (3). On the other hand, these cells demonstrate features of anergy in vitro and do not proliferate when stimulated with mitogens or recall antigens (4). They are also unusually resistant to apoptosis (5). The intracellular defect(s) responsible for this paradoxical behavior have not been fully elucidated, although chronic TNF- $\alpha$  exposure may be an etiological factor in the anergic phenotype (6), and cytokines may protect the cells from apoptosis (7). It is important to understand these features of the disease, however. The morbidity and mortality of RA are largely secondary to infections and malignancies, reflecting the immunosuppression that is intrinsic to the disease

(8). It is also possible that a defect in the regulation of apoptosis contributes to the pathogenesis of RA (9).

During development of the immune system, a number of processes occur that ensure that mature lymphocytes do not become activated by self-antigens and cause autoimmune disease. Clonal T-cell anergy represents one of these mechanisms (10). Full T-cell activation requires appropriate stimulation through both the specific T-cell receptor for antigen (TCR) and the costimulatory molecule CD28. In contrast, defective costimulation or qualitatively altered TCR signals may result in anergy, whereupon the cell becomes refractory to subsequent activating stimuli. There are a number of in vitro models of anergy induction. These include the presentation of cognate peptide on chemically fixed antigen-presenting cells (APC) (11); the pharmacological blockade of costimulatory interactions between CD28 and B7 (12); and T-cell stimulation by altered peptide ligands, which differ structurally from the cognate ligand by the introduction of a limited number of amino acid substitutions (13). Anergy can also be induced by polyclonal stimulation of T cells using bacterially derived superantigens (14), or anti-CD3 mAb's

(15), or by clonal stimulation with high concentrations of cognate peptide antigen in the presence or absence of professional APCs. Thus, the human CD4<sup>+</sup> T-cell clone, HA1.7, becomes anergic when incubated with high concentrations of its ligand, residues 306-318 of influenza virus hemagglutinin (HA 306-318) (16). Bidirectional T cell-to-T cell antigen presentation may be the critical event when anergy is induced by high concentrations of peptide, and T-cell apoptosis is an alternative outcome under these circumstances (17).

The critical feature of anergic T cells is their inability to produce IL-2 and, hence, their defective autocrine stimulation upon encounter with antigen. RA T cells share this characteristic (18), but the associated signaling defects are not yet fully characterized in either situation. Most work has been performed in anergy induced by defective costimulation in which there is evidence for defective coupling of the TCR to downstream signaling pathways with a block in ERK-1, ERK-2, and p21ras activation (19, 20). On the other hand, anergy induced by exposure to high concentrations of cognate peptide may differ from other models in that predominant defects in the calcium/calcineurin-signaling pathway have been demonstrated (21, 22), although the Ras/MAP kinase pathway has not been extensively characterized in this model. This again is reminiscent of RA T cells, in which defective calcium signaling has also been clearly demonstrated (23, 24). Although more marked in the synovium, calcium defects are also present in RA peripheral blood (25).

A clearer understanding of the molecular events associated with anergy induction on the one hand and the behavior of RA T cells on the other may suggest novel therapeutic strategies for autoimmune diseases in general and RA in particular. A number of techniques now permit a comprehensive comparison of gene transcription between tissues or cells in different states of differentiation. These include differential cDNA hybridization using cDNA microarrays (26), differential display RT-PCR (DDRT-PCR) (27), and serial analysis of gene expression (SAGE) (28). We have used DDRT-PCR to analyze changes in gene transcription in the human T-cell clone HA1.7 during anergy induction by its cognate peptide HA 306-318. In this model, APC are not present during anergy induction, considerably simplifying data interpretation. We subsequently used a differential hybridization technique to examine levels of anergy-associated transcripts in RA and, as a control, in reactive arthritis (ReA) synovium. The latter is a self-limiting condition, not characterized by defects in T-cell activation or apoptosis (29). We thereby identified transcriptional changes common to both anergic T cells and to RA synovial T cells, which may provide clues to the underlying cellular defects in RA.

## Methods

**Maintenance of the T-cell clone and induction of anergy.** The human CD4<sup>+</sup> T-cell clone HA1.7, specific for HA 306-318 (16), was maintained (10<sup>6</sup> cells/ml) in RPMI-1640

supplemented with 5% human AB serum (Sigma-Aldrich Co., Poole, United Kingdom), penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (2 mM). It was stimulated every 7 days with 1 µg/ml HA 306-318 (Sigma-Genosys, Cambridge, United Kingdom) and irradiated (30 Gy) HLA-DR1\*0101-positive PBMCs as a source of APC and every 3 to 4 days with IL-2 (10% Lymphocult T; Biotest UK Ltd., Solihull, United Kingdom). Anergy was induced as described previously (16). After a 7-day rest period during which cells were exposed to neither peptide nor IL-2, cells were washed twice in PBS and incubated for 24 hours with HA 306-318 (50 µg/ml), but without APC.

Aliquots of 10<sup>6</sup> cells were removed at 2 and 24 hours, and cell pellets were snap-frozen, then stored at -70°C for subsequent RNA extraction. An additional aliquot of cells taken at 24 hours was washed thoroughly and tested for its ability to respond to an immunogenic challenge. These cells (10<sup>4</sup>/well) were incubated with irradiated APC (10<sup>4</sup>/well), HA 306-318 (1 µg/ml), a combination of both, or IL-2. After 48 hours, [<sup>3</sup>H]thymidine (Amersham, Little Chalfont, United Kingdom) was added (1 mCi/ml) and the culture harvested 18 hours later onto fiberglass filters. Proliferation was measured as [<sup>3</sup>H]thymidine incorporation by liquid-scintillation counting.

**RNA extraction and DDRT-PCR.** Total RNA was isolated from cell pellets using the TRIzol reagent (Life Technologies Inc., Glasgow, United Kingdom) and cellular debris removed as the lower phase after chloroform extraction. The upper, aqueous phase was precipitated with isopropanol and the pellet briefly air-dried and resuspended in diethyl pyrocarbonate-treated water (100 µl). The isolated RNA was quantified spectrophotometrically and then treated with 10 U DNase I (Pharmacia Biotech AB, Uppsala, Sweden) and 33 U RNasin (Promega UK Ltd., Southampton, United Kingdom) at 37°C for 30 minutes, then at 65°C for 10 minutes for denaturation of the nuclease. Aliquots (0.6 µg) of total RNA were incubated at 65°C for 10 minutes with 6 µl of one of four oligonucleotide primer mixtures [d(T)<sub>12</sub>VG, d(T)<sub>12</sub>VC, d(T)<sub>12</sub>VA, d(T)<sub>12</sub>VT, where V = A, C, or G] at an original concentration of 10 mM, then placed on ice. Four hundred units Superscript II reverse transcriptase (Life Technologies Inc.) containing RT buffer (25 mM Tris HCl, pH 8.3, 37.5 mM KCl, 1.5 mM MgCl<sub>2</sub>), 10 mM DTT, and dNTPs (20 mM each) was added to make a total volume of 40 µl. The cDNA synthesis was carried out at 42°C for 1 hour. One microliter of each cDNA mixture was added to microplate wells, each containing the original primer mixture (1 mM final) and one of 25 random sequence oligonucleotide decamers (0.2 mM final) in a total volume of 10 µl containing PCR buffer (10 mM Tris HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1% Triton X-100), dNTPs (2 mM each), 0.5 U of *Taq* DNA polymerase (Promega UK Ltd.), and 0.5 µl α-[<sup>35</sup>S]-dATP (Amersham; 1,200 Ci/mmol). The reaction mixture was subjected to 40 cycles in a PHC-3 thermal cycler (Techne Ltd., Cam-

bridge, United Kingdom), where each cycle consisted of 94°C for 30 seconds, 41°C [for d(T)<sub>12</sub>VA and d(T)<sub>12</sub>VT primers] or 42°C [for d(T)<sub>12</sub>VG and d(T)<sub>12</sub>VC primers] for 2 minutes, and 72°C for 30 seconds. The reaction was stopped and the cDNA products from each analysis separated by electrophoresis through a 6% denaturing polyacrylamide gel. The gel was dried, and the bands were visualized after autoradiography. Each DDRT-PCR reaction was performed and analyzed in duplicate. Variations in comparative band intensity that correspond to differentially expressed transcripts were identified by visual inspection of gels and categorized into those that demonstrated absolute or relative changes in gene transcription.

**DNA cloning and sequencing.** Gel slices corresponding to cDNA fragments were excised and placed in microcentrifuge tubes containing 100 µl of water. The contents of each tube were boiled for 15 minutes and the solid debris pelleted by centrifugation. Glycogen (50 ng; Roche Diagnostics Ltd., Lewes, United Kingdom) was added to the supernatant, the DNA precipitated with ethanol, and resuspended in 10 µl of water. Reamplification of each cDNA fragment was then performed in a 40-µl reaction with 4 µl of cDNA template and the same set of oligonucleotide primers as used originally to isolate the fragment in the presence of 0.05U *Taq* DNA polymerase. The dNTPs were used at 20 µM, no radioactive isotope was added, and the same cycling parameters were used as previously.

After PCR, double-stranded cDNA was precipitated with ethanol and resuspended in sterile water (10 µl). Each PCR product was then ligated into the plasmid vector pCR2.1 (Invitrogen BV, Groningen, The Netherlands) and the mixture transformed into *Escherichia coli* INV-F “one-shot” competent cells (Invitrogen BV). Colonies were selected on LB-agar plates supplemented with ampicillin (100 mg/ml) and X-gal (1.6 mg/plate). Plasmid DNA was extracted from the bacterial colonies using the alkaline lysis method, and the presence of cloned insert DNA was verified by PCR. The oligonucleotide primers M13REV (dTCA-CACAGGAAACAGCTATGAC) and M13FOR (dCAC-GACGTTGTAACGACGGCCAG) were used in 40 cycles of the PCR where each cycle consisted of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Products were separated by electrophoresis on a 2% agarose gel, and DNA was visualized by using ethidium bromide staining under ultraviolet illumination. Sequencing of plasmid DNA was performed using the fmol DNA cycle sequencing kit (Promega UK Ltd.) with either the M13REV or M13FOR primer, end-labeled with  $\gamma$ -[<sup>32</sup>P]-ATP (10 mCi/ml; Amersham) and T4 polynucleotide kinase. Extension/termination reactions were performed in microtiter well plates with sequencing grade *Taq* DNA polymerase for 30 cycles at 95°C for 30 seconds and 70°C for 30 seconds. Sequencing reaction products were separated by electrophoresis through denaturing polyacrylamide gels and visualized using autoradiography. Sequenced

DNA was compared with the GenBank nucleotide database using the basic local alignment search tool (BLAST) algorithm program.

**Manufacture of cDNA arrays.** Specific oligonucleotide pairs for each of the cDNAs originally highlighted in the differential display were used to amplify cDNA fragments of approximately 200 bp by PCR. The cDNA fragments were purified using spin columns (Roche Diagnostics Ltd.) and the identity of the double-stranded cDNA verified by cycle sequencing in the presence of one of the PCR oligonucleotides. Sequence-verified cDNA (2 µg) was immobilized onto positively charged nylon-filter membranes (Roche Diagnostics Ltd.) using the 96-well format Hybri-Dot Manifold apparatus (Life Technologies Inc.) according to the manufacturer’s instructions.

**Probe labeling and hybridizations.** Total RNA (5 µg) extracted from resting or anergic T cells after 2 or 24 hours was incubated with 1.5 µg oligo (dT)<sub>15</sub>-primer at 95°C for 5 minutes. For radiolabeled cDNA synthesis, 400 U Superscript II reverse transcriptase (Life Technologies Inc.) containing RT buffer, 10 mM DTT, dNTP mix (50 µM dATP, dGTP, dTTP, and 0.5 µM dCTP), 40 U RNasin (Roche Diagnostics Ltd.), and 4 µl  $\alpha$ -[<sup>33</sup>P]-dCTP (2500Ci/mmol; Amersham) were added and left at 42°C for 90 minutes. The labeled cDNAs were purified using Sephadex G-50 columns (Pharmacia Biotech AB) and denatured at 100°C for 10 minutes in the presence of 10 µg human Cot1 DNA (Roche Diagnostics Ltd.) and 5 µg poly(dA) (Pharmacia Biotech AB). The complex probes were allowed to cool slowly to 45°C over 90 minutes.

Filters were prehybridized in DIG Easy Hyb buffer (Roche Diagnostics Ltd.) for 1 hour. The radioactive probes were added to the respective filters with fresh DIG Easy Hyb buffer, and hybridization was carried out at 45°C overnight. The filters were washed four times for 15 minutes at 65°C in buffer containing 0.1% SDS and 0.2 × SSC. Membranes were exposed for 3 days to Phosphor Screens (Molecular Dynamics, Sunnyvale, California, USA) and the images scanned using a Storm Scanner (Molecular Dynamics). Images were analyzed by ImageQuant software v4.2a (Molecular Dynamics), which provided a quantitative measurement corresponding directly to the intensity of the hybridization signal by assigning a pixel value to each dot on the grid. For each gene at each time point and condition (anergy or control), the pixel value was initially standardized to that of *GAPDH*. The standardized value for anergic cells was then related to that for control cells at the same point.

**Synthesis of RA and ReA synovium cDNA probes.** Synovial biopsies were obtained by arthroscopy of the knee joint from five patients with RA and five patients with ReA. RA was diagnosed according to the 1987 American Rheumatism Association criteria (30), and ReA was defined as mono- or oligoarthritis following a gastrointestinal or urogenital infection. Patients were within 12 months of symptom onset at the time of

biopsy, which was obtained after the approval of the local research ethics committee and with the patients' informed consent. Total RNA was extracted using the Hybaid Ribolyser Kit, and cDNA was synthesized using the Advantage cDNA PCR Kit (BD Clontech UK, Basingstoke, United Kingdom). Equal amounts of cDNA from each biopsy specimen were pooled to give rise to a combined cDNA population for each disease. This cDNA was radiolabeled using the random priming method with 8  $\mu$ l  $\alpha$ - $^{32}$ P]-dCTP (2500Ci/mmol; Amersham), according to the manufacturer's protocol. Hybridization to cDNA arrays was performed as described above using pooled cDNA.

**Real-time PCR quantification.** For individual synovial biopsy specimens, nonradiolabeled first-strand cDNA was synthesized as described above. For peripheral blood and synovial fluid samples, mononuclear cells were recovered using a Ficoll step gradient. Total RNA was extracted from the mononuclear cells, quantified, and nonradiolabeled first-strand cDNA synthesized as described previously with oligo(dT)<sub>15</sub> and Superscript II reverse transcriptase. We utilized a real-time PCR protocol (31) incorporating the ABI 7700 sequence detection system to facilitate SYBR-green fluorescence quantification (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). Primers for *GAPDH* (as reference gene), *Calmodulin*, *CAS*, and *BF626661* were designed to amplify PCR fragments of 80  $\pm$  5 bp long. PCR conditions for each cDNA were intensively optimized with regard to primer concentration (50–900 nM), absence of primer dimer formation, and efficiency of amplification, including equivalent amplification efficiency of target gene and *GAPDH*. All reactions used an annealing/extension temperature of 59°C and denaturation temperature of 85°C. The cDNA concentrations in different samples were then quantified by performing real-time PCR and comparing the threshold cycle for *GAPDH* and each target gene to achieve 50% maximal amplification. Primer sequences were as follows: *GAPDH* forward: d AAC AGC GAC ACC CAC TCC TC; *GAPDH* reverse: d CAT ACC AGG AAA TGA GCT TGA CAA; *Calmodulin* forward: d TAC CTT CTG TCC ACA CAC ACA AAA T; *Calmodulin* reverse: d TTT ATA TTG TAA AAC TGA TGT GTA GCT TGA TC; *CAS* forward: TGC AAT TCA ATT TCT GGC TTC A; *CAS* reverse: GAT ACT TGT CAG CGT GTT CTG GTC; *BF626661* forward: CGA TCT GGA TTC TCA TTT TCT GTT T; *BF626661* reverse: AGG AGG CTG AGT TTT TCT TCA TAT ACTT.

**Comparison of gene transcription before and after anti-TNF- $\alpha$  therapy.** PBMCs were prepared from six RA patients before therapy with the chimeric anti-TNF- $\alpha$  mAb, Infliximab (32). Repeat samples were prepared 2 weeks later, when each patient had improved symptomatically. *Calmodulin* and *CAS* transcription was measured by real-time PCR quantification in these samples as described.

**Inhibition of PBMC proliferation by pharmacological blockade of calmodulin.** PBMCs were resuspended at 10<sup>6</sup>/ml in RPMI-1640 supplemented with 5% human AB serum

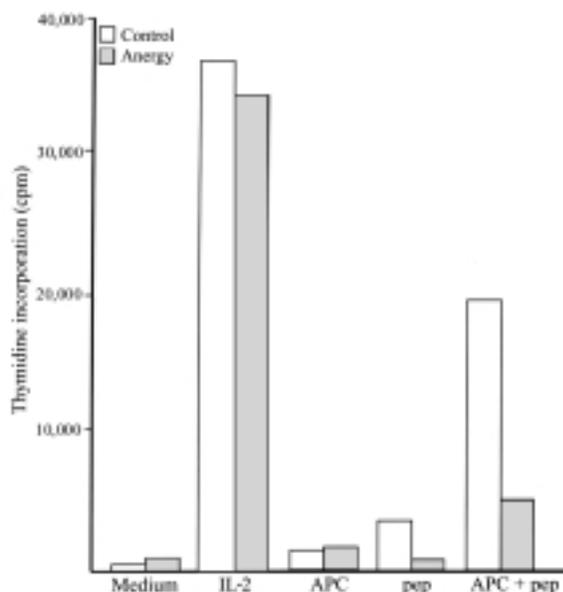
(Sigma-Aldrich Co.), penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (2 mM). On hundred microliters of cell suspension was plated into triplicate wells of a 96-well plate. Wells were made up to a final volume of 200  $\mu$ l with media alone or media containing trifluoperazine (TFP; Sigma-Aldrich Co.) at a final concentration of 1–30  $\mu$ M (33). TFP is a phenothiazine derivative that suppresses the function of the intracellular Ca<sup>2+</sup>-calmodulin complex but does not affect calcium entry into the cell (33). Cells were then incubated for 6 days at 37°C with or without the addition of 10  $\mu$ g/ml tuberculin purified protein derivative (PPD), and pulsed for the final 18 hours with 1 mCi/ml [<sup>3</sup>H]thymidine (Amersham). Proliferation was measured as [<sup>3</sup>H]thymidine incorporation by liquid scintillation counting. The proliferation index was calculated as the ratio of counts between stimulated and unstimulated cells.

## Results

**Induction of anergy in the human CD4<sup>+</sup> T-cell clone HA1.7.** The human CD4<sup>+</sup> T-cell clone, HA1.7, was incubated with peptide HA 306-318 (50  $\mu$ g/ml) for 24 hours. The anergic state of the cells was tested subsequently by an immunogenic challenge (Figure 1). Peptide-preincubated cells did not proliferate when stimulated with HA 306-318 in the presence of APC, but their viability was confirmed by responsiveness to exogenous IL-2. In contrast, control cells proliferated to both antigenic challenge and to IL-2. The inability of peptide-preincubated cells to respond to an immunogenic challenge confirmed their anergic state.

**Multiple transcriptional changes occur during anergy induction.** To identify transcriptional events occurring during the early stages of anergy induction, we used DDRT-PCR to compare RNA extracted from cells after 2 hours of incubation with peptide and after 2 hours' incubation in medium alone (control incubation). Figure 2 provides a summary of our overall strategy. We identified 79 bands that differed absolutely or quantitatively between the two conditions: 56 of these were upregulated by peptide incubation and 23 were downregulated. We decided to focus on the 40 upregulated and 12 downregulated bands representing absolute differences in transcription, and the DNA contained in these was extracted, reamplified, and cloned. Because a DDRT-PCR band often comprises more than one cDNA moiety (34), three subclones were analyzed from each clonable band. Twelve bands could not be cloned, and we ultimately identified 55 different cDNA sequences. Twenty-two corresponded to known genes and 33 were expressed sequence tags (ESTs), of which 20 gave no database matches at the nucleotide level.

**Confirmation of differential gene transcription and analysis of transcription at 24 hours.** Differential transcription was confirmed by a reverse Northern dot blot technique. PCR was used to amplify a cDNA fragment of approximately 200 bp from each of the 55 sequences and of the housekeeping transcript *GAPDH*. PCR products were

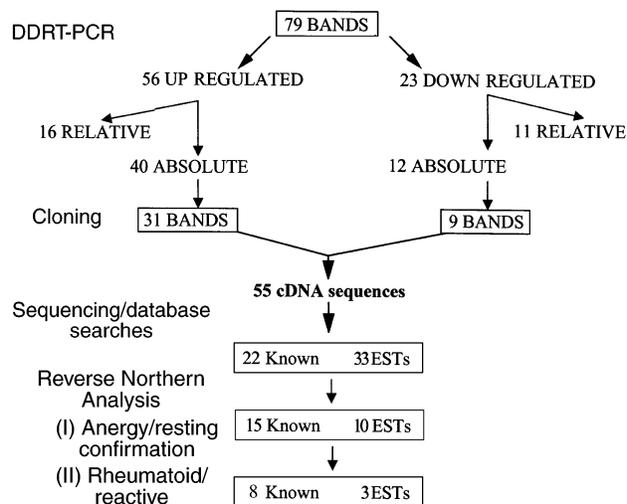


**Figure 1**  
Induction of anergy in the human CD4<sup>+</sup> T-cell clone HA1.7. HA1.7 (10<sup>6</sup>/ml) was incubated with peptide HA 306-318 (50 μg/ml) for 24 hours or with medium alone. The anergic state of the cells was subsequently tested by an immunogenic challenge. Peptide-preincubated cells (dark bars, 10<sup>4</sup>/well) did not proliferate when stimulated with HA 306-318 in the presence of APC, but their viability was confirmed by responsiveness to exogenous IL-2. In contrast, control cells (light bars, 10<sup>4</sup>/well) proliferated after either antigenic challenge or IL-2. Neither control nor anergic cells proliferated when incubated with medium, APC, or peptide alone. The figure shows proliferation after 48 hours of stimulation, measured as [<sup>3</sup>H]thymidine incorporation.

sequence verified and then immobilized onto duplicate nylon-membrane filters. Filters were subsequently hybridized to radiolabeled first-strand cDNAs prepared from peptide-incubated or control cells after 2 or 24 hours of culture. Figure 3 shows an example of a filter representing the 2-hour time point. Of the 55 sequences, 35 gave hybridization signals during reverse Northern analysis. Transcription of target genes was initially related to that of *GAPDH*, before a ratio was obtained for relative gene transcription between anergic and control resting T cells. In all, 25 (45%) of our DDRT-PCR-derived cDNAs were confirmed to represent differentially expressed transcripts as determined by the reverse Northern technique. It was noteworthy that the hybridization-based technique gave relatively modest differences in gene transcription compared with the large transcriptional differences originally identified by DDRT-PCR analysis. There are a number of factors that may contribute to this apparent discrepancy. These include the amplification step that is intrinsic to the DDRT-PCR procedure as well as the complexity of the bands in DDRT-PCR, which often contain several distinct cDNAs, including some false positives.

*Transcription of anergy-related transcripts in inflamed synovium.* We next compared transcription of the genes identified as anergy related in synovial tissue from

patients in the early stages of RA and ReA. In their initial stages, RA and ReA resemble each other histologically, although RA synovium may contain more T cells than ReA (29). The latter resolves rapidly, however, and serves as a disease control when investigating RA. Radiolabeled first-strand cDNAs from RA and ReA synovial biopsies were used to probe duplicate filters representing differentially transcribed genes. Of the 25 confirmed anergy-related transcripts, 11 were expressed in synovial tissues (Table 1). These were generally more abundant in RA or equally transcribed in the two conditions, perhaps corresponding to the generally higher number of T cells in RA synovium. Three genes were clearly downregulated in RA, however, in comparison with ReA. These were *CAS*, *Calmodulin*, and *BF626661*. *CAS* encodes for the cellular apoptosis susceptibility protein that plays a dual role in promoting growth or apoptosis at the mitotic G2/M checkpoint (35). Apoptosis is known to be defective in RA synovial T cells (5), whereas anergic T cells are unduly susceptible to apoptosis (17) and upregulate *CAS* (Table 1). *Calmodulin* is a calcium-binding protein that binds intracellular free calcium and regulates other calcium-dependent proteins (33). *BF626661* was also downregulated in both RA and in anergy. Of these three genes, the most marked difference was seen with *Calmodulin*, whose transcription in RA was less than 1% of that in ReA. In contrast, transcription of *BF626661* was tenfold lower in RA, and there was only a fourfold difference in *CAS* transcription (Table 1). We also studied *Calmodulin* and *CAS* transcript levels in individual biopsies from RA and ReA patients using real-time PCR quantification and confirmed the large difference in *Calmodulin* transcription and modest difference in *CAS* transcription as well as the paucity of *CAS* transcripts in both types of



**Figure 2**  
A schematic of the DDRT-PCR and confirmatory analysis. After differential display (DDRT-PCR) analysis of T cells undergoing anergy induction, 55 distinct cDNA sequences were isolated. These formed the basis of the array that was used to confirm differential gene expression and compare transcription in RA and ReA synovium.

synovitis (Figure 4). *Calmodulin* was also downregulated during anergy induction, potentially linking the anergic state in HA1.7 with the hyporesponsiveness of RA T cells. Genes that were equally transcribed or upregulated in RA compared with ReA included those coding for PMA-ionophore-linked activation factor of T cells (PILOT), T-plastin, calumenin, eucaryotic elongation factor 1- $\delta$ , and ADP ribosylation-like factor-1 (ARL-1). ESTs corresponding to *BF626660* and *BF626663* were both relatively abundant in RA compared to ReA (Table 1).

**Transcription of *Calmodulin* and *BF626661* in synovial fluid mononuclear cells.** Whole synovium contains a variety of cell types including fibroblasts, B cells, and plasma cells. We therefore compared transcription of *Calmodulin* and *BF626661* in matched synovial fluid mononuclear cells (SFMCs) and PBMCs from four patients with RA. We employed real-time quantitative PCR, relating transcription of our target genes to that of the housekeeping gene *GAPDH*. Figure 5 illustrates pooled data for four patients and confirms that both

*Calmodulin* and *BF626661* are downregulated in SFMCs compared with PBMCs. This variation between transcript levels in RA SFMCs and PBMCs is consistent with the proliferative and signaling defects previously documented in these cell types (4, 23–25).

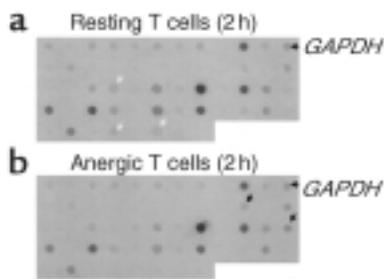
**Upregulation of *Calmodulin* transcription following anti-TNF- $\alpha$  therapy of RA.** The hyporesponsiveness of RA PBMCs is reduced after TNF- $\alpha$  blockade (36). We therefore hypothesized that, if calmodulin is related to the hyporesponsiveness of RA T cells, its transcription should increase in PBMCs after therapeutic TNF- $\alpha$  blockade. This is illustrated in Figure 6. Six patients were studied using quantitative real-time PCR. In every case *Calmodulin* transcription increased 2 weeks after a single infusion of 3 mg/kg Infliximab three- to tenfold. In contrast, transcription of *CAS* demonstrated no consistent transcriptional variation after therapy.

***Calmodulin* inhibition impairs antigen-specific proliferation of PBMCs.** The data above suggest that the hyporesponsiveness of RA T cells may be related to low levels

**Table 1**  
Transcripts common to anergic T cells and inflamed synovium

Gene ID Database Match	Gene product description/function	AN2/CO2	AN24/CO24	ReA	RA
<i>ARL-1</i> L28997	ADP ribosylation like factor-1. Formation of high-affinity membrane sites for the heterotetrameric AP-1 adaptor complex on the Golgi apparatus. Weakly stimulates phospholipase D.	2.68	0.89	0.16	0.82
<i>PILOT</i> X63741	PMA-ionophore-linked activation factor of T cells. Immediate-early transcription factor that increases FasL promoter activity. Contains a proline-rich region and three zinc finger DNA-binding domains.	2.11	0.99	1.37	1.54
<i>EEF1<math>\delta</math></i> Z21507	Eucaryotic elongation factor 1- $\delta$ . Mediates the binding of aminoacyl tRNA to the ribosome.	2.03	5.1	5.95	7.87
<i>ACLP</i> AF053944	Aortic carboxypeptidase-like protein. A non-nuclear protein important for vascular smooth muscle differentiation.	1.78	1.01	22.6	19.1
<i>CAS</i> U33286	Cellular apoptosis susceptibility gene. Dual role promoting growth or apoptosis at the mitotic G2/M checkpoint. Inhibition results in resistance to TNF- $\alpha$ -mediated apoptosis.	1.63	1.36	0.30	0.08
<i>T-plastin</i> M22299	Calcium-binding protein containing a calmodulin-like domain and an actin-binding domain. Potential role in signal transduction from membrane to cytoskeleton by modification of actin filament organization.	1.22	1.07	0.19	0.18
<i>GAPDH</i>		1	1	1	1
<i>Calmodulin</i> M27319	Calcium-binding protein that binds free intracellular calcium and regulates other calcium-dependent proteins. Role in several events associated with T-cell activation.	0.47	1.00	1.18	0.01
<i>Calumenin</i> AF013759	Calcium-binding protein predominantly localized to the endoplasmic reticulum and Golgi apparatus.	0.37	1.19	0.33	0.79
<i>BF626660</i>	EST.	0.58	1.09	0.28	0.75
<i>BF626661</i>	EST.	0.56	1.89	2.38	0.27
<i>BF626663</i>	EST.	0.29	0.50	0.16	0.45

Quantitative expression of eight known genes and three ESTs in anergic and resting T cells and in ReA and RA synovium. Hybridization filters were prepared to represent cDNA fragments of differentially expressed transcripts and the housekeeping transcript *GAPDH*. Radiolabeled cDNA probes were prepared from total RNA extracted from resting or anergic T cells or from ReA or RA synovium and hybridized to the filters, which were subsequently exposed for 3 days to Phosphor Screens. The resulting images were scanned using a Storm Scanner and analyzed as described in Methods. For all analyses, the hybridization signal was standardized against the corresponding pixel value for *GAPDH*. This is the value depicted in the table for synovial samples. T-cell samples are reported as the ratio of signals obtained from anergic to control cell probes. Thus, AN2/CO2 reflects the ratio of standardized values for anergic and control T cells after 2 hours' incubation, and AN24/CO24 reflects the ratio after 24 hours' incubation.



**Figure 3**  
Confirmation of DDRT-PCR results by reverse Northern blotting analysis. Replicate nylon-filter membranes were prepared, comprising 200-bp cDNA fragments (2  $\mu$ g of each) representing each differentially expressed transcript. Radiolabeled cDNA probes were prepared from 5  $\mu$ g total RNA extracted from (a) resting or (b) anergic T cells. The probes were hybridized to the filters overnight, and, after extensive washing, the filters were exposed for 3 days to Phosphor Screens. Transcripts downregulated during anergy induction are highlighted by white arrows. Transcripts upregulated are highlighted by black arrows. The blot represents all 55 transcripts that were considered to be differentially expressed after the initial DDRT-PCR screen, as well as the housekeeping control transcript, *GAPDH*.

of calmodulin. We therefore studied the effect of calmodulin blockade on antigen-specific proliferation of PBMCs from four control individuals. In each case, the pharmacological calmodulin antagonist TFP inhibited proliferation of T cells to PPD in a dose-dependent manner (Figure 7).

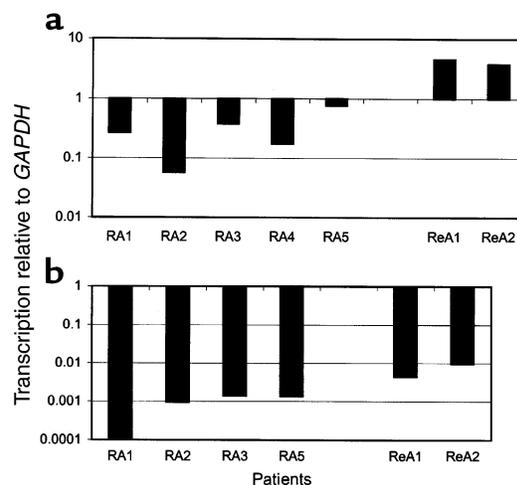
### Discussion

Synovial fluid and peripheral blood T cells in RA exhibit a number of unusual characteristics. First, despite the fact that RA is an autoimmune disease, they are hyporesponsive to both mitogens and to recall antigens (4). Second, despite high levels of proapoptotic cytokines, such as TNF- $\alpha$ , synovial fluid T cells are unduly resistant to apoptosis (5). Synoviocytes themselves also exhibit an apoptotic defect, which may contribute to the pathogenesis of the disease (9). Over the past two decades, the perception of RA as a disabling but non-fatal condition has changed dramatically, and it is now recognized to be a condition associated with considerable mortality. Death is frequently secondary to infection, and there is also an increased risk of malignancy, particularly of the immune system (8). Parallels can be drawn between the T-cell hyporesponsiveness and apoptotic defects on the one hand, and infection and malignancy risk on the other, which make it important for us to understand the basic cellular defects in RA.

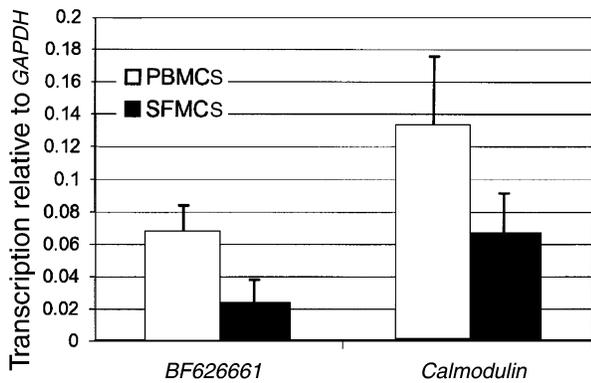
A number of powerful methods are now available to compare transcription between different tissues or between cells in different states of differentiation. SAGE (28) provides a quantitative analysis of total gene transcription but is less suitable for rapidly comparing multiple similar samples and gives minimal sequence information for each cDNA (typically 9–13 bp). cDNA microarrays (26) provide rapid analytical methods, but

may be biased toward the identification of high-abundance transcripts. In contrast, DDRT-PCR frequently identifies rare species and provides around 200 bp of 3' sequence information. We have applied DDRT-PCR to a clonal T-cell population in its resting and anergic state and subsequently used a hybridization approach to compare the transcription of anergy-related genes in RA and ReA synovial tissue. Our rationale was that because RA T cells share some characteristics with anergic T cells, an analysis of the latter may provide insights into molecular defects in the former. There are a number of *in vitro* models of anergy, but peptide-induced anergy provides two significant advantages. First, APC are not required, which greatly facilitates interpretation of the DDRT-PCR data. Second, in contrast to other anergy models, calcium-signaling pathways may be defective in peptide-induced anergy (21, 22) as they are in RA synovial T cells (23–25). We chose two time points to examine differential gene transcription: at 2 hours to represent anergy induction and at 24 hours to represent established anergy. The trigger of the anergic phenotype in RA is unknown, and it is unclear which of these time points is most relevant to the differentiation state of synovial T cells. On the other hand, the synovium contains a heterogeneous population of T cells at different stages of differentiation, and both time points may therefore be pertinent.

We identified a number of differentially regulated transcripts in anergic T cells, which were also present in RA and ReA synovium (Table 1). ReA serves as a useful disease control when investigating RA because the two conditions are histologically similar in their early phase. The former resolves without causing permanent damage, however, whereas the latter persists. From the



**Figure 4**  
*Calmodulin* and *CAS* transcription in individual biopsies. First-strand cDNA was prepared from synovial tissue from individuals with RA and with ReA. Real-time PCR was used to quantitate transcription of (a) *Calmodulin* and (b) *CAS* relative to the housekeeping transcript *GAPDH*.  $P = 0.002$  for *Calmodulin* and 0.035 for *CAS*, for RA vs. ReA (unpaired *t* test).



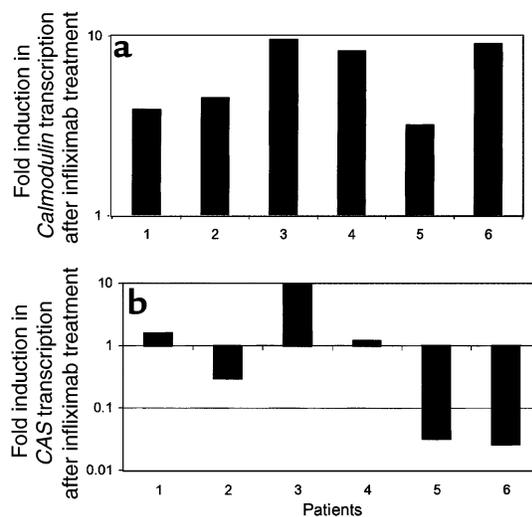
**Figure 5** Quantitation of gene transcription in mononuclear cells. First-strand cDNA was prepared from PBMCs and SFMCs from RA patients. Real-time PCR was used to quantitate transcription of *Calmodulin* and *BF626661* relative to *GAPDH* as a housekeeping transcript. The figure shows the ratio of target gene to *GAPDH* transcription in SFMCs and PBMCs (mean and SD of four patients).  $P = 0.050$  for *Calmodulin* and  $0.026$  for *BF626661* for PBMC vs. SFMC (paired *t* test).

perspective of the current analysis, some reports suggest that T cells are more numerous in RA than in ReA synovium (29). Additionally, synovium is a heterogeneous tissue, complicating the analysis of transcripts which are T-cell specific. We therefore focused our attention on three energy-related transcripts that demonstrated reduced transcription in RA compared with ReA. One of these has not been described previously and is currently undergoing further analysis. The other two transcripts were those for *Calmodulin* and *CAS*, and downregulation of these was confirmed in individual biopsies. *Calmodulin* transcription was greatly reduced in RA, by up to 99%. Furthermore, its transcription in PBMCs increased after in vivo TNF- $\alpha$  blockade, a treatment known to reverse the proliferative abnormality of RA T cells. Additionally, transcript levels were lower in RA SFMCs compared with RA PBMCs, mirroring the known proliferative and signaling defects (25). Last, pharmacological calmodulin inhibition impaired antigen-specific proliferation of PBMCs in a dose-dependent manner. While not proving causality, these observations provide compelling evidence linking the hyporesponsiveness of RA T cells to reduced *Calmodulin* transcription. Thus, calmodulin binds free calcium in cells and is critical in T-cell activation (33). It regulates the influx of extracellular calcium (37), and, although recent evidence has implicated intracellular calcium stores in the RA signaling abnormality, that does not exclude an additional and possibly synergistic defect in extracellular calcium entry (25). Furthermore, calmodulin regulates other calcium-dependent proteins including calcineurin, a phosphatase critical to the NFAT T-cell signaling pathway (38), and calcium/calmodulin-dependent protein kinase, which has also been linked to the anergic state (39). It is also implicated in cytoskeletal changes associated with T-cell activation (40) and in phosphatidyl-

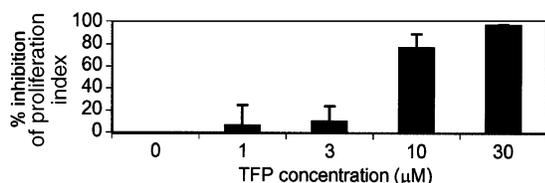
serine synthesis (41). Ultimately, calmodulin regulates genes central to T-cell proliferation (42), and it is clear that downregulation of *Calmodulin* transcription is consistent with the T-cell phenotype in RA.

*CAS* is the human homologue of the yeast chromosome segregation gene *CSE1*, which determines cell fate at the G2/M mitotic checkpoint by influencing the decision between cell growth and apoptosis (35). Its expression correlates with malignant potential in human tumors (43) but, notably, reduced levels are associated with resistance to TNF- $\alpha$ -mediated apoptosis (44). Reduced transcription in RA compared with ReA may, therefore, contribute to the defect in apoptosis observed in RA synovium in the face of high TNF- $\alpha$  levels. Both RA synoviocytes and synovial T cells demonstrate a resistance to apoptosis, however (5, 9). Additional analyses are therefore needed before attributing either defect to reduced *CAS* transcription, particularly in view of the relatively modest difference in transcript levels between RA and ReA and the overall paucity of *CAS* transcripts (Table 1 and Figure 4).

A number of transcripts were more abundant in RA compared with ReA synovium, or were equally transcribed (Table 1). These included *PILOT*, an *Egr* gene family member that increases *FasL* promoter activity and whose expression is sensitive to cyclosporine A (45). Transcription of this gene increased transiently during energy induction and was similar in RA and ReA. The gene encoding T-plastin also was equally transcribed in the two types of inflamed synovium and showed a transient and slight rise in transcription during energy induction. This is a calcium-binding protein



**Figure 6** Transcription of *Calmodulin* and *CAS* in PBMCs isolated from RA patients before and after treatment with anti-TNF- $\alpha$  mAb. First-strand cDNA was prepared from PBMCs from six patients before and 2 weeks after receiving 3 mg/kg Infliximab. Real-time PCR was used to quantitate transcription of (a) *Calmodulin* and (b) *CAS* relative to *GAPDH*. The figure shows the ratio of transcripts in the posttreatment samples relative to the pretreatment samples for each patient.



**Figure 7**

Inhibition of antigen-specific PBMC proliferation by calmodulin blockade. PBMCs were prepared from normal controls. Proliferation assays were established using PPD as a test antigen in the presence or absence of TFP, a pharmacological antagonist of calmodulin. The figure illustrates pooled data for four individuals of the percentage inhibition (mean  $\pm$  SD) of the proliferation index at each dose of TFP. The proliferation index was calculated as the ratio of counts between stimulated and unstimulated cells.

containing both a calmodulin-like and an actin-binding domain. Its structure would allow it to transduce signals from the cell membrane to the cytoskeleton, a process shown recently to be critically important during T-cell activation (46). Notably, T-plastin has previously been described only in mesenchymal cells, which may account for its expression in synovium. Its isoform, L-plastin, has been linked to T-cell activation (47), but we used a T-plastin-specific sequence for hybridization. Another calcium-binding protein, calumenin, was transiently downregulated during anergy induction but displayed only minor differences between the two types of inflammatory arthritis. It is a member of the EF-hand protein family and, in contrast to calmodulin, is present predominantly in the lumen of the endoplasmic reticulum and Golgi apparatus (48). The ESTs *BF626663* and *BF626660* showed similar transcription patterns to calumenin, although the former remained downregulated in established anergy. The gene encoding aortic carboxypeptidase-like protein (ACLP) was transiently upregulated during anergy induction but was highly transcribed in both forms of synovitis (Table 1). ACLP contains a discoidin domain and a COOH-terminal region homologous to carboxypeptidase E. It has been linked previously to vascular smooth muscle differentiation (49), which may reflect its high level of transcription in inflammatory synovitis (Table 1) where neovascularization is abundant (50). Lastly, ARL-1 is a guanine nucleotide-binding protein that generates high-affinity membrane-binding sites for the heterotetrameric AP-1 adaptor complex on the Golgi apparatus (51). Transcription was transiently increased during anergy induction and was higher in RA than in ReA.

The ESTs *BF626660* and *BF626663* are currently undergoing further analysis in our laboratory. These could represent unrecorded sequences of known transcripts because cDNA fragments isolated by DDRT-PCR preferentially locate to the 3'-untranslated region of transcripts, which are sometimes omitted from databases. To address this possibility we have prepared cDNA libraries from T-cell lines to enable us to further characterize these apparently novel cDNA fragments.

It is also important to emphasize that our DDRT-PCR analysis could not have identified posttranscriptional events. These have been shown to influence cytokine secretion by anergic HA1.7 cells (52) and are also likely to be active in synovial fluid T cells as a result of the oxidative nature of the local microenvironment (53). Notwithstanding the above, we have identified a number of genes whose transcription changes during anergy induction and differs between RA and ReA synovium. In particular, regulation of *Calmodulin* and *CAS* could explain some of the characteristic features of RA T cells and synovium, and further experiments are currently underway to more precisely define their roles. These may improve our understanding of the immunosuppression that is intrinsic to RA (8) and also the apoptotic defects that appear to play a critical role in RA pathogenesis (5, 9).

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