# Identification of Naf1/ABIN-1 among TNF-α-induced expressed genes in human synoviocytes using oligonucleotide microarrays

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**Abstract The cytokine tumor necrosis factor alpha (TNF-α) is a critical effector of the pathogenesis of rheumatoid arthritis (RA). We used oligonucleotide microarray (OM) analysis to assess TNF-α-modulated gene expression in cultured primary human synoviocytes in vitro. Genes identified include cytokines and inflammatory mediators, extracellular matrix and adhesion molecules, cell cycle and proliferation related proteins, transcription related proteins, and apoptotic mediators. OM identified 1185 differentially expressed genes in TNF-α-treated synoviocytes. The regulation of Nef-associated factor-1 (Naf1), an A20-binding, nuclear factor kappa B (NFκB) inhibitory protein was probed further given its putative role as an endogenous brake for the expression of some TNF-α-driven genes. Naf1 mRNA levels were higher in synovial biopsies from patients with active RA and seronegative arthropathy than in those from patients with osteoarthritis. These findings underscore the value of transcriptome analysis in cytokine-activated synoviocyte cultures in vitro as a means of identifying disease-associated genes in human arthritis, and implicate Naf1 as a potential modulator of TNF-α bioactivity in RA.**


*Key words:* Rheumatoid arthritis; Synoviocyte; Tumor necrosis factor alpha; Nef-associated factor-1

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease manifesting complex pathological aspects in synovial tissues, including hyperplasia of the synovial membrane, infiltration by inflammatory cells, and neovascularization. Tumor necrosis factor alpha (TNF-α) is a key inflammatory cytokine in the pathogenesis of RA [1,2]. The use of anti-TNF-α therapy has been very successful in ameliorating RA in clinical practice. However, this amelioration is only observed in a subset of the population [3]. Thus, it remains of interest to define the downstream mediators and modulators of TNF-α activity in RA.

Fibroblast-like synoviocytes (synoviocytes), are a major source of the mediators of joint destruction in RA, and are a dominant cell at the leading edge of invasion by the tumor-like pannus into adjacent cartilage and bone [4]. Propagation of synoviocytes in the presence of TNF-α provides a useful in vitro model with which to dissect out the molecular components of TNF-α bioactivity in RA [5,6]. In the present study we used this model to explore the influence of TNF-α on the synoviocyte transcriptome in vitro, with the goal of identifying RA-associated genes. Specifically, we examined the effects of TNF-α on the gene expression profile of synoviocytes by oligonucleotide microarray (OM) analysis and defined disease-associated expressed gene cohorts. We also identified Naf1 (Nef-associated factor-1), a putative nuclear factor kappa B (NFκB) inhibitor, among the TNF-α regulated genes and further examined the regulation of its expression.

## 2. Materials and methods

### 2.1. Cell culture and cell treatments

*2.1.1. Cell culture.* Primary human synoviocytes were a kind gift from Dr. Leslie Crofford, University of Michigan, Ann Arbor, MI, USA, and were isolated and cultured as previously described [7].

*2.1.2. TNF-α stimulation of synoviocytes.* Cells were serum starved in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin, henceforth referred to as serum free medium (SFM), for 24 h prior to the addition of 10 ng/ml TNF-α (R&D Systems Europe Ltd, Abingdon, UK) for the indicated time periods. Induction of differential gene expression in synoviocytes by TNF-α was routinely confirmed by analysis of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) mRNA levels by real-time polymerase chain reaction (PCR) as described below. In keeping with previous reports [8,9], TNF-α induced the expression of both genes (data not shown).

### 2.2. RNA isolation

Synovial membrane biopsies were obtained with Institutional Ethics Committee approval following informed consent from patients attending the early arthritis clinic at St Vincent's University Hospital, Dublin, Ireland. All synovial biopsy specimens were obtained from the knee at arthroscopy. Patients had been receiving non-steroidal anti-inflammatory drugs, but not disease modifying anti-rheumatic drugs. Patients were diagnosed as having RA, osteoarthritis (OA), or seronegative arthritis (SN) according to the criteria of the American College of Rheumatology [10]. RNA was isolated from tissues using the

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*Abbreviations:* TNF-α, tumor necrosis factor alpha; Naf1, Nef-associated factor-1; NFκB, nuclear factor kappa B; ABIN-1, A20-binding NFκB inhibitor; RA, rheumatoid arthritis; OA, osteoarthritis; SN, seronegative arthritis; IFN, interferon

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RNeasy system (Qiagen, UK). Total RNA was isolated from cultured cells using Trizol (Invitrogen). All procedures were carried out according to the respective manufacturer's instruction.

### 2.3. Northern blot analysis, reverse transcription (RT)-PCR and real-time PCR

Northern blotting was performed as described previously [11]. Prior to performing real-time PCR, residual chromosomal DNA was eliminated from total RNA using DNase I (Invitrogen). 2 μg of total RNA were reverse transcribed using standard procedures. Quantitative real-time PCR was carried out on cDNA using the ABI Prism 7700 sequence detection system and Taqman™ probe sets (Perkin Elmer, UK). Cycling conditions were as follows: step 1: 2 min at 50°C, step 2: 10 min at 95°C, step 3: 15 s at 95°C, step 4: 1 min at 60°C. Steps 3 and 4 were repeated for 40 cycles. Real-time PCR (Taqman™) probes were labeled with the fluorescent dyes 5′ FAM and 3′ TAMRA as quencher, with the exception of the ribosomal RNA probe which was labeled with the fluorescent dye 5′ VIC in order to facilitate multiplex analysis. All results were normalized to ribosomal RNA levels of the sample. Relative expression levels were calculated with respect to the level in a suitable control sample (see figures) and are expressed as fold increase or decrease.

### 2.4. Oligonucleotide microarray analysis

Oligonucleotide microarray analysis was performed using the Affymetrix® Gene Chip Expression Analysis system (High Wycombe, UK). Oligonucleotide microarray experiments were carried out using RNA samples isolated from cultured synoviocytes which had been stimulated with TNF-α for 0, 4 or 24 h. The entire experiment and analysis was carried out independently in triplicate. RNA samples were further purified using the RNeasy system from Qiagen. Sample preparation and subsequent analysis were carried out according to the manufacturer's instructions. The hybridization quality and array performance were assessed using the GeneChip® eukaryotic hybridization control kit and Test 2 arrays (Affymetrix®). Changes in gene expression were accessed by comparing the expression profile at 0 h with those at 4 and 24 h obtained by hybridization of the respective cRNAs to Hu95A Gene Arrays (Affymetrix®) which are representative of ~12 650 unique human expressed genes.

### 2.5. Microarray data analysis

The information generated from the replicate experiments was analyzed using GeneSpring™ Version 5.1 from Silicon Genetics. The microarray data were normalized to the distribution of all probe sets to control for systematic variation, and also to the median of each probe set to account for any differences in detection efficiency. This normalization facilitated the identification of changes in gene expression levels, which were indicative of biological variation and not variations due to measurement processes. As large numbers of replicate experiments could not be performed, the global error model was used to estimate measurement and sample-to-sample variation.

## 3. Results and discussion

### 3.1. Analysis of differential gene expression in TNF-α-stimulated synoviocytes by oligonucleotide microarray analysis

Oligonucleotide microarray analysis was performed using

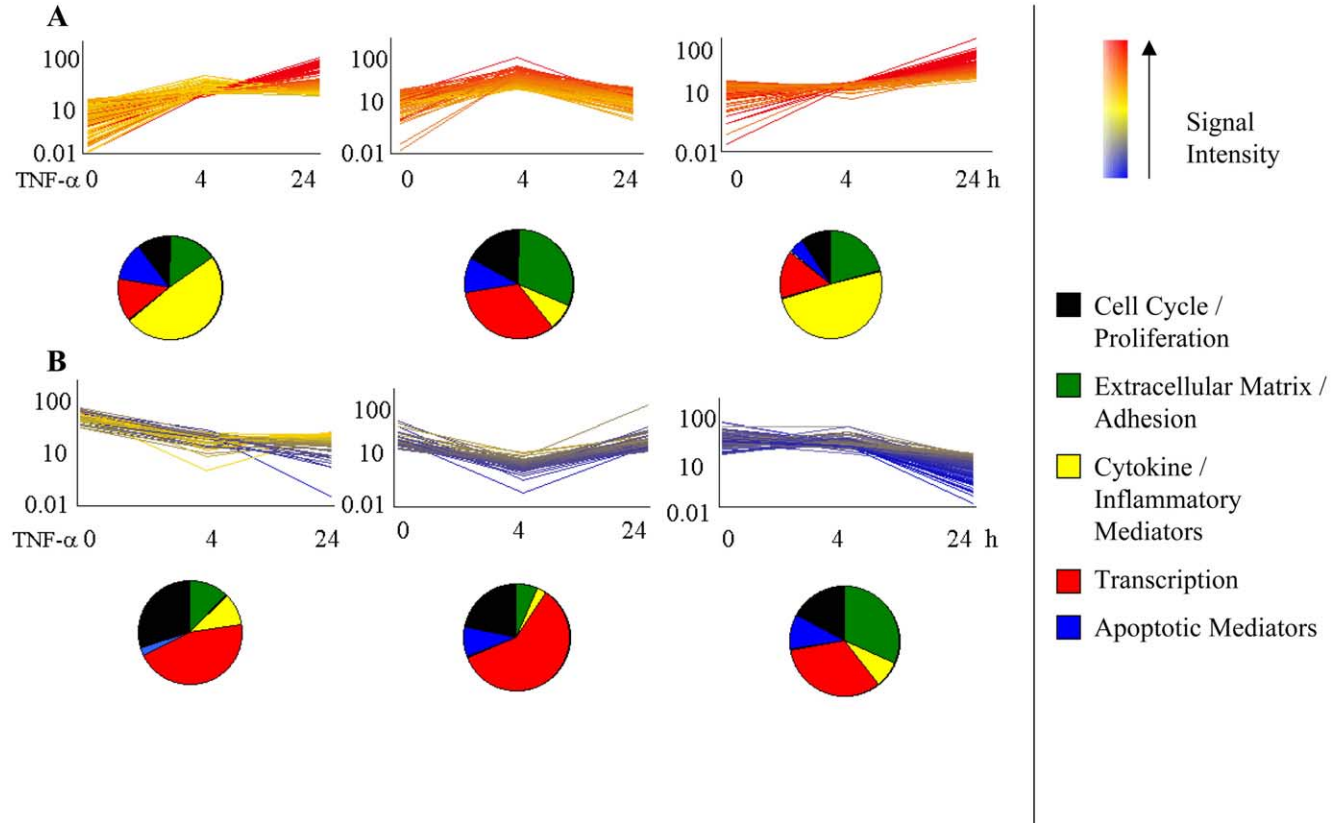



Fig. 1. A: Induced gene expression at 4 and 24 h post-TNF-α stimulation. Graphs of the log values of normalized expression ratios for genes induced ≥2-fold by TNF-α at both 4 and 24 h (*231*), ≥2-fold exclusively at 4 h (*148*), and ≥2-fold exclusively at 24 h (*184*). Pie charts represent the subsets of functionally assigned genes from the lists represented in the graphs. The number of genes functionally assigned relative to the total number in each group is defined as follows: 121/231, 59/148 and 62/184. Color coding for signal intensity and for functional assignment is indicated. B: TNF-α suppressed transcription at 4 and 24 h post-TNF-α stimulation. Graphs of the log values of normalized expression ratios for genes with ≥2-fold reduced transcription with TNF-α at both 4 and 24 h (*128*), ≥2-fold reduced exclusively at 4 h (*138*), and ≥2-fold reduction exclusively at 24 h (*356*). Pie charts represent the subsets of functionally assigned genes from the lists depicted in the graphs. The number of genes functionally assigned relative to the total number in each group is defined as follows: 37/128, 33/134 and 75/331. Color coding for signal intensity and for functional assignment is indicated.

the Affymetrix Hu95A array representing over 12 000 genes and RNA from synovial fibroblast which were unstimulated or stimulated with TNF-α for 4 or 24 h. To validate the differential expression of genes identified by OM, Northern blotting and real-time PCR were used. These techniques qualitatively confirmed modulation of gene expression by TNF-α of a representative cohort of genes. In all the expression of six genes was verified. The data on Naf1, presented below, serve as an example. Our OM analysis indicated that the expression of 231 genes was significantly induced in response to TNF-α at both 4 and 24 h, that the expression of 148 was significantly induced in response to TNF-α at 4 h only, and that the expression of 184 genes was induced only at 24 h. OM analysis also illustrated that the expression of 128 genes was significantly suppressed in response to TNF-α at both 4 and 24 h, that the expression of 138 genes was significantly suppressed in response to TNF-α at 4 h only, and that the expression of 356 genes was suppressed only at 24 h. These changes are represented graphically in Fig. 1A and B. A complete list of genes and their expression levels will be lodged at http://www.ncbi.nlm.nih.gov/geo/:GSE516. In order to dissect how changes in gene expression profiles in synoviocytes in response to TNF-α treatment may relate to disease progression in RA, five disease-associated expressed gene groups were defined within the data sets. These were cytokines and inflammatory mediators, extracellular matrix proteins and adhesion molecules, cell cycle and proliferation related proteins, transcription related proteins, and finally apoptotic mediators. The induction and suppression of the disease-associated functional groups over time are represented by the pie charts in Fig. 1A and B.

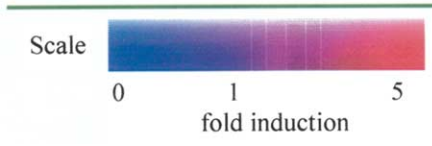
From Fig. 1A it is clear that there is a dramatic and maintained induction of cytokine and inflammatory gene expression with the addition of TNF-α to synoviocytes. The induction of expressed genes such as interleukin (IL)-8, IL-6, RANTES and MCP-1 is consistent with the current knowledge about the contribution of synoviocytes to RA. The consistent induction of substantial numbers of extracellular matrix proteins and adhesion molecules such as matrix metalloproteinases (MMPs) and VCAM is also consistent with current hypotheses on disease progression in RA. The suppression of a significant number of transcription related genes was observed, and will be the subject of further study.

### 3.2. *Disease-associated genes with potential pathophysiological roles in RA*

One distinct cohort of genes identified as differentially regulated in response to TNF-α were the interferon (IFN) respon-

Table 1
TNF-α induced IFN responsive and NFκB signalling gene transcripts

| Accession No. | Description |
|---|---|
| | **Interferon responsive Genes** |
| L05072 | Interferon regulatory factor 1 (IRF-1) |
| J03909 | Gamma-interferon-inducible protein (IP-30) |
| J04164 | Interferon-inducible protein 9-27 |
| L78440 | STAT4 |
| M13755 | Interferon-induced 17-kDa/15-kDa protein |
| M14660 | ISG-54K gene (interferon stimulated gene) encoding a 54 kDA protein |
| M30818 | Human interferon-induced cellular resistance mediator protein (MxB) |
| U72882 | Interferon-induced leucine zipper protein (IFP35) |
| X02530 | Gamma-interferon inducible early response gene (with homology to platelet p) |
| X59892 | IFN-inducible gamma2 protein |
| M97935 | Transcription factor ISGF-3 |
| L42243 | Alternatively spliced interferon receptor (IFNAR2) |
| U34605 | Retinoic acid- and interferon-inducible 58K protein RI58 |
| M16750 | Pim-1-oncogene |
| U43185 | STAT5a |
| U37518 | TRAIL |
| | **NFκB signaling complex related genes** |
| U91616 | I kappa B epsilon (IkBε) |
| M58603 | Nuclear factor kappa-B DNA binding subunit (NF-kappa-B) |
| S76638 | p50-NF-kappa B homolog |
| U20816 | Nuclear factor kappa-B2 (NF-κB2) |
| M59465 | Tumor necrosis factor alpha inducible protein A20 |




Genes were chosen and clustered on the basis of their belonging to a particular group of related genes. The levels of mRNA expression at each of the three time points (0, 4 and 24 h) are represented by three colored boxes in the color panel, the relative expression level is represented on the scale above.

sive genes. IFN-γ has the ability to alter the balance of extracellular matrix synthesis and degradation, and decreases levels of type I and type III pro-collagen mRNA in RA synoviocytes [12]. IFN-γ can also inhibit collagen synthesis and metalloproteinase production in synoviocytes that have been stimulated by TNF-α. TNF-α and IFN-γ may thus have inverse effects on many aspects of synoviocyte function, a phenomenon that has been termed mutual antagonism [12]. 16 IFN regulated and modulated genes were identified. One such IFN-induced gene, IP-30 (GILT), has been previously identified as upregulated by an SSH analysis which compared genes expressed in OA tissues with those expressed in RA tissues [13]. Also induced were target genes of the IFN-γ pathway such as TRAIL and pim-1 [14,15].

Also among the IFN-induced genes identified were the IFN-stimulated transcription factors, IFN regulatory factor-1 (IRF-1), and IFN-stimulated transcription factor-3 (ISGF3) component. IRF-1 can activate the transcription of both the IFN-α and IFN-β genes [16]. The IFN-dependent transcription factor ISGF3 acts as a multiprotein complex [17]. In its latent state ISGF3 exists as two independent components, ISGF-α and ISGF3-γ. ISGF-α comprises three polypeptides, including signal transducers and activators of transcription (STAT)1 and 2. STAT4 and STAT5a were also detected as induced in response to TNF-α (Table 1). Elevated levels of STAT4 expression have been previously demonstrated in activated macrophages present at the site of inflammation in RA [18], whereas STAT5a has been suggested to be involved in T-cell proliferation [19]. The advent of anti-IFN therapy for the treatment of rheumatoid disease has had mixed results [20–22]; thus, identifying TNF-α regulated cohort of IFN responsive genes, including downstream regulators of IFN action, may help in defining the effects of such therapies on downstream signalling events in RA.

### 3.3. *Induction of Naf1 gene expression by TNF-α in synoviocytes*

Our OM analysis revealed for the first time the expression of Naf1 in human synoviocytes and its association with TNF-α stimulation in this model system. Naf1 is the human homolog of murine A20-binding inhibitor of NFκB (ABIN-1), which binds A20 and can inhibit NFκB-dependent gene expression [23]. ABIN-1 was originally detected in a yeast two-hybrid screen [24] and has been shown to interact with the C-terminal part of A20 and to colocalize with A20 in the cell cytoplasm. Overexpression of ABIN-1 blocks NFκB activation by TNF-α, and it is thought that this may occur at the level of TRAF2 [25]. It had been postulated that through its interaction with A20, ABIN-1 may modulate the inhibitory effect of A20 on NFκB signalling [24]. However, recent evidence suggests that the interaction of ABIN-1 with A20 may not be required for NFκB inhibition by either protein, in vitro [26]. Thus, the exact mechanism of NFκB inhibition by A20 and its interacting proteins in vivo is still unclear. The kinetics of Naf1 induction was examined by quantitative PCR after stimulation with TNF-α. The expression of Naf1 in response to TNF-α was maximal at 8 h over the course of 24 h as illustrated in Fig. 2. Previous array studies examining synoviocyte gene expression in response to TNF-α did not detect Naf1 [27]. However, this is probably due to the fact that 24 h was the only time point examined, and the kinetics of Naf1 induction indicate that expression of Naf1 is maximal at 8 h. A recent study has shown induction of Naf1 in TNF-α-stimulated HeLa cells and inhibition of its expression following disruption of the p65 NFκB subunit, indicating that the expression of Naf1 is NFκB dependent [28]. These findings are consistent with those of others [29,30]. In this study we also found induction of the expression of A20 in response to TNF-α in synoviocytes (Table 1). Therefore, it may be suggested that parallel induction of Naf1 and A20 in inflamed synoviocytes offers potential targets through which the NFκB pathway could be regulated therapeutically.

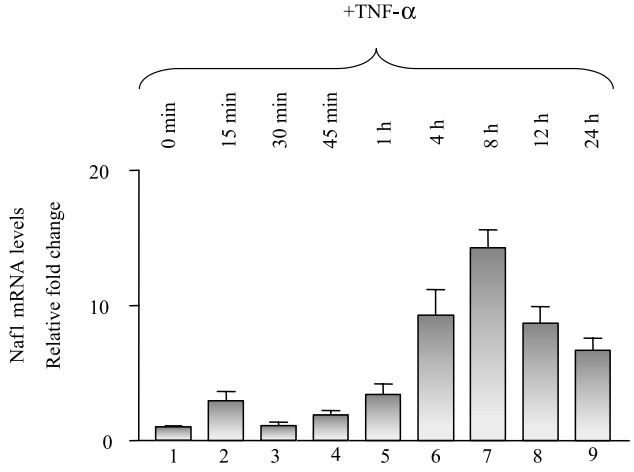



Fig. 2. Kinetics of induction of Naf1 mRNA in synoviocytes in response to TNF-α. TNF-α (10 ng/ml) was added for 15 min–24 h to synoviocyte cultures that had been serum starved overnight. Naf1 mRNA expression levels were determined by quantitative real-time PCR. These results are an average of five separate duplicate experiments. Error bars represent S.E.M.

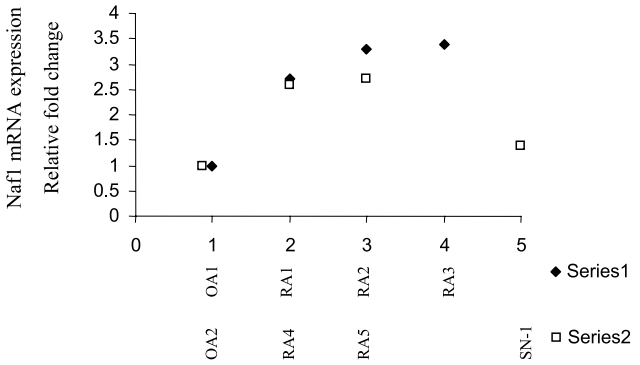



Fig. 3. Analysis of Naf1 mRNA expression levels in tissue samples from patients with arthritis by quantitative real-time PCR. Each series represents an independent group of patient samples. Fold change is relative to the OA samples.

### 3.4. *Analysis of Naf1 mRNA levels in tissue biopsies from arthritis patients*

Naf1 mRNA levels were also analyzed in tissue biopsies from two types of inflammatory arthritis, RA and SN (negative for rheumatoid factor) and compared to expression levels in tissue samples from patients suffering from the non-inflammatory arthritis, OA. Two independent analyzes were carried out using quantitative real-time PCR and the results are presented as series 1 and 2 in Fig. 3. Relatively high levels of Naf1 expression were observed in patients suffering from inflammatory arthritis compared to those with non-inflamma-

tory arthritis (Fig. 3). These results link high levels of expression of Naf1 with inflammatory disease states. As elevated levels of NFκB are associated with inflammatory arthritis, it may appear contradictory that elevated levels of Naf1 expression (an NFκB inhibitor) are also detected. However, it may be suggested that these elevated levels of Naf1 play a role in negative feedback regulation of NFκB expression, and therefore suggest a possible contribution of this gene product to modulating the TNF-α/NFκB inflammatory drive in RA in humans.

Our studies have shown Naf1 to be a TNF-α inducible, disease-associated gene. A recent study has shown that Naf1 can also bind the MAP kinase ERK2 and cause its retention in the cytoplasm, which in turn leads to a reduction of ERK2 nuclear signalling [31]. Thus, Naf1 may attenuate both NFκB and ERK signalling in human synoviocytes. The effects of Naf1 on the regulation of ERK signalling and the expression of other A20 interacting NFκB regulators such as ABIN-2 in the context of rheumatoid disease will be further investigated.

In conclusion, this study has identified cohorts of cytokines and inflammatory mediators, extracellular matrix and adhesion molecules, cell cycle related proteins, transcription related proteins, and apoptotic mediators, whose expression is associated with an experimental model of RA progression. Of particular interest is the association of elevated Naf1 expression with RA. It suggests that natural inhibitors of the TNF-α–NFκB signalling pathway might have value as a future therapeutic target.

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