

Transcriptional profiling of leukocytes from rheumatoid arthritis patients before and after anti-tumor necrosis factor therapy: A comparison of anti-nuclear antibody positive and negative subsets

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Abstract. Anti-nuclear antibodies (ANAs) may be induced in patients with rheumatoid arthritis (RA) receiving anti-tumor necrosis factor (TNF) therapy with TNF inhibitors (TNFi), etanercept, infliximab or adalimumab. In the present study, 11 patients who were TNFi drug naive were started on TNFi at a time of high disease activity. Of these, all cases were positive for rheumatoid factor and 9 cases tested were positive for anti-citrullinated peptide (anti-CCP) antibodies prior to TNFi treatment. Peripheral blood mononuclear cells (PBMCs) and serum were collected from all patients before and after TNFi therapy. Serum was assayed for ANAs over time. Total cellular RNA was extracted from PBMCs and assessed using Illumina arrays. Gene expression profiles were examined for alterations in key effector pathways. After 3 or more months on TNFi, 6 patients converted to ANA-positivity. Analysis of transcripts from patients with RA who converted to ANA-positivity after 3 months on TNFi identified complex gene expression profiles that reflected a reduction in cell adhesion, cell stress and lipid metabolism transcripts. In summary, unique transcriptional profiles in PBMCs from patients with RA were observed after TNFi therapy. This pilot study suggests that transcriptional profiling is a precise method of measuring the impact of TNFi therapies and reveals novel pathways that likely influence the immune response.

Introduction

Tumor necrosis factor (TNF)- α inhibitors (TNFi) have proven efficacy in the treatment of active rheumatoid arthritis (RA) and other chronic inflammatory diseases (1-5). Different forms of TNFi have been marketed, such as the monoclonal antibodies (mAbs) to TNF, infliximab and adalimumab or the soluble receptor etanercept. Infliximab is a chimeric murine anti-TNF mAb with an Fc domain from human IgG1 and adalimumab is a completely humanized anti-TNF mAb of the IgG1 isotype (1,3,6). Etanercept is a soluble TNF receptor fusion protein consisting of a dimer of the extracellular domain of the human p75 TNF receptor (CD120b) combined with human Fc and hinge regions from IgG1 (1,5). Each of these three TNFi can bind to soluble and membrane-bound forms of TNF and by virtue of their IgG1 Fc domains, the mAbs mediate cell killing either by the activation of complement or by apoptosis, whereas the soluble receptor acts to neutralize TNF. Importantly, the preponderance of evidence suggests that etanercept and adalimumab are weakly immunogenic, whereas the reports for infliximab are variable with frequencies from zero to over half of patients developing anti-chimeric antibodies depending on the dose of therapy (1,3,5,6).

After several months of treatment with TNFi, reduced levels of autoantibodies associated with RA, such as rheumatoid factor (RF) and anti-citrullinated peptides or proteins (anti-CCP) antibodies, are detected in the serum (7-9). The reduced levels of RA-associated autoantibodies correlate with improved clinical measures. Although the mechanisms are not completely understood, TNFi are likely to down-regulate inflammatory cytokines and localized apoptosis in the rheumatoid synovium, which could limit the availability of autoantigen for the generation of RF and anti-CCP antibodies (10).

A side effect of TNFi is the induction of new autoantibodies such as anti-nuclear antibodies (ANAs) and anti-double stranded DNA (anti-dsDNA) antibodies (3,11-14). These antibodies are thought to be induced by an upregulation of B-cell responses in the absence of TNF and the antigen specificity driven by increased apoptosis in the periphery, which might also be related to an inability to clear dead cells. The

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downregulation of C-reactive protein might contribute to the lack of clearance of cell debris and might further promote autoreactivity in the periphery (15). The development of ANAs is common in patients after treatment with infliximab, with reports of one-third to >90% of patients converting to ANA positivity compared with approximately half of patients treated with etanercept converting to ANA positivity (3,13). While less data exists on adalimumab, the data available are similarly variable in reported percentages (12). Anti-dsDNA generation is present in most TNFi-treated patients at half the rate of ANA induction. Widely variable reported rates could in part be a reflection of the differences in methods used for autoantibody assays by clinical laboratories. Notably, the development of ANA or anti-dsDNA autoantibodies is only rarely associated with the development of other lupus-associated antibodies or clinical evidence of lupus-like syndrome in RA patients (3).

Gene expression profiles of whole blood or peripheral blood mononuclear cell (PBMC) subsets obtained from RA patients before and after treatment with TNFi have been reported by several investigators (16-22). The focus of these studies has been on the identification of a gene expression profile that correlates with a clinical response to TNFi therapy. The aim of the present study was to examine the gene expression profile of patients with RA converting to an ANA-positive phenotype after TNFi treatment. Thus, the present pilot study was carried out in order to determine whether shifts in transcriptional profiles after only 3 months of TNFi treatment that correlated with ANA-positive conversion could be detected. The focus was on differences in effector pathways, such as cell adhesion and cytokines, which might be predicted to be altered after TNFi treatment.

Materials and methods

Patients. The study protocol was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center (Dallas, TX, USA). Written informed consent was obtained from patients with RA whose physician had made the clinical decision to begin a TNFi drug for the first time. All RA patients fulfilled the American College of Rheumatology 1987 classification criteria for RA (23). The patient's age, gender, duration of RA and disease activity score (DAS-28) were recorded (24). Peripheral blood was collected at each visit in heparinized tubes for the isolation of PBMCs and serum was collected before and after TNFi therapy with etanercept (n=6), infliximab (n=4) and adalimumab (n=1). Autoantibody levels were determined in routine assays carried out by the hospital. ANAs were determined by ImmunoFluorescence Antibody (IFA) assay and levels reported as titers using an ANA test system with IFA slides containing epithelial cells transfected with specific target DNA sequences. All reagents and controls were purchased from Immuno Concepts (immunoconcepts.com), including Fluorescent Hep-2000 ANA Slides (2040-Ro), SSA/Ro positive control (2035-Ro), titratable control serum (2026), negative control serum (2031) and fluorescent antibody reagent (3009). Patient serum was screened for antibodies to nuclear antigens and results were visualized by fluorescent microscopy. RF was assayed by nephelometry (turbidimetry) and reported as IU/ml using a particle-enhanced immunoassay

with latex-bound heat inactivated IgG, which was bound by RF antibodies in patient serum to form antigen-antibody complexes. The resulting agglutination reaction was read on a Siemens Advia 1200 System (Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA) using methods previously reported in detail (25,26). Values <15 IU/ml were considered to be normal (negative) for RF. Anti-CCP antibody levels were determined by a routine ELISA (QUANTA Lite CCP3 IgG/IgA kit; 704550; Inova Diagnostics; inovadx.com) for IgG and IgA and reported as U/ml. Briefly, anti-CCP antibodies in patient serum were bound to wells of a microtiter plate coated with synthetic CCP, washed to remove unbound serum components and subsequently treated with anti-human IgG/IgA antibody labeled with horse radish peroxidase. The anti-CCP was detected by the addition of a substrate, such as 3,3',5,5' tetramethylbenzidine, and the color was read using an ELISA plate reader. Values <5 U/ml were considered to be negative. Details regarding the contribution of this assay to the diagnosis of RA have been detailed elsewhere (27-29).

PBMC RNA preparation, chip hybridization and analysis. Peripheral blood samples were collected in heparinized tubes in order to obtain PBMCs and processed using a rapid density-gradient centrifugation over Ficoll-Hypaque as previously described (30). PBMCs were immediately centrifuged and total cellular RNA was prepared from PBMCs in TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the samples were frozen at -80°C until further processing by the microarray core of University of Texas Southwestern Medical Center (30). The RNA extraction, microarray analysis and quality control steps were performed following validated protocols of the Immunology DNA Microarray Core (microarray.swmed.edu). Purified RNA was extracted over RNeasy Qiagen columns (Qiagen, Inc., Valencia, CA, USA) (30). RNA samples that were limited in amount or did not pass the quality control check on the bioanalyzer were excluded from further analysis. cRNA was hybridized onto Illumina chips (Illumina, Inc., San Diego, CA, USA) using the standard protocols according to the manufacturer's instructions.

Data was processed with BeadStudio 1.5.0.34 software (Illumina, Inc.) and differentially expressed genes were identified using GeneSpring 7.3.1 Analysis Platform software (Agilent Technologies, Inc., Redwood City, CA, USA) (30). Data from each array was normalized and routine filtering was carried out to exclude absent and low signals. In addition, the cross-gene error model was applied to filter for reliable genes by control strength. Out of 48,000 probe sets, 32,074 passed filtering criteria for the Human WG-6 BeadChips and 26,993 of 48,701 probe sets passed filtering criteria for the Human V2 BeadChips.

To maintain consistency in nomenclature for various analyses, transcripts are annotated using gene symbols to identify transcripts. Data on specific genes, transcripts and proteins were gathered from a variety of sources as referenced, and from public databases, including DAVID Functional Annotation Bioinformatics Microarray Analysis (david.ncifcrf.gov), ArrayExpress (ebi.ac.uk/arrayexpress), Human GeneCards (genecards.org) and OMIM (omim.org/). Unless otherwise indicated, a 1.5-fold change (FC) between pre- and post-cutoff

Table I. Characteristics of RA patients treated with anti-TNF inhibitors.

Patient no.	Age (years)	Gender	Years of RA	RF (IU/ml)	Anti-CCP (U/ml)	Anti-TNF
ANA negative						
101	57	F	13	171	>100	Etanercept
102	36	F	6	27	16	Etanercept
104	30	F	3	444	ND	Etanercept
105	58	M	7	357	ND	Infliximab
106	64	M	15	56	143	Etanercept
Average	49	3:2	8.8	211	86.3	
ANA positive						
103	50	F	2	2260	100	Adalimumab
107	59	F	29	296	58	Etanercept
108	79	M	2	244	93	Infliximab
109	70	F	10	150	>100	Etanercept
110	54	F	1	46	>100	Infliximab
111	51	F	11	802	>100	Infliximab
Average	60.5	5:1	9.2	633	91.8	

Data on the demographic and disease characteristics of RA patients, including age, gender, disease duration (years of RA), serum RF levels (positivity >15 IU/ml) and anti-CCP levels (positivity >5 U/ml) were measured in routine assays by the clinical laboratory. The TNF inhibitor for each individual is shown. All patients were negative for ANAs at the initiation of the study. After at least 3 months of anti-TNF- α therapy, the ANA assays were repeated and the RA patients grouped into ANA- or ANA+ converters for data analysis. RA, rheumatoid arthritis; TNF, tumor necrosis factor; RF, rheumatoid factor; anti-CCP, anti-citrullinated peptide; ANAs, anti-nuclear antibodies; ND, not detected.

was set to identify differentially regulated transcripts in the analyses described (30).

Statistical analysis. Data was subjected to statistical analysis including one-way analysis of variance (ANOVA), Welch's t-test or Mann Whitney t-test as indicated in combination with multiple testing corrections such as the Benjamini and Hochberg (BH) false discovery rate ($P < 0.05$) or the Bonferroni family-wise error rate ($P < 0.05$) to control for genes that might appear in the analysis by chance as indicated in the results. Data are presented as the normalized mean signal intensity (MSI) or as the relative percentage of normalized data for the comparison of gene expression (GE) levels after therapy to those before therapy (post-therapy MSI/pre-therapy MSI \times 100; before therapy GE=100%) were presented graphically using GraphPad Prism version 6 Software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Alterations in RA leukocyte transcriptomes after TNFi therapy. The patients with RA comprised 8 females and 3 males with a mean disease duration of 9 years. Serum was assessed for RF, anti-CCP antibodies and ANAs using standard clinical assays. As shown in Table I, of the 11 RA patients assessed, all were positive for RF, 9 were positive for anti-CCP antibodies and all were ANA-negative at the initiation of the study. After at least 3 months of TNFi treatment, 6 patients converted to ANA positivity. Initial analysis of transcripts from samples taken from patients with RA converting to ANA-positive after 3 months on TNFi therapy identified 112 transcripts that were

differentially regulated with a change of >1.5 fold in expression after TNFi treatment compared with before. Of these, 42 transcripts were upregulated and 70 were downregulated following the initiation of therapy. Of the 112 transcripts, 64 were statistically significant by ANOVA. Of the 64, 52 transcripts passed the Benjamini and Hochberg multiple testing correction and 17 differentially regulated transcripts were significant when the stringent Bonferroni correction was applied, including several expressed sequence tags that were not studied further.

The proteins encoded by the most differentially expressed transcripts were not well-known immunoregulatory molecules, but were molecules involved in cell adhesion, cell stress and the metabolome. For example, two patients (nos. 107 and 108) displayed increased expression levels of five transcripts before treatment as compared with the expression levels in the remainder of the samples (Fig. 1). These included transcripts for vezatin (*VEZT*) and ephrin receptor A3 (*EPHA3*) that encode proteins involved in cell adhesion; protein tyrosine phosphatase, non-receptor type 7 (*PTPN7*) that encodes for a phosphatase regulating macrophage mitogen-activated protein kinases (MAPK) and TNF production; max-like dimerization protein X (*MLX*) that encodes for a stress sensor as part of the MYC/MAX/MLX network; and acyl-CoA thioesterase 8 (*ACOT8*) that encodes for an enzyme that regulates lipid metabolism. Expression of these transcripts was downregulated after TNFi therapy for all ANA-positive patients and markedly downregulated in two ANA-positive patients as illustrated in Fig. 1. Other phosphatases, for example many of the dual specificity phosphatase (DUSP) family members including *DUSP3* (Fig. 1), were expressed at lower levels

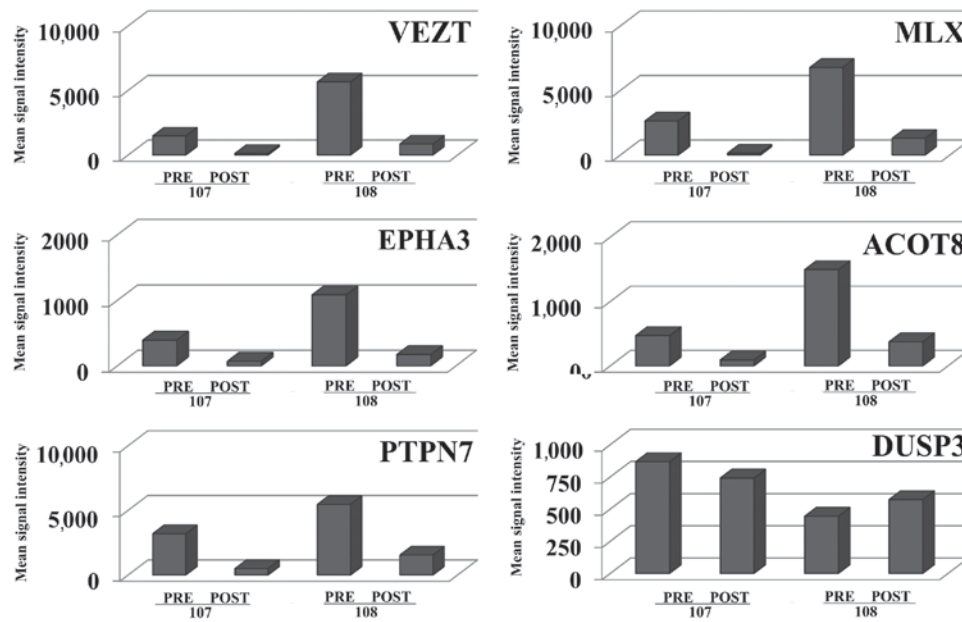


Figure 1. Transcripts demonstrating highly differential expression were linked to cell adhesion, cell stress and the metabolome. Transcripts that were expressed at increased levels in ANA-positive RA patients and were strongly downregulated in samples taken after tumor necrosis factor inhibitor therapy in two RA patients (nos. 107 and 108) are shown. Differential expression of transcripts for all ANA-positive converters included ACOT8 (4.27-FC), DNAJC7 (2.9 FC), EPHA3 (6.28 FC), MLX (6.57 FC), PARVB (4.46), PPT1 (3.2 FC), PTPN7 (4.23 FC), RAB5C (4.45), RBP3 (3.23 FC) and VEZT (7.28 FC). Pre, prior to therapy; Post, after therapy; ANA, anti-nuclear antibody; FC, fold change; ACOT, acyl-CoA thioesterase 8; DNAJC7, DnaJ (Hsp40) homolog, subfamily C, member 7; EPHA3, ephrin receptor A3; MLX, max-like dimerization protein X; PARVB, β -parvin; PPT1, palmitoyl-protein thioesterase 1; PTPN7, protein tyrosine phosphatase, non-receptor type 7; RAB5C, ras-related protein rab-5C; RBP3, retinol binding protein 3, interstitial; VEZT, vezatin.

than *PTPN7* and did not demonstrate the marked up- or downregulation observed for *PTPN7* in the ANA-positive patient samples. These transcripts were detected at lower levels in the remaining RA patient samples, suggesting that the two RA patients might represent a unique subgroup of ANA-positive converters or might have had much more active disease at the time of collection of the pre-treatment samples. However, following the initiation of TNFi therapy a marked reduction in expression was observed in contrast to other expressed genes. The remaining differentially regulated transcripts exhibited a dynamic pattern and included DnaJ (Hsp40) homolog, subfamily C, member 7 (*DNAJC7*), β -parvin (*PARVB*), palmitoyl-protein thioesterase 1 (*PPT1*), ras-related protein rab-5C (*RAB5C*) and retinol binding protein 3, interstitial (*RBP3*) in all of the ANA-positive samples after TNFi therapy (Fig. 2). The ANA-negative RA patient samples expressed low levels of these transcripts with no statistically significant differences in gene expression between before vs. after TNFi therapy groups, although there was a consistent trend toward downregulation of transcripts as shown in Fig. 2.

No correlation exists between ANA subgroups and transcriptional profiles associated with responders to TNFi therapy. Subsequently, an ‘eight gene signature’ was analyzed that has previously been shown to predict clinical responders to infliximab for patients with RA in a study by Julia *et al* (21). In the present study, 5 of the 8 predictive transcripts were detected, including interleukin 2 receptor β (*IL2RB*), granulysin (*GPLY*), solute carrier family 2 (facilitated glucose transporter), member 3 (*SLC2A3*; also known as *GLUT3*), cathelicidin antimicrobial peptide (*CAMP*) and toll-like receptor 5 (*TLR5*) in both

ANA-positive and ANA-negative subsets of RA patient samples as shown in Fig. 3. There were no significant differences between the before and after treatment groups. Levels of the other three predictive ‘responder transcripts’, max dimerization protein 4 (*MXD4*), SH2 domain containing 1B (*SH2D1B*) or major histocompatibility complex, class II, DR beta 3 (*HLA-DRB3*) were below the threshold settings for the analyses. Significant levels of *HLA-DRB3* transcripts were detected in one ANA-negative RA patient sample, and for this patient *HLA-DRB3* transcript levels decreased following TNFi therapy (2381 pre-therapy vs. 1848 post-therapy MSI). Overall, these transcripts were similar to the majority of expressed transcripts in that lower expression levels were detected by the probe sets in most patients initially and there was a trend toward downregulation of transcripts in the ANA-negative samples whilst the ANA-positive samples demonstrated a varied or dynamic pattern of transcript levels between the before and after therapy samples.

ANA subgroups express unique transcriptional profiles. Likewise, differences in patterns were observed for the ANA-positive samples compared with the ANA-negative samples for interferon (IFN)-inducible (IFI) transcripts and transcripts associated with immune cell subsets after TNFi therapy (Figs. 4 and 5). Fig. 4 shows that a subset of patients in the ANA-negative group expressed increased levels of IFN-regulated transcripts after TNFi therapy and that the remainder in this group expressed variable levels, consistent with activation of IFN-inducible pathways. By contrast, little change was detected in IFI transcripts in ANA-positive patients. The IFI transcripts demonstrated a pattern that deviated from the observed transcriptional profiles observed in comparisons of statistically significant transcripts between ANA groups.

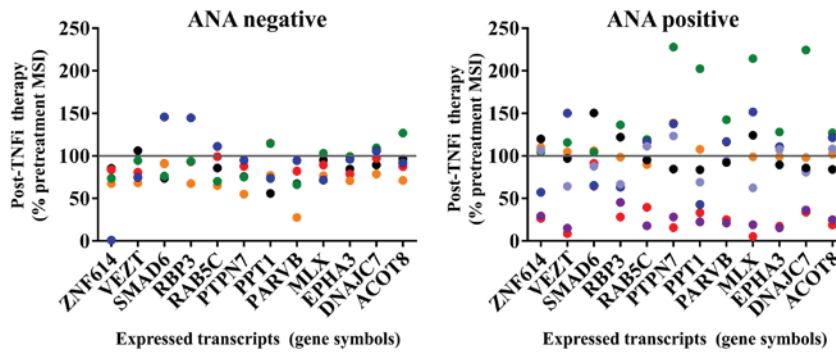


Figure 2. Dynamic gene expression profiles in ANA-positive RA samples were observed after TNFi therapy. Post treatment gene expression levels are expressed as the percentage MSI of pre-treatment levels in individual patient samples. The ANA-negative RA patient samples expressed low levels of these transcripts with no statistically significant differences in gene expression, although this group trended toward downregulation after therapy. A line at the 100 percent mark indicates the pre-treatment levels for each sample. Key to color points: ANA negative group: Patient 101 is black, 102 is red, 104 is orange, 105 is green and 106 is blue. ANA positive group: Patient 103 is black, 107 is red, 108 is mauve, 109 is orange, 110 is green and two post-treatment visits are shown for patient 111, visit 1 blue and visit 2 periwinkle. ANA, anti-nuclear antibody; RA, rheumatoid arthritis; TNFi, tumor necrosis factor inhibitor; MSI, mean signal intensity; ZNF614, zinc finger protein 614; YEZT, vezatin; RBP3, retinol binding protein 3, interstitial; RAB5C, ras-related protein rab-5C; PTPN7, protein tyrosine phosphatase, non-receptor type 7; PPT1, palmitoyl-protein thioesterase 1; PARVB, β -parvin; MLX, max-like dimerization protein X; EPHA3, ephrin receptor A3; DNAJC7, DnaJ (Hsp40) homolog, subfamily C, member 7; ACOT8, acyl-CoA thioesterase 8.

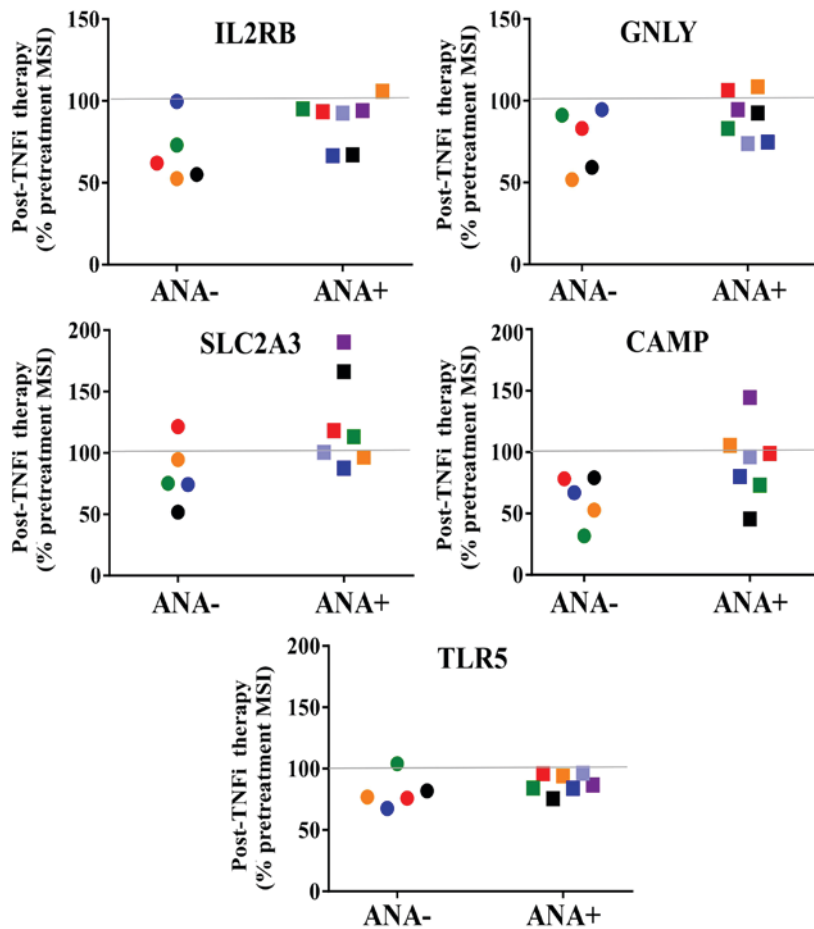


Figure 3. Five transcripts from the 'eight gene signature' predictive of responders to TNFi therapy. Post-treatment results for ANA- and ANA+ rheumatoid arthritis patient groups are shown for 5 predictive transcripts present in the samples. For comparison of ANA- and ANA+, all pre-treatment MSI values were set at 100% for each individual sample. The ANA- group expressed lower levels of transcripts, which were not significantly different. The line at the 100% mark denotes the pre-treatment levels for all samples. ANA-, anti-nuclear antibody negative; ANA+, anti-nuclear antibody positive; TNFi, tumor necrosis factor inhibitor; MSI, mean signal intensity; IL2RB, interleukin 2 receptor, β ; GNLY, granulysin; SLC2A3, solute carrier family 2 (facilitated glucose transporter), member 3; CAMP, cathelicidin antimicrobial peptide; TLR5, toll-like receptor 5.

Major leukocyte subset markers were also examined, such as the myeloid cell marker *CD14*, the T-cell marker

CD3Z (i.e., *CD247* or T cell receptor ζ chain) and cytolytic lymphocyte marker *CD8A* (*CD8* α chain). *CD74*, the HLA

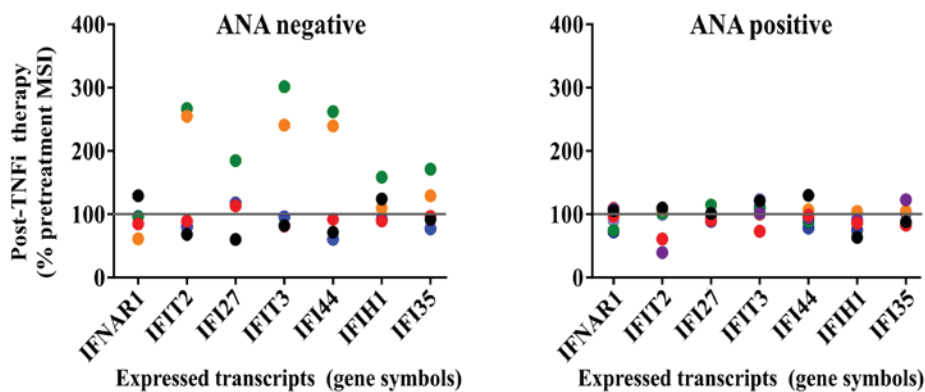


Figure 4. IFI transcripts are elevated in a subset of ANA negative patients after TNFi therapy. Post-treatment results for ANA negative and ANA positive RA patient groups are shown for IFN-regulated transcripts. All pre-treatment MSI values were set at 100% for each individual sample. A subset of samples from the ANA negative group exhibited increased expression of IFI transcripts; however, this difference was not statistically significant for comparisons of the before and after therapy groups. The ANA positive group demonstrated less overall IFI gene expression and post-treatment IFI transcript levels were similar to pre-treatment levels. IFNAR1, interferon alpha and beta receptor subunit 1; IFIT, interferon induced protein with tetratricopeptide repeats; IFI, interferon-inducible; IFIH1, interferon induced with helicase C domain 1; ANA, anti-nuclear antibody; TNFi, tumor necrosis factor inhibitor; MSI, mean signal intensity.

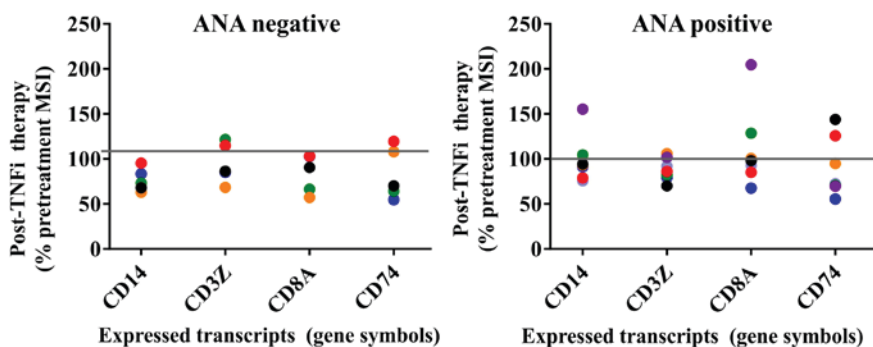


Figure 5. Transcripts for PBMC subset markers and invariant chain expression before and after TNFi therapy. Post-treatment results for ANA negative and ANA positive rheumatoid arthritis patient groups are shown for PBMC cell surface subset markers CD14, CD3Z, CD8A and CD74 transcripts. All pre-treatment MSI values were set at 100% for each individual sample. The ANA negative group demonstrated overall greater consistency in post-treatment transcript levels. The line at the 100% mark indicates the pre-treatment levels for all samples. PBMC, peripheral blood mononuclear cell; TNFi, tumor necrosis factor inhibitor; ANA, anti-nuclear antibody; MSI, mean signal intensity. CD14, cluster of differentiation 14; CD3Z, CD247 or T cell receptor ζ chain; CD8A, CD8 alpha chain; CD74, cluster of differentiation 74.

Class II invariant chain was also included for comparison. The majority of samples trended toward downregulation after TNFi therapy in the two ANA groups; however, once again the ANA-positive group displayed greater variation with both up- and downregulated gene expression. Transcripts for toll-like receptors (TLRs) including TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR8 were not significantly altered in either ANA group, although the mean signal intensity of all TLRs with the exception of TLR1 was near the threshold of detection for the assay (data not shown). Similar results were noted for the Fc receptors *FCER1*, *FCGR2A*, *FCGR2B* and *FCGR3A*.

Discussion

We observed unique differential expression of transcriptional profiles in PBMCs from patients with RA after TNFi therapy and compared our findings to previous reports of TNFi gene signatures. Although our study was conducted with isolated PBMCs, activation markers of leukocyte subsets were not readily discernable in our dataset. However, we were able to

distinguish differential gene expression that correlated with increased metabolism, cell stress and unique adhesion molecules in groups and in individual patients. Thus, we were able to distinguish differential transcriptional profiles that were consistent with ongoing alterations in the immune response after TNFi treatment.

We have previously shown that myeloid cells express the highest levels of IFI transcripts in PBMC subsets from patients with lupus, which is now known as the 'interferon signature' (30). In the current study, *CD14* transcripts were downregulated in most samples in both ANA groups, consistent with myeloid cell activation. Yet the ANA-positive group exhibited decreased expression of IFI transcripts and these PBMCs displayed higher metabolomic activity as demonstrated by the highest differentially expressed transcript levels. It has been previously demonstrated that TNF and IFNs cross-regulate each other (31). Thus, increased circulating TNF levels is one possible explanation for the lack of increased IFI-inducible transcript expression after TNFi therapy in the ANA-positive group. Alternatively, it could be associated with

the minor circulating populations of inflammatory (CD16^{bright}) monocytes and dendritic cells that are expanded in autoimmune diseases (30,31). These cells might be depleted, sequestered in tissues or have undergone differentiation and thus not be responsive to increased signaling by IFNs. We attempted to examine transcript levels for markers that are associated with inflammatory monocytes and dendritic cells such as CD16, CD169 (also known as Siglec-1) or chemokine receptors such as CXCR3; however, only low levels of transcripts could be detected. Thus, this observation will require further studies of PBMCs from TNFi-treated patients assessed by a combination of flow cytometry and gene expression assays to confirm this mechanism.

A pattern of transcripts that were profoundly downregulated after TNFi therapy was observed in two patients who turned ANA-positive (Fig. 1). One transcript, vezatin (*VEZT*), encodes for a ubiquitous transmembrane protein that is a member of the cadherin family. Vezatin has been shown to be an adherens anchor as part of the cadherin and catenin complex (32,33). The role of this protein in leukocyte function has yet to be established; however, a study suggested that it can be cleaved from apoptotic cells by caspase 8 along with the IL21 receptor (34). We carried out imaging and flow cytometry experiments to confirm the expression of vezatin in PBMCs and found that it was expressed at similar levels in a small study of PBMCs from healthy controls and patients with RA (data not shown). Future studies of RA patients with active disease and documented therapies will be required to replicate the findings from our transcriptional analysis at the protein level.

Several other transcripts such as *MLX*, *EPHA3*, *ACOT8* and *PTPN7* exhibited similar expression profiles to *VEZT*. These transcripts belong to a diverse group of proteins. *EPHA3* originates from 'erythropoietin-producing hepatocellular' receptor and it belongs to a family of receptor tyrosine kinases that are activated by membrane proteins called ephrins. Elevated expression of the cell surface *EPHA3* receptor can be induced by TNF, and expression of *EPHA3* receptors is increased on leukocytes, endothelial cells and epithelial cells early in the inflammatory response, disrupting normal adhesion and altering cytoskeletal organization, thereby promoting trafficking into inflammatory sites (35,36). Other adhesion-related transcripts in the most highly differentially expressed gene list included *EGFL5*, which encodes for the epidermal growth factor-like family member 5 that appears to interact with integrins to regulate endothelial cell function (37). Also, a well-known cell adhesion gene, *PARVB*, encodes β -parvin (also known as affixin) that interacts with integrin-linked kinase and is involved in initial cell interactions with the extracellular matrix, cell migration and in cell survival (38,39).

Another differentially expressed transcript, *RAB5C* encodes for the small GTPase Rab5C, which is involved with Rab7 in the proper docking and fusion of intracellular vesicles such as early to late endosome conversion, to generate appropriate endocytic compartments (40). Notably, IFN γ upregulates transcription and protein expression of Rab5 family members in macrophages and these proteins have been implicated in intracellular membrane functions, including the killing of pathogens and Rac-dependent cell motility (41,42).

The differentially expressed transcript of *ACOT8*, also known as peroxisomal acyl-CoA thioesterase-1 (*PTE-1*) or *PTE-2*, encodes for the type II acyl-Coenzyme A thioesterase 8 (PTE-2 protein) that has a broad tissue distribution in mouse and man, resides in peroxisomes and is important for fatty acid metabolism. PTE-2 is highly conserved from yeast to man and it has been observed that fibrates and fasting regulate *ACOT8* gene expression via peroxisome proliferator-activated receptor (PPAR) α (43-46). Acyl-CoA thioesterases catalyze the hydrolysis of acyl-CoAs, thus releasing CoASH and free fatty acids. PTE-2 activity is regulated by and dependent on coenzyme A levels (43). It has been proposed that PTE-2 may have a key role in peroxisomal lipid metabolism releasing fatty acids of specific lengths in the β -oxidation pathway from predominantly medium length acyl-CoAs for export (46). Overexpression of PTE-2 is associated with increased PPAR γ expression and results in lipid accumulation, which is considered to contribute to an adipogenic phenotype (47). *PPT1* is another of the most highly expressed genes that is involved in lipid metabolism. *PPT1* encodes palmitoyl protein thioesterase 1 (PPT1 protein), a small glycoprotein with enzyme activity that removes thioester-linked fatty acyl groups (i.e. palmitate) from cysteines and contributes to the catabolism of lipidated protein in lysosomes (48,49).

The PTPN7 protein is a phosphatase that has been shown to regulate TNF production by downregulating MAPKs including ERK1, ERK2 and p38 MAPK in macrophages (50). In this previous study, stimulation with lipopolysaccharide (LPS) reduced PTPN7 which corresponded with increased TNF production. The role of PTPN7 in the regulation of MAPKs was confirmed by use of small interfering RNA and overexpression of exogenous PTPN7. By contrast, we examined the transcripts for a number of other phosphatases; many were DUSPs, which are not susceptible to direct LPS-induced effects (51) and their expression was consistent with *DUSP3* transcripts as shown in Fig. 1 and across all ANA-positive samples. These studies suggest that elevated *PTPN7* gene expression levels might reflect a compensatory mechanism to regulate TNF secretion.

Another highly differentially expressed transcript was for the *MLX* protein. *MLX* has been shown to primarily localize to the cytoplasm and respond to glucose and similar stimuli, serving as a metabolic sensor in the MYC/MAX/MLX network. *MLX* forms heterodimers with other transcription factor partners, which then translocate to the nucleus and suppress E-box containing promoters regulating cell growth, differentiation, nutrient uptake and stress responses (52,53). Interestingly, *MLX* has been implicated as a potential candidate susceptibility gene for Takayasu's arteritis (54). Notably, other members of this family, such as *MXII*, were also downregulated in the ANA-positive group. By contrast, *MYCN* demonstrated overall less variability in signal intensity before and after TNFi treatment (mean MSI before therapy 24,851 vs. 24,440 after therapy for all ANA-positive samples). Another member of this network, *MXD4* has been reported to be differentially regulated in the 'eight gene signature study' of predictive gene expression in TNFi responders (21).

The highly differentially expressed transcript *DNAJC7* is a gene regulating cell growth arrest and apoptosis. *DNAJC7* encodes for a heat shock protein 40 family member known as

DNAJC7 or TPR2. DNAJC7 is a binding partner and prolongs the life of p53 (55,56). DNAJC7 functions by displacing MDM2 from p53/MDM2 complexes and binding p53 (56,57). The p53 protein activates a number of genes involved in cell cycle arrest, senescence, apoptosis and other functions (58).

Finally, another highly differentially expressed transcript *SMAD6* encodes the Smad6 protein, which along with Smad7 comprise the inhibitory Smad family, provides negative feedback regulation in the signaling pathways of transforming growth factor- β and the growth factors known as bone morphogenetic proteins (BMPs) (59-62). Smad6 is a transcriptional repressor and has been shown to inhibit both cytoplasmic and nuclear BMP activity (63,64).

Possible functions for the remaining most differentially expressed genes *RBP3* and zinc finger protein 614 (*ZNF614*) remain unknown. *RBP3* encodes for the retinol binding protein 3 which is highly expressed on photoreceptor outer segments and is critical for the exchange of 11-*cis* retinol and all-*trans* retinol between photoreceptors and retinal pigment epithelium (65). Currently, there is little information available regarding the expression or function of RBP3 in peripheral PBMCs, although autoantibodies to RBP3 have been described (65). *ZNF614* encodes for a protein which possibly functions as a transcriptional regulator. More research on the function of ZNF614 will be necessary to determine a potential role in immune regulation.

After 3 months of therapy a canonical '8 gene responder signature' that was previously reported did not segregate groups in our RA samples (21). While the previous validation studies assessed samples at approximately the same time (after 14 weeks), differences including the profiling of whole blood and RA patients on concomitant methotrexate treatment with TNFi compared with methotrexate monotherapy alone may have affected the results (16). Thus, sampling our patients at later timepoints might have provided additional validation of the 'clinical responder transcriptional profile' observed in the previous study.

We compared the gene expression profiles from our dataset with 'RA gene expression signatures' from previous reports. Gene expression that was predictive of RA as reported by Edwards *et al* was analyzed for our dataset (66). No clear difference was observed in the expression of the transcripts reported by Edwards *et al* in individual RA patients when results before and after TNFi therapy were compared (data not shown). This is not unexpected given the differences in methods and platforms for these studies. In the future, standardized approaches for sample preparation and analysis will improve overall reproducibility between studies.

These observations suggest that TNFi treatment potentially downregulates transcripts for molecules involved in cell metabolism, cell adhesion and the cell stress response in a subset of patients with RA who develop ANA positivity after TNFi therapy. The most differentially expressed transcripts from patients remaining ANA-negative were mostly downregulated after therapy and similar results were observed in the analysis of a canonical TNF response predictor gene signature. Gene expression profiles from patients with RA converting to ANA positivity during therapy were more complex and demonstrated a dynamic gene expression pattern with both up- and downregulated transcripts for the same transcript obtained from different

individuals. Although all of the RA patients had clinically active disease at the initiation of therapy, we propose that the dynamic pattern observed post-treatment might be indicative of increased activation in the ANA-positive group, as has previously been suggested for B cells (67). Thus, these results warrant further studies to evaluate whether TNFi-treated ANA-positive RA patients demonstrate other indicators of increased peripheral immune activation and cell death compared with the ANA-negative group. These results suggest that transcriptional profiling is a more precise method of measuring the impact of TNF blockade on the cross-regulation between TNF and type I IFNs that can contribute to lupus-like symptoms.

Whereas samples from patients remaining ANA-negative demonstrated less overall differential regulation in assessed gene expression, the samples from patients with RA who converted to ANA-positivity during therapy were more complex, with two RA patients demonstrating striking increases in gene expression that reflected activation of cell adhesion, cell stress and lipid metabolism pathways, and these transcript levels were markedly reduced after treatment with TNFi therapy. Further studies will be required in order to attribute this unique gene expression profile to the effects on systemic inflammation; however, it is well-known that TNF upregulates parallel pathways of cell adhesion and metabolomics in immune responses. Therefore, these studies suggest novel markers and mechanisms that might contribute to RA and to the effects of TNFi therapy.

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