Peripheral Blood Expression of Nuclear Factor-κB-Regulated Genes Is Associated with Rheumatoid Arthritis Disease Activity and Responds Differentially to Anti-Tumor Necrosis Factor-α versus Methotrexate

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ABSTRACT. ***Objective.*** To evaluate peripheral blood expression of genes regulated by nuclear factor-κB (NF-κB), a key mediator of tumor necrosis factor-α (TNF-α) signaling, in patients with rheumatoid arthritis (RA) before and during treatment with anti-TNF-α or methotrexate (MTX). We analyzed association of gene expression with disease activity, rheumatoid factor (RF), age, sex, disease duration, treatment modality, and clinical response.

Methods. Sixty patients consented for RNA analysis at baseline and after 2 and 6 weeks of treatment. Disease activity was quantified using Disease Activity Score (DAS28) and C-reactive protein (CRP). Expression of 67 TNF-α-responsive, NF-κB-regulated genes was measured using Affymetrix arrays and RT-PCR.

Results. Expression of 34 genes was associated with DAS28-CRP, notably S100A12/calgranulin C, IL7R, and aquaporin 3. No association was observed with age, sex, RF, or disease duration. Expression of 16 genes changed in a manner that differed significantly between treatment groups. Eleven were reduced in anti-TNF-α-treated patients relative to MTX, while 5 were increased. The majority of these observations were confirmed using RT-PCR. Gene expression was not associated significantly with change in disease activity.

Conclusion. NF-κB-dependent gene expression in peripheral leukocytes is highly correlated with RA activity as measured by DAS28-CRP. Expression of many genes responds differentially to anti-TNF-α versus MTX, suggesting fundamentally different effects on the NF-κB pathway. This peripheral blood expression signature provides candidate markers that could lead to development of a simple, minimally invasive pharmacodynamic assay for RA treatments directed at the NF-κB pathway. Combination of gene expression data with clinical scores and serum markers may provide more sensitive and predictive measures of RA disease activity. (First Release August 1 2007; J Rheumatol 2007;34:1817–22)

Key Indexing Terms:
RHEUMATOID ARTHRITIS METHOTREXATE TUMOR NECROSIS FACTOR-α INHIBITOR TRANSCRIPT EXPRESSION ANALYSIS TRANSCRIPTION FACTOR NUCLEAR FACTOR-κB

Tumor necrosis factor-α (TNF-α) inhibitors are an efficacious therapy for rheumatoid arthritis (RA) and several other inflammatory conditions. TNF-α is a key proinflammatory cytokine and a central driver of RA pathophysiology. It is secreted by inflamed synoviocytes, and has both direct (e.g., promotion of osteoclast differentiation) and indirect (e.g., upregulation of chemokine production, leading to increased monocyte recruitment to the diseased joint) effects on the disease process.

Unlike many disease modifying therapies, for example, methotrexate (MTX) or leflunomide, anti-TNF-α therapies have a narrowly focused mechanism of action: all currently approved agents are biological therapeutics that bind TNF-α, preventing it from interacting with cell-surface receptors TNFR1 and TNFR2[1]. TNF-α binding to TNFR1 activates the nuclear factor-κB (NF-κB) signal transduction pathway. NF-κB, a transcription factor, remains inactive in a cytoplasmic complex with IκA and IκB until activation of TNFR1 promotes phosphorylation of IκB by IκKB; upon phosphorylation IκB is ubiquitinated and degraded, releasing NF-κB to

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translocate into the cell nucleus and activate transcription of a wide range of target genes[2].

NF-κB activation of disease-related cytokines and proteases has been experimentally demonstrated in RA synovial tissues[3], and TNF-α inhibitors have been shown to inhibit many NF-κB-mediated processes in diseased joints[4]. It is not known, however, whether TNF-α inhibitors significantly alter NF-κB-dependent gene expression patterns in non-disease tissues. In the case of peripheral blood leukocytes, at least 2 factors predict that such changes might be difficult to detect. First, although serum TNF-α levels are generally elevated in patients with RA relative to healthy individuals, absolute levels are much lower in the general circulation than at the disease site, suggesting a lower signal intensity. Additionally, the NF-κB signal transduction mechanism integrates signals originating at a large number of receptors, including TLR4, IL1R, VEGFR, and many others, such that elimination of just one agonist (TNF-α) might not elicit large changes in the overall behavior of the system.

Peripheral blood biomarkers of pathway-level changes resulting from inhibition of NF-κB signaling would be of significant value in elucidation of pharmacokinetic-pharmacodynamic (PK/PD) relationships for novel RA therapies that target other molecules in this pathway (e.g., IκKB, p38[5]), allowing them to be more easily studied and ultimately perhaps more safely utilized. To develop such biomarkers, we compared peripheral blood gene expression in RA patients beginning treatment with TNF-α inhibitors to that of patients beginning therapy with MTX.

MATERIALS AND METHODS

Patients. The study group included 60 patients, 30 starting treatment with MTX and 30 starting treatment with an anti-TNF-α therapy (etanercept, adalimumab, or infliximab; Table 1). Patients were invited to participate and gave informed consent for analysis of RNA expression when initiation of new RA therapy was indicated based on arthritis activity; they were not randomized to study medications. Patients met American College of Rheumatology (ACR) criteria for RA and had active disease (> 6 swollen and > 6 tender joints). Patients who were already taking MTX at study start (anti-TNF-α group only) were required to have maintained a stable dose for 12 weeks; for patients already taking prednisone a stable dose of < 10 mg/day for 4 weeks was required. At baseline, only one new RA medication, either MTX or anti-TNF-α, was started. Blood samples were obtained at study start (baseline) and 2 and 6 weeks after treatment initiation. Patients underwent disease activity evaluations including assessment of the ACR core set [painful and swollen joint count, pain index, patient and physician global assessment, Health Assessment Questionnaire and C-reactive protein (CRP) testing] and serum rheumatoid factor (RF) at each timepoint. Disease activity was quantitated using Disease Activity Score (DAS28)-CRP (available from: http://www.das-score.nl/www.das-score.nl/index.html). Clinical response was assessed as change in DAS28-CRP from baseline to 12 weeks (last observation carried forward method).

Table 1. Characteristics of patient population. All variables are assessed at study baseline unless otherwise indicated; values are mean ± SD unless otherwise indicated.

	Anti-TNF-α* (n = 30)	MTX (n = 30)**
Age, yrs	53.1 ± 14.8	53.5 ± 14.0
Disease duration, yrs	11.8 ± 11.4	12.8 ± 8.6
Percent female	80	80
RF-positive, %	55	83
Concomitant MTX, %	57	NA
Concomitant corticosteroid, %	37	53
Baseline DAS	5.1 ± 0.9	5.6 ± 1.1
Change in DAS (Week 12)†	–2.2 ± 1.2	–1.3 ± 1.2

* Enbrel, n = 23, 50 mg/week; Humira, n = 6, 40 mg/2 weeks; Remicade, n = 1, 3 mg/kg/6 weeks. ** Starting MTX dose ranged from 7.5 to 15 mg/week. † $p < 0.001$ for difference between treatment groups. MTX: methotrexate, DAS: Disease Activity Score (DAS28-CRP), NA: not applicable.

Gene expression profiling. Peripheral blood was drawn into PAXgene (PreAnalytix) tubes and stored at –80°C; RNA was isolated using PreAnalytix PAXgene™ 96 Blood RNA Kit (Qiagen, Valencia, CA, USA). Total peripheral blood RNA was subjected to PNA-based globin mRNA Reduction Protocol (Applied Biosystems, Foster City, CA, USA) prior to T7 labeling using an Invitrogen cDNA synthesis kit and Affymetrix IVT kit for cRNA. Affymetrix Gene Chip U133 Plus 2 hybridization, washing, and scanning followed manufacturer's protocols. Probesets with median MAS2.0 values < 100 were excluded from analysis. The set of TNF-α-responsive, NF-κB-dependent genes for analysis was established by review of relevant *in vitro* experimental results[6-8].

For reverse transcription-polymerase chain reaction (RT-PCR) analyses, first-strand cDNA synthesis was performed using a reverse transcription system (Applied Biosystems) according to the manufacturer's protocol, except that both oligo-dT and random hexamers were used for priming. The quality of resulting cDNA samples was assessed by analysis of 18S and $ß_2$-microglobulin transcripts by Real-Time PCR® using the ABI-Prism® 7700 (Applied Biosystems). Primers and MGB Eclipse® probes for Real-Time PCR® analysis of NF-κB-regulated genes were designed and validated by Nanogen Inc. (Bothell, WA, USA). Patient samples were assayed using these reagents and an ABI-Prism 7900. Data analysis used SDS software v2.1 (Applied Biosystems); transcript abundance for each sample was normalized to a designated "housekeeping" gene ($ß_2$-microglobulin) as described in Applied Biosystems User Bulletin No. 2.

Statistical analysis. Affymetrix gene expression values (MAS2.0) were natural-log transformed prior to analysis. Association of baseline gene expression data with clinical variables was assessed using univariate linear regression. Tests of association between treatment modality (anti-TNF-α or MTX) and changes in gene expression used gene expression data from both 2 and 6-week timepoints. These analyses used multivariate logistic regression, controlling for baseline gene expression, and observed changes in disease activity. Tests of association between changes in gene expression and change in disease activity used gene expression data from 2 and 6-week timepoints and disease activity (DAS28-CRP) scores assessed at 12 weeks. These analyses used multivariate linear regression and were controlled for baseline gene expression level and treatment modality. P values were adjusted for multiple comparisons within each analysis using the step-up procedure of Benjamini and Hochberg[9]. RT-PCR data were analyzed in a manner identical to the Affymetrix Gene Chip data. Hierarchical clustering of gene expression data and generation of heatmap graphics used Gene Pattern software[10].

RESULTS

Patients. Sixty patients were enrolled and had at least one followup visit where an RNA sample was obtained, 30 in each treatment group. One anti-TNF-α patient did not attend the 6-week followup, while 5 patients (3 MTX and 2 anti-TNF-α) did not have a 2-week followup. Eighteen patients did not attend the 12-week followup visit (11 anti-TNF-α and 7

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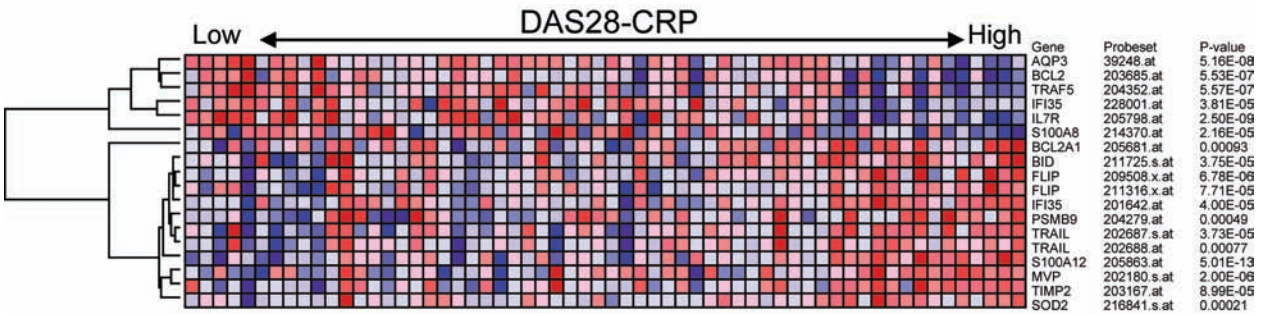
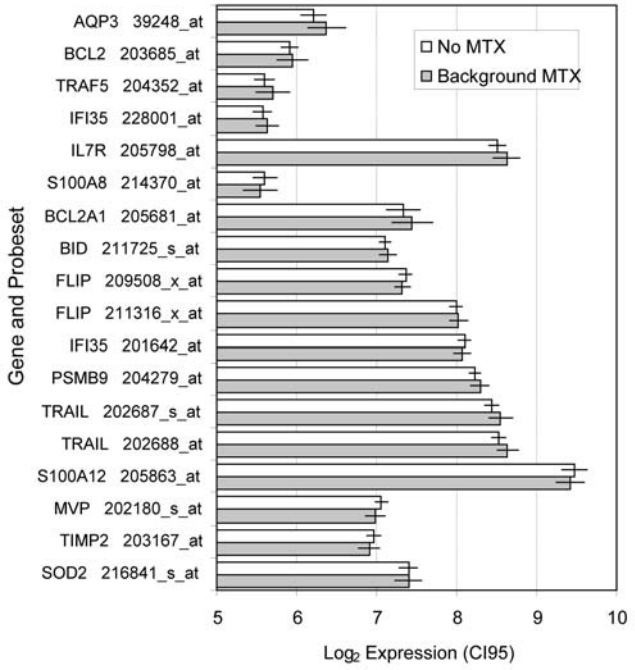


Figure 1. Heatmap illustrating differential expression of NF-κB-regulated transcripts associated (adjusted $p < 0.001$) with RA disease activity (DAS28-CRP) at study baseline. Color indicates higher (red) versus lower (blue) transcript abundance. Patient samples are ordered from lowest (left) to highest (right) Disease Activity Score.



MTX); for these patients the 6-week disease activity data were carried forward. Seventeen patients starting anti-TNF-α were taking a stable dose of MTX at study start; their duration of prior MTX treatment ranged from 4 to 167 months (median 33 mo). Additionally, 27 patients (16 MTX and 11 anti-TNF-α) were taking a stable dose of prednisone (Table 1). The anti-TNF-α and MTX treatment groups were similar with respect to demographic characteristics and baseline disease activity; as expected, they did differ significantly with respect to clinical response (Table 1); clinical responses did not differ based on concomitant medication.

Gene expression profiling. Our literature review resulted in a list of 98 genes that were TNF-α-responsive, NF-κB-depend-

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Figure 2. Baseline gene expression (mean ± 95% CI) in patients who were taking a stable dose of MTX at study start (n = 17; median duration of exposure 33 mo) versus those who were not (n = 43). Genes shown are the same ones represented in Figure 1. No statistically significant differences were observed between the 2 groups (adjusted $p > 0.05$).

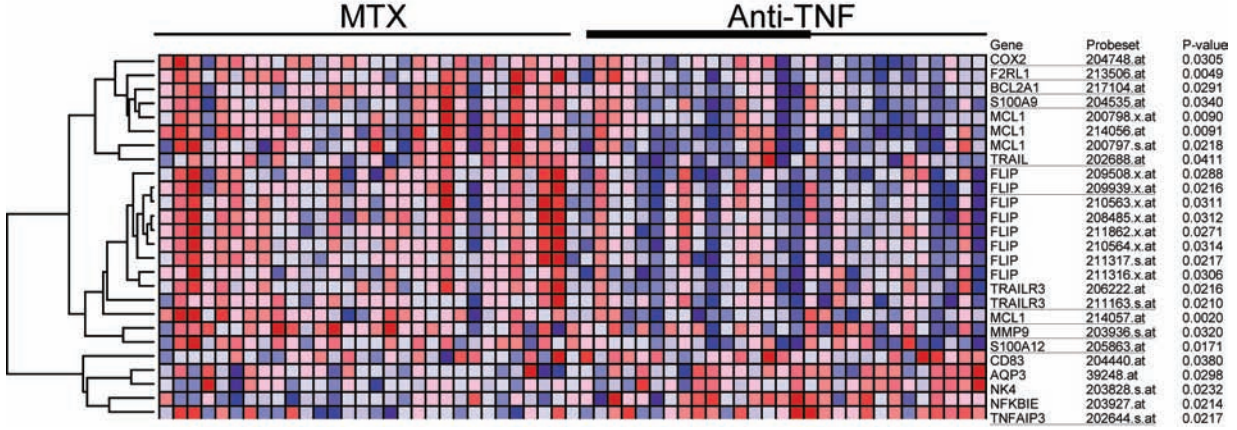

Figure 3. Heatmap illustrating significant (adjusted $p < 0.05$) differential effect of anti-TNF-α versus MTX on NF-κB-regulated gene expression. Heavy underline indicates those anti-TNF-α-treated patients who were receiving a stable dose of MTX at study baseline. Color indicates increase (red) versus decrease (blue) in gene expression at 6-week timepoint relative to baseline.

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ent and represented on the Affymetrix U133 Plus 2 chip. A total of 67 genes (110 probesets) were expressed above our detection cutoff (median MAS2.0 > 100) and were used in subsequent analyses (a list of all genes, probesets, and median intensities is available from the authors upon request).

Patient disease activity at baseline was significantly associated with expression level of 34 genes (adjusted $p < 0.05$). Of the 16 most strongly associated genes ($p < 0.0001$; Figure 1), 6 were inversely correlated with DAS28-CRP and 10 were positively correlated. We additionally examined associations of baseline gene expression with sex, RF positivity, disease duration, baseline treatment with disease modifying antirheumatic drugs (DMARD; patients taking a stable dose of MTX versus those who were not), and clinical response (percentage change in DAS28-CRP after 12 weeks of treatment). None of these analyses revealed significant associations (adjusted $p > 0.05$ for all probesets). Figure 2 illustrates the similarity in baseline gene expression between patients taking a stable dose of MTX versus those who were not taking MTX at study baseline.

Changes in NF-κB-mediated gene expression following treatment initiation differed notably between treatment groups. At 2 and 6 weeks, expression of 11 genes was significantly reduced in the anti-TNF-α group relative to MTX, while 5 genes were significantly increased (Week 6 data are shown in Figure 3). When both treatment groups were analyzed together, no gene expression changes were significantly associated with change in disease activity. Considering that this might be explained in part by the need to control the analysis for the significant difference in magnitude of response between treatment groups, or possibly by lack of relevance of NF-κB-regulated genes to MTX efficacy, we repeated this analysis using only the anti-TNF-α-treated group, but still did not observe significant associations with response. We also performed analyses controlling for concomitant use of corticosteroids and MTX (allowed in the anti-TNF-α group), but observed no significant effect on changes in gene expression (adjusted $p > 0.05$ for all genes). Figure 4 illustrates changes in gene expression from baseline to Week 6 in anti-TNF-α-treated patients who did ($n = 17$) versus those who did not ($n = 13$) take a stable dose of MTX prior to and during the study period.

RT-PCR analyses. To corroborate our key findings using a non-hybridization-based assay, we designed RT-PCR reagents complementary to probeset sequences in 11 genes whose expression pattern differed significantly between treatment groups (underlined in Figure 3). Eight of 11 RT-PCR assays recapitulated the pattern of change seen in the Affymetrix data (Figure 5A), with expression reduced in anti-TNF-α patients and increased or unchanged in MTX patients. Two of the 8 did not reach statistical significance when treatment groups were compared controlling for differences in clinical change (Figure 5B).

DISCUSSION

Our focused analysis of NF-κB-regulated genes revealed a strong peripheral blood gene expression signature of RA disease activity. Fully half the genes studied (34/67) were significantly associated with DAS28 at study baseline. Among the strongest associations were calgranulins A and C (S100A8 and A12). Synovial calgranulin levels have been shown to differentiate patients with erosive RA versus nonerosive RA[11], and their peripheral blood expression has been shown to differentiate RA patients from healthy controls[12], but the observation that peripheral expression directly correlates with disease activity has not previously been made.

We did not observe significant association between gene expression levels and baseline therapy (Figure 2); the 17 patients receiving a stable dose of MTX did not differ significantly from those who were not so treated. This may be because MTX does not have a known target upstream of NF-κB, such that it would not be expected to directly modulate activity of this transcription factor. Additionally, the wide range of MTX treatment duration (4–167 mo) likely obscures resolution of effects unique to, for example, newly-treated patients.

We observed differential responses of many NF-κB-regulated genes to anti-TNF-α versus MTX therapies; this indicates that, despite the fact that both are efficacious treatments for RA, these 2 DMARD have fundamentally different effects on one of the central signal transduction systems in RA pathophysiology. The majority of genes were downregulated in response to TNF-α inhibition. However, a subset, including the NF-κB complex regulatory protein NEMO (NFKBIE), were observed to increase (Figure 3), suggesting that these genes may be subject to NF-κB-mediated inhibition of expression. As was the case for baseline gene expression levels, this effect was not altered by concomitant therapy with MTX. Changes in gene expression observed between baseline and 6-week timepoints not differ significantly between the 57% of anti-TNF-α patients receiving background MTX and those receiving anti-TNF-α only (Figure 4; adjusted $p > 0.05$ for all genes). We were able to reproduce the observed pattern of regulation in 8 of 11 such genes using RT-PCR. In each case we observed robust downregulation of gene expression in the anti-TNF-α-treated group, although in 2 cases the comparison to the MTX-treated group was not statistically significant (Figure 5).

The gene sets we observed to associate with baseline disease activity and to change following anti-TNF-α treatment had several common members, including S100A12, AQP3, BCL2A1, and FLIP. Despite this, we did not observe any genes to change in a manner that was associated significantly with change in disease activity following treatment. There may be multiple reasons for this. First, the need to control our analyses for the strong association between treatment group and disease response may have obscured an underlying relationship between gene expression changes and magnitude of

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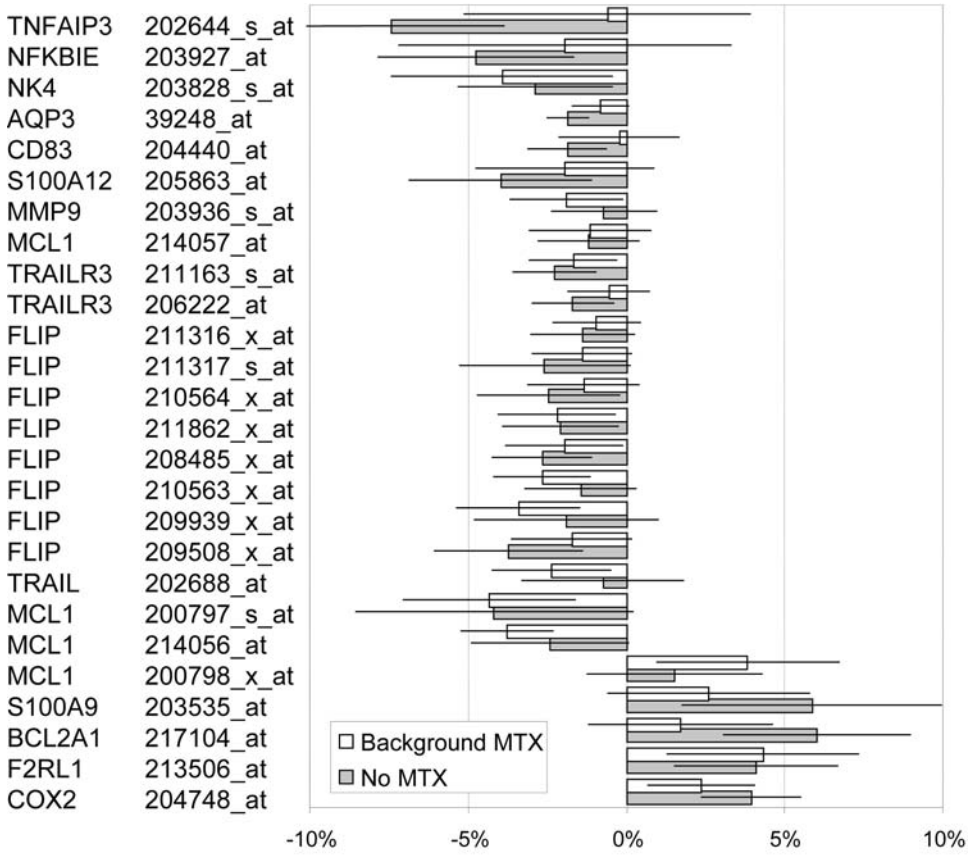

Figure 4. Changes in gene expression (mean ± 95% CI) from baseline to 6 weeks in anti-TNF-α-treated patients who did (n = 17) versus those who did not (n = 13) take a stable dose of MTX prior to and during the study period. Genes shown are the same ones represented in Figure 3. No statistically significant differences were observed between the 2 groups (adjusted $p > 0.05$).

disease response. Additionally, in this short-term study we assessed response after only 12 weeks of treatment. Particularly in the case of MTX patients, a longer followup period might be required to observe the full extent of clinical response. Nevertheless, our results do suggest that some tissue-level gene regulation patterns associated with disease pathophysiology, such as elevated NF-κB-regulated expression of FLIP in RA synovial fibroblasts[13], may be recapitulated in peripheral blood leukocytes, where we observed FLIP expression was positively correlated with disease activity and was downregulated in response to anti-TNF-α therapy (Figure 1).

Our approach to biomarker discovery was designed to yield high-confidence biomarkers of inhibition of the NF-κB pathway that are straightforward to apply in a clinical research setting. Our data analysis strategy was to maximize power and minimize false-positive results. By considering only 110 probesets, rather than all 17,057 Affymetrix Gene Chip U133 Plus 2 probesets with median expression values > 100, we greatly reduced the statistical burden of multiple hypothesis testing; by focusing on previously validated TNF-α- and NF-κB-responsive genes, we ensured that any positive findings would have strong *a priori* biological validation. Utilizing RNA from peripheral blood collected directly into RNA stabilization reagent (PAXgene tube) provides a simple, clinically feasible assay that avoids potential gene-expression artifacts associated with cell activation during, for example, peripheral blood mononuclear cell isolation protocols. Finally, translation of the most robust Affymetrix Gene Chip results into RT-PCR assays allows these transcripts to be assayed in clinical samples without the cost and potential regulatory implications of whole-genome array profiling.

Owing to the modest size and nonrandomized nature of our patient cohort, these results should be viewed as hypothesis-generating; they provide a clearly defined set of candidate biomarkers for validation in future randomized trials. In addition to potentially facilitating development of new RA therapeutics, gene expression biomarkers like these may ultimately be combined with serum CRP and clinical evaluations to yield more robust, treatment-responsive metrics of RA disease activity.

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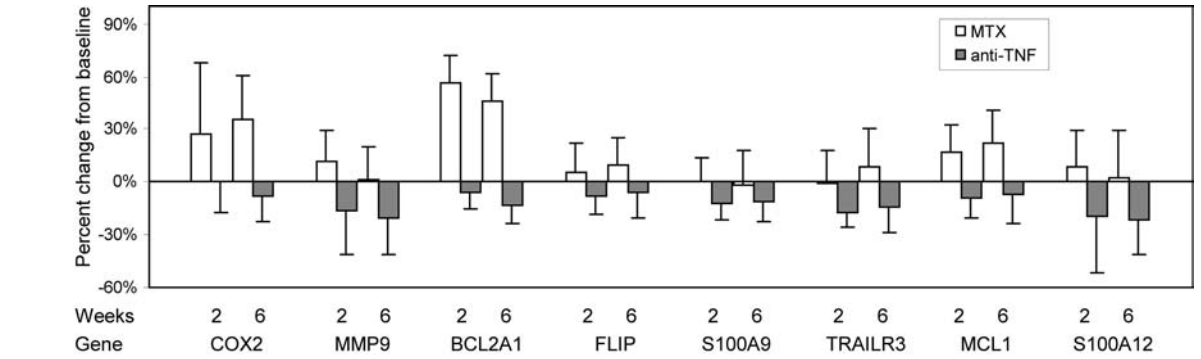

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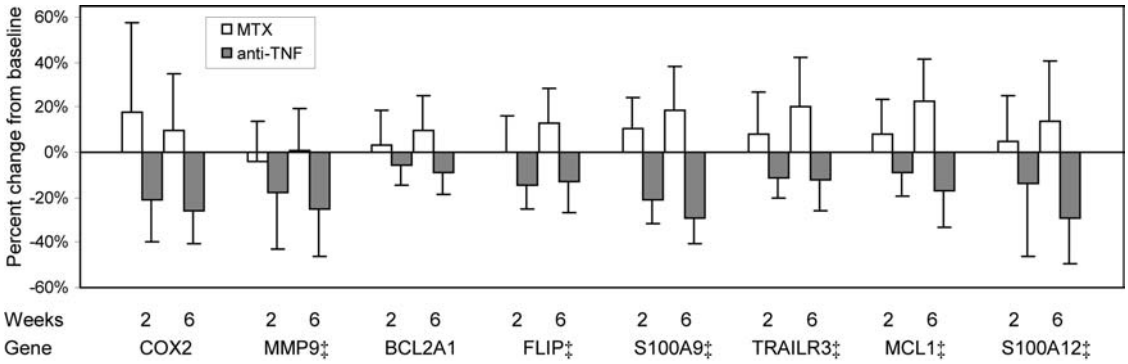

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Figure 5. Changes in peripheral blood expression of NF-κB-regulated transcripts, relative to baseline, in patients treated with anti-TNF-α and MTX. A. Affymetrix gene expression data (Gene Chip U133v2); treatment groups differ significantly following adjustment for differences in clinical change (adjusted $p < 0.05$ for all genes). B. Verification of Gene Chip results using quantitative RT-PCR; ‡significant ($p < 0.05$) difference in gene expression between treatment groups.

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