Concise report

Interferon gene expression signature in rheumatoid arthritis neutrophils correlates with a good response to TNFi therapy

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Abstract

Objective. The aim of this study was to use whole transcriptome sequencing (RNA-Seq) of RA neutrophils to identify pre-therapy gene expression signatures that correlate with disease activity or response to TNF inhibitor (TNFi) therapy.

Methods. Neutrophils were isolated from the venous blood of RA patients ($n = 20$) pre-TNFi therapy and from healthy controls ($n = 6$). RNA was poly(A) selected and sequenced on the Illumina HiSeq 2000 platform. Reads were mapped to the human genome (hg19) using TopHat and differential expression analysis was carried out using edgeR (5% false discovery rate). Signalling pathway analysis was carried out using Ingenuity Pathway Analysis (IPA) software. IFN signalling was confirmed by western blotting for phosphorylated signal transducer and activator of transcription (STAT) proteins. Response to TNFi was measured at 12 weeks using change in the 28-item DAS (DAS28).

Results. Pathway analysis with IPA predicted activation of IFN signalling in RA neutrophils, identifying 178 IFN-response genes regulated by IFN-α, IFN-β or IFN-γ ($P < 0.01$). IPA also predicted activation of STAT1, STAT2 and STAT3 transcription factors in RA neutrophils ($P < 0.01$), which was confirmed by western blotting. Expression of IFN-response genes was heterogeneous and patients could be categorized as IFN-high or IFN-low. Patients in the IFN-high group achieved a better response to TNFi therapy [ΔDAS28, $P = 0.05$, odds ratio (OR) 1.4 (95% CI 1.005, 1.950)] than patients in the IFN-low group. The level of expression of IFN-response genes (IFN score) predicted a good response [European League Against Rheumatism (EULAR) criteria] to TNFi using receiver operating characteristic curve analysis (area under the curve 0.76).

Conclusion. IFN-response genes are significantly up-regulated in RA neutrophils compared with healthy controls. Higher IFN-response gene expression in RA neutrophils correlates with a good response to TNFi therapy.

Key words: rheumatoid arthritis, neutrophil, interferon, gene expression, RNA-Seq.

Introduction

Delay in achieving adequate disease control in RA can lead to irreversible damage, disability and poor quality of life. Recent technological advances have enabled researchers to take genome-wide approaches to understanding the molecular causes of disease heterogeneity and provide opportunities to discover novel biomarkers of drug resistance in RA [1]. Neutrophils play an important role in the development and progression of RA through the release of proteases that damage host tissue and via the synthesis and secretion of cytokines, chemokines and leucotrienes, which orchestrate the activation of other inflammatory cells [2, 3]. RA neutrophils have an activated phenotype that is down-regulated by TNF inhibitors (TNFi) in line with clinical improvements in disease activity [4]. These changes in neutrophil function include changes in gene expression that mirror dynamic changes observed when neutrophils are stimulated with cytokines

in vitro [5]. Thus quantification of these changes in neutrophil gene expression may serve as a good surrogate marker of disease activity in RA.

Peripheral blood provides a source of patient material that is more convenient and less invasive than synovial tissue in the search for candidate biomarkers of disease response to therapy. However, measuring transcriptome changes in whole blood leucocyte samples is not without problems and may have led to important markers being missed. For example, interpretation of results from whole blood preparations may be confounded by dynamic variations in leucocyte subpopulations or activation states that occur both within individuals and during changes in disease activity [1]. Studying homogeneous cell types may therefore be preferable, at least for initial exploratory investigations. The abundance of neutrophils in peripheral blood, together with their dynamic regulation of gene expression in response to external stimuli [5] and TNFi therapy [4], makes them good candidates to investigate for potential biomarkers. This study used whole transcriptome sequencing (RNA-Seq) to determine which signalling pathways are activated in RA peripheral blood neutrophils and to identify any molecular biomarkers that may correlate with response, or lack of response, to TNFi therapy.

Patients and methods

Patients and controls

Participants gave written informed consent according to the Declaration of Helsinki. Ethics approval was obtained from the University of Liverpool Committee on Research Ethics and Sefton Adults. Patients fulfilled ACR criteria for RA [6] and were about to begin TNFi therapy. Patient clinical characteristics pre- and post-therapy are shown in supplementary Table S1, available at *Rheumatology* Online. Response to therapy was based on the decrease in the 28-item DAS (DAS28) score measured after 12 weeks according to EULAR guidelines [6].

Isolation of neutrophils

Blood was collected into lithium–heparin vacutainers from RA patients ($n = 20$) and healthy controls ($n = 6$). Neutrophils were isolated within 30 min of blood collection, as previously described [5].

Isolation of RNA

RNA was isolated using TRIzol–chloroform (Invitrogen, Paisley, UK) precipitation, as previously described [5], and cleaned using an RNeasy mini kit (Qiagen, Manchester, UK), including a DNA digestion step. RNA integrity was assessed using a 2100 Bioanalyser RNA Nano chip (Agilent Technologies, Wokingham, UK) and was routinely found to be $\geqslant 7.0$.

Library generation and sequencing

Total RNA was enriched for mRNA using poly(A) selection. Standard Illumina (San Diego, CA, USA) protocols were used to generate 50 bp single-end read libraries that were sequenced on the Illumina HiSeq 2000 platform.

Read mapping and gene annotation

Reads were mapped to the human genome (hg19) using TopHat version 2.0.4 and gene expression [reads per kilobase per million (RPKM)] values were calculated using Cufflinks version 2.0.2 [7]. A minimum RPKM threshold of expression of $\geqslant 0.3$ was applied to the data in order to minimize the risk of including false positives against discarding true positives from the dataset [5]. Count data were generated using HTSeq version 0.5 [8]. Statistical analysis was carried out using edgeR version 3.0.8 [9] with a 5% false discovery rate (FDR).

Bioinformatics

Bioinformatics analysis was carried out using Ingenuity Pathway Analysis (IPA; Qiagen Silicon Valley, Redwood City, CA USA; http://www.ingenuity.com), as previously described [5]. Hierarchical cluster analysis was carried out using MeV version 4.8 [10] using Manhattan clustering and average linkage.

Western blotting of phosphorylated proteins

Freshly isolated RA and healthy control neutrophils were rapidly lysed in boiling Laemmli buffer containing phosphatase inhibitor cocktail II (Calbiochem, Nottingham, UK). Protein samples (equivalent to 10^5 cells) were separated by SDS-PAGE (10% gel) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Watford, UK). Primary antibodies were phospho-STAT1 (Tyr701), phospho-STAT2 (Tyr690), phospho-STAT3 (Ser705), phospho-STAT5 (Tyr 694) (all 1:1000; Cell Signalling Technologies, Danvers, MA, USA), and actin (1:10 000; Abcam, Cambridge, UK). Secondary antibodies were anti-rabbit IgG (Cell Signalling Technologies) and anti-mouse IgG (Sigma, Gillingham, UK) horseradish peroxidase (HRP)-linked antibodies (1:5000). Bound antibodies were detected using the electrochemiluminescence system (Millipore) on carefully exposed film (Amersham, Amersham, UK) to avoid saturation.

Statistical analysis

Statistical analyses were carried out using SPSS version 20 (IBM, Armonk, NY, USA) and GraphPad Prism version 4 (GraphPad Software, La Jolla, CA, USA) using Student's *t*-test except where stated. Receiver operating characteristic (ROC) area under the curve (AUC) analysis was carried out using GraphPad Prism version 4.

Results

Analysis of transcriptome sequencing of RA and healthy neutrophils

Transcriptome sequencing (RNA-Seq) was carried out on mRNA purified from freshly isolated RA and healthy control neutrophils. In order to identify which signalling pathways were elevated in RA, individuals were grouped into two categories—RA ($n = 20$) and healthy controls ($n = 6$)—and the fold change in gene expression (up- or down-regulated) in the RA patients compared with healthy

Fig. 1 The IFN signature in RA neutrophils
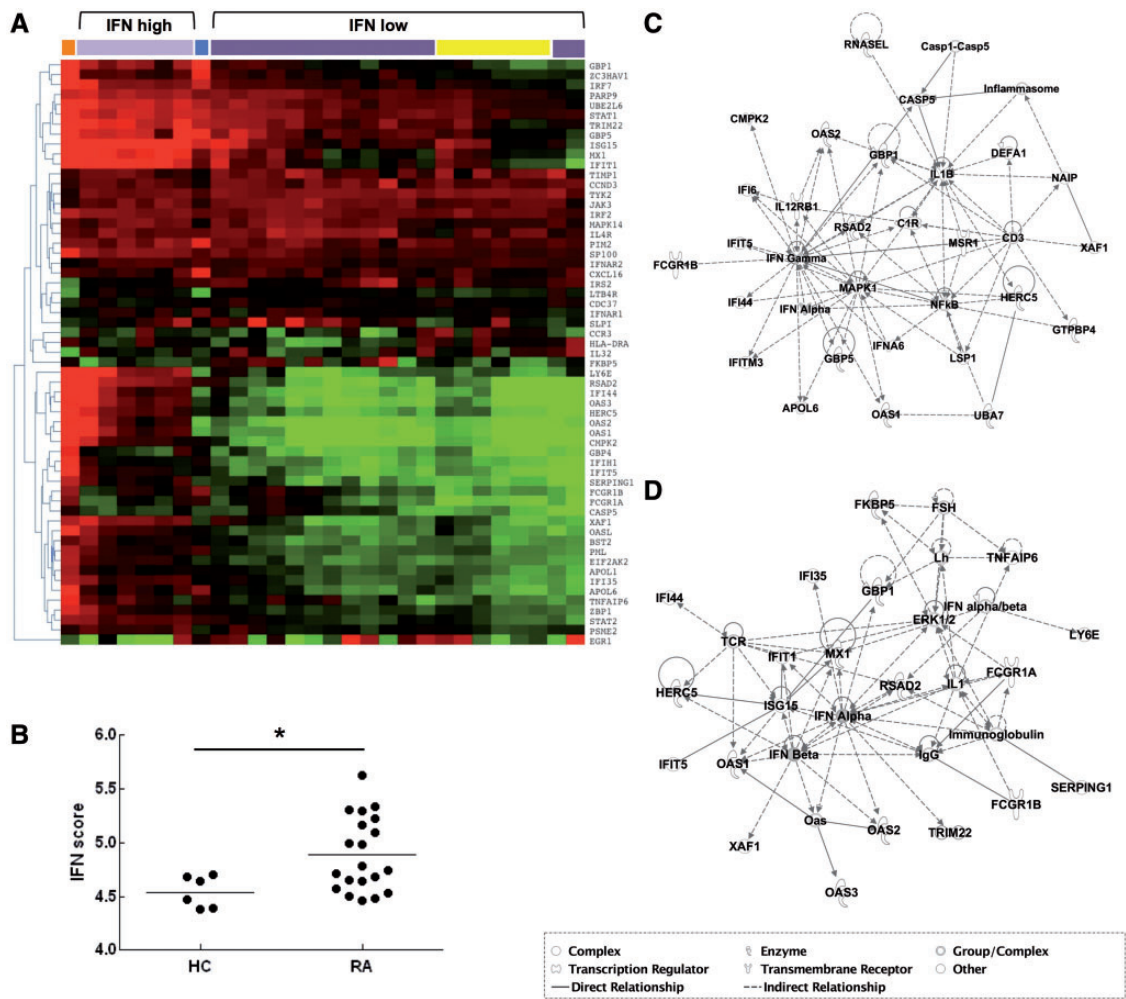
(A) Expression of IFN-regulated genes from 20 RA (purple bars), 6 healthy control (yellow bar), IFN-α-treated (orange bar) and IFN-γ-treated (blue bar) neutrophils. (Expression level: green = low, black = medium, red = high.) (B) The IFN signature (IFN score) was significantly higher in RA patients (*$P < 0.05$) compared with healthy controls (HC). Ingenuity analysis identified two gene networks, regulated by (C) IFN-α and IFN-γ (shaded grey) or (D) IFN-α and IFN-β (shaded grey).

controls was calculated. These data were then analysed using IPA applying a ⩾ 1.5-fold change in gene expression threshold, which identified 666 genes more highly expressed in RA compared with 223 genes more highly expressed in controls. IPA predicted that the most differently regulated signalling pathway in RA neutrophils was IFN signalling ($P = 1.33^{e−06}$), and that the upstream regulators most likely to be influencing gene expression in RA neutrophils were IFN-αA2 ($P = 2.24^{e−31}$), IFN-β1 ($P = 2.01^{e−18}$) and IFN-γ ($P = 1.89^{e−27}$).

Expression of IFN signature in RA neutrophils

IPA predicted a total of 178 genes from our dataset to be regulated by IFN-αA2, IFN-β1 or IFN-γ (see supplementary Table S2, available at Rheumatology Online). In order to obtain a visual representation of the expression profiles of

these 178 IFN-response genes we performed cluster analysis of the RPKM values for these transcripts in each individual sample using MeV (data not shown). In addition, we prepared and sequenced RNA from healthy control neutrophils stimulated in vitro for 1 h with type I IFN (IFN-αA2, 10 ng/ml) or type II IFN (IFN-γ, 10 ng/ml) and included these samples in our cluster analysis. Patients with RA clustered into two populations, largely defined by a group of 59 genes (Fig. 1A). Cluster 1 (light purple bar) was bordered on either side by the samples treated in vitro with IFN-αA2 (orange bar) and IFN-γ (blue bar) samples, while cluster 2 (dark purple bar) also contained the healthy controls (yellow bar), which clustered together. We calculated an IFN score based on the mean RPKM values ($\log_2$) for 178 IFN-regulated genes. The IFN score for the RA patients was significantly higher than for

healthy controls (Fig. 1B; $P < 0.05$). The IFN score for cluster 1 of the RA patients was significantly higher than for cluster 2 ($P < 0.01$), and we therefore designated these clusters IFN-high and IFN-low, respectively. Ingenuity analysis predicted two IFN-regulated networks: Fig. 1C includes 13 genes from the dataset and is regulated by IFN-α and IFN-γ and Fig. 1D includes 20 genes from the dataset regulated by IFN-α and IFN-β.

STAT activation

IFN signalling is characterized by the activation of signal transducer and activation of transcription (STAT) family proteins. IFN-γ signals through homodimers of STAT1, whereas IFN-α and IFN-β predominantly activate STAT1/2 heterodimers, but can additionally activate STAT3 and STAT5. Ingenuity predicted that STAT1 ($P = 1.99^{e-23}$), STAT2 ($P = 6.12^{e-13}$) and STAT3 ($P = 1.02^{e-20}$) were activated in the RA patients. In order to confirm this we carried out western blot analysis for phosphorylated STAT proteins on neutrophil protein lysates prepared alongside the RNA samples that were sequenced. Phosphorylation of STAT1 was significantly higher in RA patients ($P < 0.05$; Fig. 2A; RA $n = 10$, HC $n = 4$). We also observed increased phosphorylation of STAT3 and STAT5 in RA patients, although this did not reach statistical significance. STAT2 phosphorylation was detected in 3 of 10 RA patients (data not shown). A representative blot of protein lysates from three IFN-high patients (RA1–3), two IFN-low patients (RA4–5) and a healthy control is shown in Fig. 2B. The IFN score for each individual is shown.

Correlation of IFN signature with disease activity

There was a strong positive correlation between the pre-therapy IFN score and the change in DAS28 after 12 weeks of TNFi (Fig. 2C; $P = 0.02$ Pearson's correlation, $r = 0.51$) . Comparison of the change in DAS28 scores measured before and 12 weeks after therapy revealed that patients in the IFN-high group responded better to TNFi therapy than patients in the IFN-low group [Fig. 2D; $P = 0.05$ Student's *t*-test, OR 1.4 (95% CI 1.005, 1.950)].

Correlation of IFN score with response to TNFi therapy

ROC AUC analysis was used to evaluate the ability of the IFN score to predict response to TNFi therapy in the patient cohort. This analysis revealed an AUC of 0.64 to predict response based on a decrease in DAS28 $\geqslant 1.2$ (data not shown). However, by categorizing the response to TNFi therapy using the EULAR criteria of good *vs* moderate or no response [6], the AUC increased to 0.76 (Fig. 2E).

Discussion

This study has shown for the first time that RA neutrophils exhibit an IFN signature that indicates activation *in vivo* by type I and type II IFNs. Intriguingly, our study has identified a significant correlation between pre-therapy levels of IFN-regulated gene expression and change in disease activity at 12 weeks of therapy with TNFi. We show that

FIG. 2 Activation of STAT proteins in RA neutrophils and prediction of response to TNFi
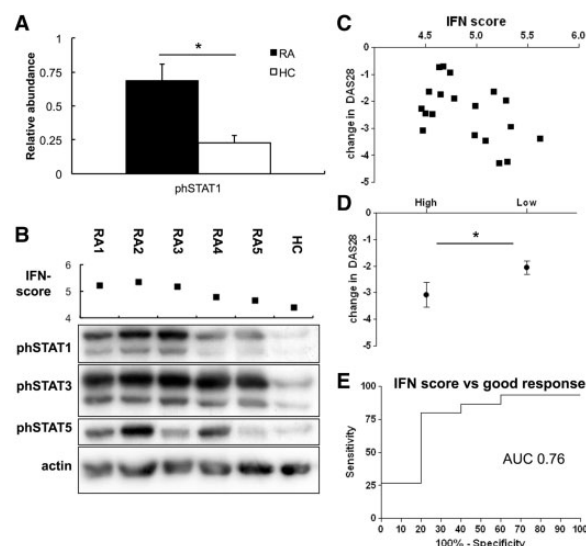
(**A** and **B**) RA neutrophils ($n = 10$) showed significant activation of STAT1 compared with healthy control (HC) neutrophils ($n = 4$, *$P < 0.05$). STAT3 and STAT5 proteins were also activated in RA neutrophils compared with healthy controls ($P > 0.05$). (**C**) Pre-therapy IFN score correlated significantly ($P = 0.02$, $r = 0.51$) with change in DAS28 following 12 weeks of TNFi therapy. (**D**) IFN-high RA patients responded better to TNFi therapy than IFN-low RA patients (*$P = 0.05$). (**E**) Receiver operating characteristics curves for the IFN score as a predictor of response to TNFi therapy based on the EULAR good response criteria was 0.76. STAT: signal transducer and activator of transcription; DAS28: 28-item DAS; TNFi: TNF inhibitor; EULAR: European League Against Rheumatism.

patients with a high IFN signature subsequently achieved the greatest response to a TNFi and have demonstrated that the pre-therapy IFN signature correlates with a good response to a TNFi using ROC curve analysis, with an AUC of 0.76. These results warrant further investigation in a larger cohort of RA patients to add further validation to the data. In particular, the number of EULAR non-responders should be increased in this validation cohort.

Activation of neutrophils by IFNs has previously been identified using microarray analysis in polyarticular juvenile RA [11]. Microarrays have also identified an IFN signature in whole blood samples taken from patients with RA [12–16] and an association between IFN activity in RA plasma and response to TNFi [17]. Some, but not all, of these studies have identified a significant link between IFN-regulated gene expression and response to biologics. These recent studies have all observed heterogeneity in the levels of IFN-regulated gene expression across a population of RA patients, with ~25–30% of patients in each study exhibiting an IFN-high gene expression

signature. Our data support and considerably extend this observation. Interestingly, the response to rituximab has been associated with an IFN-low signature [18]. Rituximab is licensed for use in RA patients who have failed TNFi, and while our study only included four TNFi non-responders, all had an IFN-low gene expression signature.

Our investigation is the first RNA-Seq study of a purified cell population in RA patients. This approach offers advantages over the study of whole blood or peripheral blood mononuclear cells (PBMCs), which do not take into account the considerable variation in leucocyte subtypes in whole blood preparations and the distinct and highly variable gene expression profiles of these cells [19]. As the proportion of each leucocyte subtype in peripheral blood may vary considerably between individuals and during therapy, this biological variation is likely to affect the outcome of studies in which the RNA is isolated from mixed cell populations [1]. In addition, inflammatory stimuli, such as cytokines, may have opposing effects on gene expression in different leucocytes [20]. This is particularly important for low-abundance genes, when changes of expression in one cell type may be saturated by signals from other cells. Gene expression studies of other purified blood leucocyte populations have not yet been reported in RA, but it should be noted that IFNs may influence monocyte activation in other rheumatic diseases such as SLE and SS. The identification of an IFN gene expression signature in RA whole blood may therefore represent gene expression in both neutrophils and other PBMCs.

While a wide range of biologics is now available for patients with RA, the lack of biomarkers to predict response can lead to potentially avoidable joint damage or risk of preventable adverse events if the wrong biologic is chosen. The identification of an IFN signature that correlates with a good clinical response to TNFi may therefore be useful in helping clinicians and patients to choose the optimal biologic after failure of DMARD therapy. The abundance of neutrophils in peripheral blood, together with their dynamic regulation of gene expression during active and resolved inflammation, makes them attractive candidates for the development of biomarker assays for drug response.

Rheumatology key messages

- Peripheral blood neutrophils in RA are activated by type I and type II IFNs.
- RA neutrophils have activated STAT signalling and IFN-regulated gene expression.
- Pre-therapy IFN-regulated gene expression in neutrophils correlates with a good response to TNF inhibitor in RA.

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Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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