Expression of STAT3-regulated genes in circulating CD4+ T cells discriminates rheumatoid arthritis independently of clinical parameters in early arthritis

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Abstract

Objectives. Dysregulated signal transduction and activator of transcription-3 (STAT3) signalling in CD4+ T cells has been proposed as an early pathophysiological event in RA. We sought further evidence for this observation, and to determine its clinical relevance.

Methods. Microarray technology was used to measure gene expression in purified peripheral blood CD4+ T cells from treatment-naïve RA patients and disease controls newly recruited from an early arthritis clinic. Analysis focused on 12 previously proposed transcripts, and concurrent STAT3 pathway activation was determined in the same cells by flow cytometry. A pooled analysis of previous and current gene expression findings incorporated detailed clinical parameters and employed multivariate analysis.

Results. In an independent cohort of 161 patients, expression of 11 of 12 proposed signature genes differed significantly between RA patients and controls, robustly validating the earlier findings. Differential regulation was most pronounced for the STAT3 target genes PIM1, BCL3 and SOCS3 (>1.3-fold difference; P < 0.005), each of whose expression correlated strongly with paired intracellular phospho-STAT3. In a meta-analysis of 279 patients the same three genes accounted for the majority of the signature’s ability to discriminate RA patients, which was found to be independent of age, joint involvement or acute phase response.

Conclusion. The STAT3-mediated dysregulation of BCL3, SOCS3 and PIM1 in circulating CD4+ T cells is a discriminatory feature of early RA that occurs independently of acute phase response. The mechanistic and functional implications of this observation at a cellular level warrant clarification.

Key words: rheumatoid arthritis, T lymphocytes, gene expression

Rheumatology key messages

• Three STAT3-regulated genes discriminate RA independently of clinical parameters in early arthritis CD4+ lymphocytes.
• The mechanistic relevance of these genes’ activation amongst early RA CD4+ lymphocytes awaits clarification.

Introduction

RA is a chronic disease of immune dysregulation the pathogenesis of which remains incompletely understood [1]. An orchestrating role for CD4+ T cells is suggested by a number of lines of evidence, including accumulating data from genetic association studies [2, 3], analyses of diseased synovia [4] and the observed therapeutic efficacy of co-stimulation blockade [5]. We previously identified a 12-gene CD4+ T cell expression signature in early arthritis...
arthritist patients that predicted a diagnosis of RA [6]. This
signature comprised an over-representation of genes
regulated by signal transduction and activator of tran-
scription-3 (STAT3), each of whose expression correlated
with paired serum levels of IL-6, itself a prominent inducer
of STAT3 signalling [7]. Using flow cytometry we recently
confirmed the importance of IL-6-mediated STAT3 activa-
tion in CD4+ T cells (in contrast to other circulating cyto-
kines and leucocytes) as an early event in the clinical
phase of RA, and suggested its potential value as a diag-
nostic biomarker [8].

Aberrant STAT3 signalling has a well-documented role
in tumorigenesis via induction of pro-survival and cell
cycle pathways [9-11]. Our observations add to accumu-
lating evidence that analogous mechanisms of STAT3
dysregulation might sustain autoimmunity [12, 13].
Through a more sophisticated understanding of IL-6/
STAT3 signalling at a cellular level, therapies that go
beyond generic blockade of the IL-6 inflammatory cas-
cade, instead targeting disease-specific mechanisms,
may be uncovered [14].

The current investigation sought to validate the rele-
ance of our previously described CD4+ T cell gene signa-
nature in a distinct early arthritis cohort. In particular, we
determined the extent to which expression of STAT3-
regulated genes was independently associated with a
diagnosis of RA when considered alongside clinical par-
eters such as inflammation.

**Methods**

**Patients**

During 2012-13, consecutive patients were recruited from
the Newcastle Early Arthritis Cohort, which has been
described in detail elsewhere [6, 15, 16], and peripheral
blood was obtained prior to commencement of therapy.
Initial diagnoses were validated at follow-up visits over a
median period of 20 months (range 13-25) as described
[8], and with reference to 2010 ACR/EULAR classification
criteria for RA [17]. All patients gave written, informed con-
sent for inclusion into the study, which was approved by
the local Regional Ethics Committee.

**Twelve-gene expression signature measurement in
CD4+ T cells of the independent cohort**

Total RNA was extracted from CD4+ T cells positively se-
lected from monocyte-depleted whole blood within 4 h of
blood draw as previously described [6]. cRNA generated
from 250 ng total RNA (Illumina TotalPrep RNA
Amplification Kit) was hybridized to the Illumina Human
HT12v4 BeadChip (Illumina, San Diego, CA, USA). After
quality control using established methods previously out-
lined [6], data relating exclusively to the 12 signature
genes previously identified [8] were extracted for detailed
analysis. Expression data used for this experiment are
available in the Gene Expression Omnibus database
(GEO: http://www.ncbi.nlm.nih.gov/geo; accession
number GSE80513). Since the HT12v4 BeadChip annota-
tion differed slightly from the WG6v3 array used for our
original work, unique Illumina NuId references were used
instead to map probes of identical sequence for this
purpose.

**Flow cytometric determination of STAT3 pathway
activation in CD4+ T cells**

Phosflow cytometry was performed on freshly drawn, un-
stimulated whole blood obtained contemporaneously with
that used for CD4+ T cell RNA extraction. Anti-CD4-APC-
eFluor 780 (SK3) (eBioscience Ltd, Hatfield, UK), anti-Stat3 (pY705)-Alexa Fluor 647 (4/P-STAT3) and anti-
CD3-Pacific Blue (UCHT1) (both BD Biosciences, Oxford,
UK) were used along with appropriate buffers and controls
in the staining protocol as previously described [8]. Data
were collected on a BD FACSCanto II (BD Biosciences,
Oxford, UK) and analysed using FlowJo (Treestar,
Ashland, OR, USA).

**Combined cohort microarray analysis**

Since baseline and follow-up diagnostic classification of
patients in the previously described cohort [6] was under-
taken with reference to the 1987 ACR criteria [18], retro-
spective application of the 2010 ACR/EULAR classification
criteria was applied [17], so that both co-
horts were similarly classified. Thirteen of 62 patients pre-
iously classified at baseline with undifferentiated arthritis
became 2010-RA, and 6 of 47 1987-RA patients did not
fulfil the 2010 criteria. Next, a de novo pipeline for the
normalization and quality control of independently derived
raw microarray datasets from the previous and current
patient cohorts (GEO accession numbers GSE20098 and
GSE80513, respectively) was employed as previously
described, demonstrably accounting for anticipated
batch effects [19].

**Statistical analysis**

Hierarchical clustering (Euclidian distance metric; Ward’s
linkage method) was performed and visualized in R
programming environment (https://r-project.org). Mann-
Whitney U and Kruskal Wallis tests were used
for two-group and multiple group univariate analyses, re-
spectively, along with chi-squared ($\chi^2$) and
Khomogorov-
Smirnov tests as indicated in the text. Bivariate correla-
tions were determined using Spearman’s Rho, and logis-
tic regression was used for multivariate analyses with
validated diagnostic outcome as the dependent variable,
and independent variables as detailed in the text.
Receiver-operating characteristic curves for competing
logistic regression models were constructed and differ-
ences in their areas under the curve compared using
t-tests. In addition, scatterplots overlaid with non-
parametric density plots [20] were used to depict
separation of comparator groups attributable to normal-
gene expression, using SAS Institute JMP statistical
visualization software (version 13; Cary, NC, USA).
Results

Baseline clinical characteristics of newly recruited patients

Some 161 early arthritis patients were enrolled into the study, of whom 47 (29%) were diagnosed with RA and the remainder with alternative diagnoses; their baseline clinical characteristics are summarized in Table 1. Early RA patients differed, on average, from other early arthritis clinic attendees by a higher acute phase response, more swollen and tender joints, circulating autoantibodies (RF ACPA) and older age.

Independent validation of STAT3-regulated CD4+ T cell signature in early RA

In our independent cohort of 161 treatment-naïve early arthritis clinic attendees, significant differences in normalized expression were seen for 11 of the 12 previously identified signature genes between RA and non-RA CD4+ T cells (Table 2). Only thee (PIM1, BCL3 and SOCS3) achieved the 1.2-fold difference between comparator groups set as a threshold in our original study [6], but they did so comfortably with >1.3-fold differences being observed in each case (Table 2 and Fig. 1A/C150C). Indeed, it was notable that these three genes were ranked amongst the top 25 differentially expressed by fold-difference out of a total of 30 458 non-redundant, filtered probes in the source microarray dataset, something that would be highly unlikely to have occurred by chance (P = 9.8 \times 10^{-15}, one-sample Kolmogorov-Smirnov test).

The three highlighted genes are known to be regulated by STAT3 [21–23]. We therefore hypothesized that their normalized expression would in turn depend upon constitutive STAT3 phosphorylation in CD4+ T cells, as measured using flow cytometry of contemporaneously obtained fresh blood samples. Intracellular phospho-STAT3 measurements indeed correlated strikingly with paired BCL3, SOCS3 and PIM1 gene expression in ex vivo CD4+ T cells of early arthritis patients (Fig. 1D–F), but not with that of other genes in the signature such as PDCD1 or IGFL2, which are not known to be induced by STAT3 (supplementary Fig. S1, available at Rheumatology online). These data confirm the importance of STAT3 signalling as a mediator of BCL3, SOCS3 and PIM1 gene induction in early RA.

Twelve-gene signature’s ability to discriminate early RA in combined cohort accounted for by a three-gene subset

Our previously described 12-gene CD4+ T cell signature was originally identified in untreated early RA patients defined prior to the publication of modified classification criteria for the condition. In our independent RA cohort, defined under the new classification system, a subset of three genes (BCL3, SOCS3 and PIM1) was clearly up-regulated. To investigate whether this held true following diagnostic re-classification of the previous cohort, and to increase the statistical power of our study, a pooled analysis of microarray data was carried out. Some 101 of 279 (36%) in the combined cohort were diagnosed with RA, and these individuals were again distinguishable from other early arthritis clinic attendees at baseline by their

### Table 1 Baseline clinical characteristics of patients according to diagnosis

| Diagnosis | RA (n = 47) | Non-RA (n = 114) | P-value |
|-----------|------------|-----------------|---------|
| Age, years | 60 (21–87) | 51 (17–92) | <0.001 |
| % Female | 74 | 68 | ns |
| Symptom duration | 12 (2–52) | 12 (2–52) | ns |
| TJC28 | 6 (1–20) | 2 (0–25) | 0.001 |
| SJC28 | 1 (1–17) | 0 (0–9) | 0.002 |
| CRP, g/l | 10 (<5–66) | 5 (<5–189) | 0.005 |
| ESR | 21 (4–86) | 9 (1–113) | 0.004 |
| %RF+ | 64 | 10 | <0.001 |
| %ACPA+ | 64 | 0 | <0.001 |
| DAS28 | 4.32 (2.24–7.15) | n/a | n/a |
| Non-RA diagnoses, number (% of 114) | PsA, 20 (17) | Other SpA, 18 (16) | Other IA, 8 (7) |
| | Crystal, 10 (9) | Other | OA, 25 (22) |
| | Other non-IA, 33 (29) | | |

Values are median (range) unless otherwise stated. aBaseline diagnosis was confirmed at median 20 months (range 13–25). bMann-Whitney U test or chi-squared test with Yates’ continuity correction for continuous and dichotomous data respectively. TJC: tender joint count; SJC: swollen joint count; ns: not significant; n/a: not applicable; IA: inflammatory arthritis.
Expression of STAT3-regulated genes

TABLE 2  Normalized expression values of indicated transcripts in early arthritis patient diagnostic groups of the independent cohort

| Gene | Symbol | RefSeq | Illumina Probe ID | RA (n = 47) | Non-RA (n = 114) | Fold-change<sup>b</sup> | P-value<sup>c</sup> |
|------|--------|--------|-------------------|-------------|------------------|---------------------|-----------------|
| BCL3 | NM_005178 | ILMN_1710514 | 867 | 585 | 1.48 | <0.001 |
| PIM1 | NM_002648 | ILMN_1815023 | 3567 | 2633 | 1.35 | <0.001 |
| SOCS3 | NM_003955 | ILMN_1781001 | 850 | 614 | 1.38 | 0.004 |
| LDHA | NM_005566 | ILMN_1807106 | 7493 | 6572 | 1.14 | <0.001 |
| GPRIN3 | CR743148<sup>d</sup> | ILMN_1901616 | 377 | 322 | 1.17 | 0.004 |
| MUC1 | NM_01444391 | ILMN_1756992 | 310 | 283 | 1.10 | 0.013 |
| PDCD1 | NM_005018 | ILMN_1806725 | 168 | 159 | 1.06 | 0.029 |
| SBNO2 | NM_014963 | ILMN_1808811 | 193 | 180 | 1.07 | 0.011 |
| IGFL2 | NM_001002915 | ILMN_1790227 | 142 | 135 | 1.05 | 0.017 |
| LOC731186 | XM_001128760 | ILMN_1900154 | 148 | 145 | 1.02 | 0.033 |
| CMAHP | NR_002174 | ILMN_1704084 | 222 | 217 | 1.02 | ns |
| NOG | NM_005450 | ILMN_1652287 | 143 | 150 | –1.05 | 0.007 |

RefSeq accession numbers and Illumina probe IDs are given (see supplementary Table S1, available at Rheumatology online for probe sequences). *Median normalized gene expression values presented. †Linearized fold-change relative to non-RA group given. ‡Mann–Whitney U test. #Transcript CR743148 has been retired from the National Center for Biotechnology Information, but the expressed sequence tag corresponds to splice variant(s) within the GPRIN3 gene (chromosome 4.90). Genes demonstrating >1.2-fold differences indicated in boldface. ns: not significant.

Fig. 1 Expression of STAT3-regulated genes in circulating CD4+ lymphocytes of an independent early arthritis cohort

(A–C) Normalized gene expression of three STAT3-regulated genes in circulating CD4+ T cells of an independent early arthritis cohort of 161 patients presenting with RA or other arthritides (Non-RA). Mann–Whitney U tests used to determine P-values; FC denotes fold-change. (D–F) Bivariate correlation between the same genes’ normalized expression and pSTAT3 measurements in paired circulating CD4+ T cells of early arthritis clinic attendees. Spearman’s Rho correlation coefficients and associated P-values are depicted. STAT3: signal transduction and activator of transcription-3; pSTAT: phospho-STAT3.

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1253
older age, increased number of tender and swollen joints, and higher acute phase responses; detailed characteristics of the combined cohort are summarized in supplementary Table S1, available at Rheumatology online. Fig. 2A depicts the results of hierarchical clustering based on the 12-gene signature in the combined cohort, discriminating a subset of individuals enriched for a diagnosis of RA [includes 68/101 (67%) RA patients in the overall population compared with 40/178 (22%) non-RA patients; $P < 0.001$, $\chi^2$ test]. Again, only the expression of BCL3, SOCS3 and PIM1 stood out as being $>1.2$-fold up-regulated in early RA on univariate analysis of this combined cohort (supplementary Table S2, available at Rheumatology online). Hierarchical clustering confirmed that these three genes alone accounted for the majority of the previously noted clustering effect, once more discriminating a subgroup that was significantly enriched for RA ($\chi^2 P < 0.001$; Fig. 2B). Moreover, segregation of RA patients from disease controls is evident when representing the data in 2D space according to their normalized expression (Fig. 2C). These data indicate that BCL3, SOCS3 and PIM1 account for the majority of the previously described 12-gene signature’s discriminatory ability with respect to a diagnosis of RA in the setting of an early arthritis clinic.

Association of gene expression with RA is independent of baseline clinical parameters

Considering the potentially confounding influence of age, swollen or tender joint count, and, in particular, acute
Expression of STAT3-regulated genes

Table 3: Summaries of logistic regression outputs in respect of RA vs non-RA diagnoses

|       | B      | SE     | Wald  | df | P-value | Exp[B] (95% CI) |
|-------|--------|--------|-------|----|---------|----------------|
| BCL3  | 0.001  | 0.001  | 6.964 | 1  | 0.008   | 1.001 (1.000-1.002) |
| Age   | 0.037  | 0.011  | 11.831| 1  | 0.001   | 1.034 (1.016-1.059) |
| CRP   | –0.005 | 0.006  | 0.546 | 1  | 0.460   | 0.995 (0.983-1.008) |
| ESR   | 0.007  | 0.007  | 1.001 | 1  | 0.317   | 1.007 (0.993-1.022) |
| TJC28 | 0.034  | 0.020  | 2.967 | 1  | 0.085   | 1.034 (0.995-1.075) |
| SJC28 | 0.227  | 0.059  | 14.860| 1  | <0.001  | 1.255 (1.118-1.409) |
| Constant | –4.399 | 0.728  | 36.494| 1  | <0.001  | 0.012 |

|       | B      | SE     | Wald  | df | P-value | Exp[B] (95% CI) |
|-------|--------|--------|-------|----|---------|----------------|
| PIM1  | 0.000  | 0.000  | 7.996 | 1  | 0.005   | 1.000 (1.000-1.001) |
| Age   | 0.034  | 0.011  | 10.258| 1  | 0.001   | 1.034 (1.013-1.056) |
| CRP   | –0.006 | 0.006  | 1.019 | 1  | 0.313   | 0.994 (0.981-1.006) |
| ESR   | 0.008  | 0.007  | 1.203 | 1  | 0.273   | 1.008 (0.994-1.022) |
| TJC28 | 0.036  | 0.020  | 3.320 | 1  | 0.068   | 1.036 (0.997-1.077) |
| SJC28 | 0.214  | 0.058  | 13.621| 1  | <0.001  | 1.239 (1.106-1.388) |
| Constant | –4.512 | 0.734  | 37.843| 1  | <0.001  | 0.011 |

|       | B      | SE     | Wald  | df | P-value | Exp[B] (95% CI) |
|-------|--------|--------|-------|----|---------|----------------|
| SOCS3 | 0.001  | 0.001  | 5.930 | 1  | 0.015   | 1.001 (1.000-1.002) |
| Age   | 0.038  | 0.011  | 12.479| 1  | <0.001  | 1.038 (1.017-1.060) |
| CRP   | –0.007 | 0.006  | 1.186 | 1  | 0.276   | 0.993 (0.981-1.006) |
| ESR   | 0.009  | 0.007  | 1.648 | 1  | 0.099   | 1.009 (0.995-1.024) |
| TJC28 | 0.031  | 0.019  | 2.587 | 1  | 0.108   | 1.032 (0.993-1.072) |
| SJC28 | 0.150  | 0.082  | 3.327 | 1  | 0.068   | 1.162 (0.989-1.365) |
| Constant | –4.424 | 0.737  | 36.009| 1  | <0.001  | 0.012 |

BCL3, PIM1 and SOCS3 (boldface) are, respectively, considered as independent candidate variables alongside age, CRP, ESR, TJC and SJC. TJC: tender joint count; SJC: swollen joint count.

phase response (Table 1 and supplementary Tables S1 and S3, available at Rheumatology online), we used logistic regression to confirm that the up-regulation of BCL3, PIM1 and SOCS3 observed in early RA was independent of these clinical parameters (P < 0.05 for each gene; Table 3). To quantify the relative additive value of a three-gene (comprising BCL3, SOCS3 and PIM1) or 12-gene signature over clinical parameters alone, results of multivariate analyses summarized as composite receiver-operating characteristic curves were compared (Fig. 3). Hence, by including the 12-gene signature in a model that included age, swollen joint count, tender joint count, CRP and ESR, a statistically significant area under the curve increase from 0.78–0.87 was achieved (P < 0.001). Interestingly, however, the three-gene signature accounted for a substantial component of this effect (area under the curve increase 0.78–0.83; P = 0.007). Considered together, these findings suggest that up-regulated expression of BCL3, SOCS3 and PIM1 in circulating CD4+ T cells of early RA patients is independent of potentially confounding clinical parameters, and accounts for much of the previously described 12-gene signature’s discriminatory ability for early RA.

Discussion

In this validation study, we confirmed the ability of our previously proposed CD4+ T cell gene expression signature to identify RA patients amongst unselected, treatment-naïve early arthritis clinic attendees. Normalized gene expression differed significantly between RA patients and disease controls for 11 out of the 12 signature genes, but the fold-changes were most striking for BCL3, PIM1 and SOCS3. Each of these genes is known to be regulated by STAT3, and their expression correlated significantly with paired CD4+ T cell phospho-STAT3 levels. Indeed, hierarchical clustering suggested that the ability of the 12-gene signature to discriminate RA patients in this replication cohort was almost entirely accounted for by these three genes alone. The reproducibility of this component of the original gene signature in two independent studies separated by 5 years is remarkable given the heterogeneity of the patient population presenting to early arthritis clinics. Furthermore, the signature is robust to replacement of 1987 ACR classification criteria [18] used to define RA in our previous analysis with updated criteria developed for use specifically in the setting of early disease [17]. Finally, our analysis demonstrated that the associations of BCL3, PIM1 and SOCS3 gene expression with diagnostic outcome are independent of clinical parameters such as age and systemic inflammation. Rather than being mere bystander phenomena, our data could indicate a direct role for STAT3-regulated gene induction in RA pathogenesis, for example via altered T cell effector function. This possibility remains the subject of ongoing investigation.

A growing body of evidence now highlights IL-6-mediated dysregulation of CD4+ T cell STAT3 signalling during RA development [24, 25]. Amongst RA patients who experience good therapeutic responses to the anti-IL-6 receptor monoclonal antibody tocilizumab, concurrent down-regulation of STAT3-regulated genes by
CD4+ T cells has been eloquently demonstrated—including that of BCL3, PIM1 and SOCS3 [26]. Such data fuel optimism that cellular biomarkers of STAT3 pathway activation might have clinical value for the development of stratified treatment approaches [27].

Even more tantalizing is the possibility, suggested by our data, that the identified IL-6-mediated transcriptional programme might itself mark a molecular mechanism by which susceptible CD4+ T cells switch to adopt a pathogenic phenotype. Such speculation stems from a functional consideration of the three component signature genes we have identified. BCL3 is an atypical IκB family member which has, until recently, been little studied in human T cell biology [28, 29]. Particularly implicated in the development of T follicular helper cells [30, 31], it appears to represent a common element upon which a range of dysregulated cellular pathways converge [32], and may play a role in restraining the plasticity of the CD4+ T cell effector phenotype [33]. PIM1, one of a family of three serine/threonine-dependent kinases, has been implicated as an early mediator of Th1 commitment [34]; it was recently suggested as a novel therapeutic target in skin psoriasis [35]. SOCS3 is a negative regulator of STAT3 signalling, and whether its up-regulation in early RA reflects a direct failure of this regulatory system is unknown, but it is notable that spontaneous inflammatory arthritis develops in mice following mutation of the molecule’s IL-6 receptor binding site [36].

Taken together, it is of interest that the robust three-gene CD4+ T cell signature we now validate parallels the STAT3-dependent transcriptional pattern observed in the malignant Sézary cells of individuals with the leukaemic variant of cutaneous T cell lymphoma, in which up-regulated PIM1 and SOCS3 is specifically described [37, 38]. By contrast, all three signature genes are down-regulated in circulating CD4+ T cells of patients with latent tuberculosis infection when compared with those with active infection [39]. The extent to which their induction by IL-6 promotes autoimmunity by sustaining a pathogenic, pro-proliferative cell phenotype—indeed, whether their modulation might favour tolerance induction—warrants concerted investigation. Such studies may reveal targetable disease mechanisms relevant to autoimmune diseases beyond RA alone.

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Supplementary data
Supplementary data are available at Rheumatology online.

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Expression of STAT3-regulated genes

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