EXTENDED REPORT

IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis

Amy E Anderson,1 Arthur G Pratt,1 Mamdouh A K Sedhom,2 John Paul Doran,1 Christine Routledge,1 Ben Hargreaves,1 Philip M Brown,1 Kim-Anh Lê Cao,2 John D Isaacs,1 Ranjeny Thomas2

ABSTRACT

Objectives A previously identified signal transduction and activator of transcription-3 (STAT3) target-enriched gene signature in circulating CD4+ T cells of patients with early rheumatoid arthritis (RA) was prominent in autoantibody-negative individuals. Here, interleukin (IL)-6-mediated STAT signalling was investigated in circulating lymphocytes of an independent early arthritis patient cohort, seeking further insight into RA pathogenesis and biomarkers of potential clinical utility.

Methods Constitutive and IL-6-induced expression of phosphorylated STAT1 (pSTAT1) and pSTAT3 was determined in T and B cells using Phosflow cytometric analysis in patients with RA and controls. Contemporaneous levels of serum cytokines were measured by immunoassay. Induced gene expression was measured in cultured CD4+T cells by quantitative real-time PCR.

Results Among circulating lymphocytes of 187 patients with early arthritis, constitutive pSTAT3 correlated with serum IL-6 levels maximally in CD4+ T cells. Increased constitutive pSTAT3, but not pSTAT1, was observed in circulating CD4+ T cells of patients with early anticitrullinated peptide autoantibody (ACPA)-negative RA compared with disease controls, and these levels decreased alongside markers of disease activity with IL-6R-targeted treatment. Among patients presenting with seronegative undifferentiated arthritis (UA) the ratio of constitutive pSTAT3:pSTAT1 in CD4+ T cells contributed substantially to an algorithm for predicting progression to classifiable RA during a median of 20 months follow-up (area under receiver operator characteristic curve=0.84; p<0.001).

Conclusions Our findings support a particular role for IL-6-driven CD4+ T cell activation via STAT3 during the induction of RA, particularly as a feature of ACPA-negative arthritis. CD4+ T cell pSTAT measurements show promise as biomarkers of UA–RA progression and now require independent validation.

INTRODUCTION

Anti-citrullinated peptide autoantibody (ACPA)-negative undifferentiated arthritis (UA) remains a unique diagnostic challenge in the early arthritis (EA) clinic. We previously identified a gene expression ‘signature’ in circulating CD4+ T cells of EA clinic attendees, which was most prominent in patients with ACPA-seronegative rheumatoid arthritis (RA), and predicted UA–RA progression.2 In that work, signal transducer and activator of transcription (STAT)3-regulated genes were over-represented in the signature’s make-up, their expression correlating with paired serum interleukin (IL)-6 concentrations independently of C reactive protein (CRP) and tumour necrosis factor (TNF).2 Similar patterns were not seen for other serum mediators that signal via STAT3. The importance of IL-6-mediated STAT3 signalling among CD4+ T cells during the induction of experimental arthritis, and in established human RA, has been independently highlighted,3,4 and the therapeutic efficacy of IL-6 receptor (IL-6R) blockade in RA management emphasises the pathway’s importance in disease pathogenesis.5–8 However, the cellular mechanisms by which IL-6 drives the induction and perpetuation of human disease remain to be resolved.

IL-6 shares with other gp130-related cytokines its reliance for signal transduction on the ubiquitously expressed, membrane-bound β-receptor subunit gp130, but must also bind to its α-subunit IL-6R (CD126) in either membrane or soluble form (classical or trans signalling, respectively).9–11 Although downstream signalling occurs primarily via STAT3, STAT1 is also used,12 and cross-regulation between these pathways appears to be an important determinant of cellular responses to gp130-related cytokines.13,14

The aims of the current study were to validate our initial observations in an independent EA patient cohort and to develop a more tractable assay to facilitate replication by other research groups. In so doing, we sought a better understanding of IL-6 signalling in lymphocytes as an early pathological event in the natural history of RA, compared with other arthritides.

METHODS

Subjects

Biological material was obtained from consecutive, consenting patients referred from primary care with recent onset arthritis and recruited from the Newcastle Early Arthritis Clinic (NEAC), UK, during 2012–2013. Patients had not been exposed to disease-modifying antirheumatic drugs or corticosteroids. In this cohort, a working clinical diagnosis was assigned by a rheumatologist at the first consultation as previously described,2 with RA being diagnosed with reference to 2010 European League Against Rheumatism/American College of Rheumatology classification criteria.13 Diagnoses...
were validated or, in the case of UA, updated at follow-up visits
over a median period of 20 months (range 13–25). Patients with
established RA treated with the anti-IL-6R monoclonal antibody
tocilizumab was recruited from the Freeman Hospital
Musculoskeletal Unit. Additional patients with RA (similarly ful-
filling 2010 classification criteria) and healthy volunteers were
recruited from the Princess Alexandra Hospital and community,
respectively, in Brisbane, Australia. They received a combination
of methotrexate, sulfasalazine and hydroxychloroquine, unless
contraindicated, immediately after diagnosis of RA. Treatment
was intensified according to a response-driven step-up algorithm.17

All subjects gave written informed consent before inclusion
into the study, which was approved by local ethics committees
at the respective institutions. Patients with pre-existing rheuma-
tological diagnoses, and all those aged <16 years, were excluded.
The disease activity score in 28 joints (DAS28; erythrocyte sedi-
mentation rate, ESR) was used throughout the study to deter-
mine RA disease activity.

Multiparameter flow cytometric analysis of whole blood
The following antibodies were used for Phosflow and IL-6R
phenotype analysis in samples processed at Newcastle:
anti-CD3-Pacific Blue (UCHT1), anti-CD19-FITC (4G7),
anti-CD14-APC (HIB19), anti-Stat3 (pY705)-Alexa Fluor 647
(4/P-STAT3) and anti-Stat1 (pY701)-Alexa Fluor 647 (4a; all
BD Biosciences, Oxford, UK); anti-CD4-APC-eFluor 780 (SK3;
eBioscience, Hatfield, UK); IL-6R-Fluorescein (17506; R&D
Systems Europe, Abingdon, UK). Phosflow was performed on
whole blood, which was either left unstimulated or stimulated
with 100 ng/mL IL-6 (PeproTech EC, London, UK) for 15 min
at 37°C. BD Phosflow Lyse/Fix and BD Phosflow Perm Buffer
III (both BD Biosciences) were used as per the manufacturers’
instructions. IL-6R expression was assessed in whole blood
using BD FACS Lysing Solution (BD Biosciences) as per the
manufacturers’ instructions. Data were collected on a BD
FACSCanto II (BD Biosciences) and analysed using FlowJo
(Treestar, Ashland, Oregon, USA). The protocol followed for
samples processed in Brisbane was similar, using
anti-CD3-Pacific blue (UCHT1), and anti-Stat3 (pY705)-PE
(4/P-STAT3; BD Biosciences), with a Gallios flow cytometer and
Kaluza software for data acquisition/analysis (both Beckman
Coulter). Flow-Set Pro Fluorospheres (Beckman Coulter) were
used to normalise median fluorescence intensities (MFI) for
pSTAT3 measurements between data sets.

Serum cytokine measurement
Between 09:30 and 12:30, baseline serum was drawn and
frozen at −80°C until use. Serum IL-6, TNF and soluble IL-6R
(sIL-6R) were measured using a highly sensitive, validated elec-
trochemiluminescence immunoassorbance detection system (Meso
Scale Discovery, Gaithersberg, Massachusetts, USA) as
described.2 Soluble gp130 (sgp130) and IL-23 measurements
were made using a Quantikine ELISA kit (R&D systems,
Minneapolis, Michigan, USA). Manufacturer guidance was
adhered to, but a cocktail of non-human sera (Heteroblock,
Bozeman, Missouri, USA) was added to each assay at an opti-
mised final concentration of 32 μg/mL to correct for potential
assay interference by heterophilic antibodies present in sera.19

Gene expression assays in cultured CD4+ T cells
High-purity CD4+ T cell isolation from whole blood of healthy
volunteers, cell culture in the presence of IL-6/sIL-6R, RNA
extraction and quantitative real-time PCR for measurement of
STAT3 target gene expression were performed as previously
described.2 Raw gene expression data were normalised relative
to the housekeeping gene 18S as 2−ΔΔCt values to determine fold
induction.

Calculation of Leiden Prediction Score
Using a range of readily available clinical and serological para-
meters, the Leiden Prediction Score was developed to stratify individuals’ risk of UA–RA progression with reference to the
1987 RA classification criteria, but its value for the same
purpose when applying the 2010 criteria is diminished.20 In
the absence of a validated alternative prediction tool for this setting,
we devised a modification to the algorithm described by its
authors,19 23 incorporating pSTAT1 and pSTAT3 parameters as
outlined in the text and using methodology outlined in the
online supplementary text: ‘Prediction Rule’.

Statistical analyses
The majority of analyses were carried out using SPSS (Chicago,
Illinois, USA), with statistical significance defined by α=5%. Non-parametric analyses of variance (Kruskal–Wallis test;
Dunn’s posthoc pairwise analyses) and Mann–Whitney U tests
were used for multiple-group and two-group independent compar-
isons, respectively, and Wilcoxon matched pairs test for
paired comparisons. Spearman’s rank correlations and analyses
of covariance were performed for bivariate comparisons; for
multiple or logistic regression dependent variables were selected
as indicated in the text, and skewed data were log10 trans-
formed. Additional receiver operator characteristic (ROC) ana-
lyses, including paired area under curve (AUC) comparisons by
DeLong’s non-parametric method24 and bootstrap cross-
validation (1000 bootstrap samples), were conducted using the
pROC statistical package25 (http://cran.r-project.org). Diagnostic
evaluations were calculated from contingency tables using http://
statpages.org.

RESULTS
Patient groups
A total of 187 NEAC attendees were recruited into the study.
Among this cohort, definitive diagnoses could be assigned by
rheumatologists at first consultation for 152 individuals (81%).
This number increased to 184 (98%) during follow-up.
Diagnoses were further categorised as RA (ACPA positive or
negative), non-RA inflammatory arthritis (non-RA IA) or
non-IA/arthralgia (NIA), as previously described,2 and clinical
criteria are provided in the online supplementary table
S1. The non-RA IA group comprised
psoriatic arthritis (36%), reactive or self-limiting IA (22%),
other spondyloarthropathy including ankylosing spondylitis
(11%), crystal arthropathy (18%) or other IA (13%). Twenty-seven (40%) of the NIA group were diagnosed with
probable osteoarthritis. A total of 22 additional patients with
RA and 13 healthy controls were recruited from Brisbane, con-
tributing only to experiments presented in figure 1E, F; their
clinical details are provided in the online supplementary table
S2. Details of tocilizumab-treated patients are given in the
online supplementary table S3.

IL-6-mediated STAT3 phosphorylation induces RA ‘signature
genes’ in CD4+ T cells in vitro and is a potential biomarker
The measurement of gene expression in peripheral blood
subsets used in our previous investigation is technically challeng-
ning, and we therefore sought a more tractable tool for clinical
Whole blood flow cytometric analysis presented a promising solution. We first confirmed that IL-6-mediated activation of primary CD4+ T cells could, via STAT3 phosphorylation, induce the same genes that featured prominently in our transcriptional signature. Maximal STAT3 phosphorylation was observed 15 min after IL-6 exposure (figure 1A), and upregulation of its exemplar targets SOCS3, BCL3 and SBNO2 reached significance at 1 h (figure 1B–D). We next confirmed that detectable differences in pSTAT3 were present between distinct patient populations. We measured constitutive pSTAT3 in circulating T cells (CD3+) of patients with early untreated RA, patients with treated RA and healthy volunteers (see online supplementary table S2A). Significantly higher CD3+ pSTAT3 expression was seen in patients with early RA compared with both healthy controls and patients with treated RA (figure 1E). Consistent with this, CD3+ T cell pSTAT3 measurements in prospectively followed patients with early RA treated with disease-modifying antirheumatic drugs (DMARDs) diminished over time (figure 1F; see online supplementary table S2B for additional clinical detail).

### Table 1

<table>
<thead>
<tr>
<th>Newcastle early arthritis clinic patients stratified by diagnostic outcome at the end of follow-up</th>
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<tr>
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<tr>
<td>CRP (g/L)</td>
</tr>
<tr>
<td>ACPA+, %</td>
</tr>
<tr>
<td>RF+, %</td>
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<tr>
<td>DAS28</td>
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</table>

Except where indicated, median and range are given. *Kruskal–Wallis ANOVA analysis confined to three groups with confirmed inflammatory diagnoses at inception: ACPA−RA, ACPA+RA and non-RA inflammatory arthritis. †Kruskal–Wallis ANOVA, five groups, including OA/non-inflammatory arthralgia and UA.

ACPA, anticitrullinated peptide autoantibody; ANOVA, analysis of variance; CRP, C reactive protein DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; UA, undifferentiated arthritis.

**Figure 1**

(A–D) CD4+ T cells isolated from whole blood of eight healthy volunteers were cultured with 50 ng/mL final concentration recombinant interleukin (IL)-6 and an equimolar concentration of soluble IL-6 receptor (sIL-6R). Intracellular phosphorylated signal transduction and activator of transcription-3 (pSTAT3) expression was measured by flow cytometric analysis from one aliquot (A), with induced expression of early rheumatoid arthritis (RA) ‘signature’ STAT3 target genes SOCS3, BCL3 and SBNO2 being measured in another aliquot for each individual (B–D); preliminary work established that peak expression was reached at 15 and 60 min, depicted here for pSTAT and transcripts, respectively. (E) pSTAT3 was measured in circulating CD3+T cells of patients with early RA, established RA counterparts and healthy donors as described; additional clinical information supplied in the online supplementary table S1. (F) Constitutive CD3+ T cell pSTAT3 was measured serially in 11 newly diagnosed patients with RA before (pre) and 2–4 months (median 12 weeks) post initiation of disease-modifying antirheumatic drug therapy. Wilcoxon signed-rank test (A–D and F) or non-parametric analysis of variance with Dunn’s posthoc pairwise analyses (E): *p<0.05, **p <0.01 and ***p<0.001. Error bars indicate interquartile ranges. MFI, median fluorescence intensity.
Strong and independent association between circulating CD4+ T cell pSTAT3 and paired serum IL-6 in EA

Constitutive pSTAT1, pSTAT3 and IL-6R expression in lymphocyte subsets was measured alongside paired serum IL-6, IL-6R and sgp130 concentrations in 187 NEAC patients (all diagnoses; table 1). Constitutive pSTAT3 expression was highest in CD4+ T cells (figure 2A). A similar pattern was seen for pSTAT1 (figure 2B). Constitutive pSTAT3 correlated with paired serum IL-6 levels most strongly in CD4+ T cells (r=0.60) and, to a lesser extent, CD8+ T cells (r=0.36), but not B cells (figure 2C; also online supplementary figure S1A–C). The relationship was similar irrespective of diagnostic category (figure 2D; online supplementary figure S1D–E). Importantly, multiple regression analysis (using CD4+ T cell pSTAT3 as the independent variable) indicated that the association with IL-6 remained after correction for age, CRP and circulating TNF, which does not signal primarily via STAT3 (β=0.44; p<0.001; online supplementary table S4), providing strong evidence for a causal relationship between IL-6 and STAT3 activation. No such correlations were seen in respect of pSTAT1 in any lymphocyte subset (figure 2E; online supplementary figure S1F–H). As was the case with IL-10 in our previous study, IL-23 (also known to signal via STAT3) was undetectable in all but a small number of NEAC patients’ sera (<10%; detection threshold 39 pg/mL; not shown) and did not correlate with pSTAT3. These results indicate that, in EA, CD4+ T lymphocytes are most sensitive to circulating IL-6. No correlation was seen between pSTAT1/3 in any subset and either circulating IL-6R or sgp130 (see online supplementary figure S2), excluding an overt influence of these mediators of trans IL-6 signalling on STAT activation in circulating lymphocytes. Moreover, strikingly higher levels of membrane IL-6R expression were expressed by circulating CD4+ T cells than by CD8+ T cells or CD19+ B cells (figure 2F).

Reduction in constitutive CD4+ pSTAT3 after anti-IL-6R blockade mirrors RA disease activity

To confirm the in vivo role of IL-6 signalling in STAT3 activation in RA, CD4+ T cell pSTAT3 was monitored in four patients with established, active RA in whom tocilizumab therapy was initiated. Clinical characteristics of the patients are provided in the online supplementary table S3. All four patients responded clinically to tocilizumab, and we observed that peripheral blood CD4+ T cell pSTAT3 declined with treatment (figure 3A) in a manner that more closely resembled serial disease activity scores (DAS28; figure 3B) than did a cruder measure of response, such as CRP (figure 3C). These data provide direct in vivo evidence that IL-6-mediated CD4+ T cell STAT3 activation is associated with disease activity. Furthermore, pSTAT3 measurement may have value as a biomarker for monitoring disease activity in RA.

Figure 2 (A) Constitutive phosphorylated signal transduction and activator of transcription-3 (pSTAT3) expression is maximal in CD4+ T cells compared with CD8+ and CD19+ cells in the peripheral blood of patients with early arthritis, and (B) a similar pattern is seen for pSTAT1. (C) Relationship between constitutive pSTAT3 and paired serum interleukin (IL)-6 is strongest in the CD4+ T cell lymphocyte population; significant differences are seen between best-fit line slopes (analyses of covariance (ANCOVA) p<0.001). (D) Comparable strength of association between constitutive pSTAT3 in CD4+ T cells and paired circulating IL-6 levels in patients with early rheumatoid arthritis (RA; black) and a disease control group comprising non-RA inflammatory arthritis (grey); no difference in the slope of the best-fit lines is seen between the comparator groups (ANCOVA). (E) Serum IL-6 does not correlate with basal pSTAT1 in any lymphocyte subset. (F) Surface IL-6R expression is maximal in the CD4+ T lymphocyte subset. ***p<0.001, non-parametric analysis of variance with Dunn’s posthoc pairwise analyses; Spearman correlation coefficients (rho) and associated p values are depicted. Correlation plots are also presented individually in the online supplementary figure S1, for further clarity. MFI, median fluorescence intensity.
Constitutive and induced CD4+ T cell pSTAT3 discriminates early ACPA-negative RA, reflecting serum IL-6 levels

Constitutive and IL-6-induced pSTAT1 and pSTAT3 expression was measured in lymphocytes of 184 EA clinic attendees, stratified by diagnostic category. Neither pSTAT1 nor pSTAT3 differed between diagnostic groups among CD8+ T cells or CD19+ B cells (see online supplementary figure S3A–D). In CD4+ T cells, basal pSTAT3 was significantly higher in patients with ACPA-negative RA than in non-RA IA and NIA controls (figure 4A,B; the same pattern was observed when patients with RA were stratified according to whether they were seronegative for both rheumatoid factor and ACPA; online supplementary figure S3E). The expected reciprocal pattern was seen with respect to induced pSTAT3 in CD4+ T cells on ex vivo IL-6 stimulation of whole blood (figure 4A, C; online supplementary figure S3F), reflecting reduced STAT3 phosphorylation capacity in vitro where constitutive pSTAT3 is increased. The relationship between serum IL-6 concentration and diagnostic category mirrored that of CD4+ T cell pSTAT3, being highest among patients with ACPA-negative RA (median 8.6 pg/mL; figure 4D). No differences were seen in constitutive CD4+ T cell pSTAT1 between diagnostic outcome groups (see online supplementary figure S4). Given the relatively high surface expression of IL-6R by CD4+ T cells (figure 2F), we investigated whether differential expression of this receptor according to diagnostic category could explain the observed differences in CD4+ T cell pSTAT3 levels, but found this not to be the case (figure 4E). Neither sIL-6R nor sgp130 differed between patients grouped according to diagnostic category (see online supplementary figure S5). These results indicate that IL-6 drives STAT3 phosphorylation prominently (and in preference to STAT1 phosphorylation) in circulating CD4+ T lymphocytes of patients with early ACPA-negative RA.

Constitutive CD4+ T cell pSTAT measurement has predictive value in diagnosing UA

We explored the potential of CD4+ T cell pSTAT3 as a diagnostic biomarker for EA patients with unclassifiable disease. Among 32 of 35 such patients with UA for whom outcome diagnoses became available, 12 had evolved to classifiable RA over a median of 20-months follow-up, compared with the remaining 20 assigned alternative (non-RA) diagnoses. Despite equivalent baseline clinical parameters (including inflammatory markers; online supplementary table S5) and circulating IL-6 levels (figure 5A), pSTAT3 in total CD4+ T cells differed significantly between the two outcome groups at baseline, being highest in UA–RA patients (p=0.02; figure 5B). Although no difference was seen between the two groups in respect of CD4+ T cell pSTAT1 or surface IL-6R expression (see online supplementary figure S6), the ratio of constitutive CD4+ T cell pSTAT3 to pSTAT1 ([MFI_{pSTAT3}]/[MFI_{pSTAT1}]) was again found to be highest in the UA–RA group (p=0.008; figure 5C).

The use of revised RA classification criteria15 has diminished the prevalence of ACPA in early UA,24 and this was reflected in our small cohort, all but one of whom were ACPA negative (see online supplementary table S5). The Leiden prediction rule, developed for use prior to this revision in classification, assigns a heavy predictive weighting to ACPA-positive individuals with respect to progression from UA to RA.14 Therefore, we reasoned that substitution of the prediction rule’s ACPA component with an indicator of constitutive CD4+ T cell pSTAT3/1 signalling might enhance its utility for predicting ACPA-seronegative UA–RA progression under 2010 classification criteria. Indeed, the predictive power of the published Leiden rule for an RA outcome in our small patient group was modest (AUC 0.67, 95% CI 0.47 to 0.87; figure 5D), consistent with previous reports.1 2 22 By comparison, the predictive power of constitutive CD4+ T cell pSTAT3 (MFI; AUC=0.77, 95% CI 0.61 to 0.95) or, as depicted in figure 5D, the pSTAT3:pSTAT1 ratio (AUC 0.78; 95% CI 0.61 to 0.95) appeared greater, although differences were not significant (p=0.4).

Maximal predictive utility for RA was achieved when the Leiden rule was modified to incorporate pSTAT3:pSTAT1 in place of ACPA status (see online supplementary text: ‘Prediction Rule’; AUC 0.84; 95% CI 0.68 to 1.0). This was significantly enhanced over that of the Leiden score alone (p=0.04; figure 5D). To address a potential over-fitting issue, a bootstrap cross-validation approach was also performed and gave similar AUC values, indicating that they are unlikely to be grossly inflated (see online supplementary table S6). An optimum cut-off value for the composite metric of 9.5 for determining likely progression of UA to RA had positive and negative predictive values of 0.82 (95% CI 0.54 to 0.96) and 0.86 (0.71 to 0.93), respectively (see also online supplementary table S7).

DISCUSSION

We report the first detailed, prospective analysis of IL-6 pathway activation parameters in a well-characterised EA patient cohort. Of particular clinical relevance, we find that measurement of the constitutive pSTAT3:pSTAT1 ratio in CD4+ T cells by flow cytometry holds promise as a predictive biomarker for RA progression among newly presenting ACPA-negative UA patients. Moreover, its incorporation into a prediction algorithm for evolving RA, itself published prior to revised classification criteria, yields a potentially valuable tool for predicting UA–RA progression in the post-2010 era. Our finding warrants replication in diverse EA cohorts as a priority. Importantly, our new

Figure 3 Longitudinal analysis of total CD4+ phosphorylated signal transduction and activator of transcription-3 (pSTAT3) (A), disease activity score in 28 joints (DAS28) (B) and C reactive protein (CRP; C) in four patients with established rheumatoid arthritis. Measurements were made immediately before, and 1 month after, initial treatments with tocilizumab. Additional clinical information is available in the online supplementary table S4. MFI, median fluorescence intensity.
data confirm the importance of IL-6-mediated CD4+ T cell STAT3 signalling in early RA and highlight flow cytometry as a method by which IL-6/STAT3 pathway activation might be used as a diagnostic biomarker. With this in mind, the use of the pSTAT3:pSTAT1 ratio was preferred over pSTAT3 MFI alone, representing a metric that can be more easily standardised for use between laboratories and technologies.

The activation of STAT3 by IL-6 in circulating CD4+ T cells raises intriguing questions as to the pathway’s role in RA induction. Our observation that cytokine-mediated T cell activation accompanies the earliest clinical phase of RA recalls previous evidence that such mechanisms might underpin disease pathogenesis independently of antigen recognition.27 28 An IA mouse model characterised by mutated IL-6 signalling machinery has recently added support to this concept.29–31 We noted that peripheral blood CD4+ T cells from patients with untreated RA and non-RA IA were similarly ‘sensitive’ to circulating IL-6 (figure 2D) excluding differential cytokine responsiveness as the driver of distinctive cellular STAT3 phosphorylation between disease phenotypes. However, CD4+ pSTAT3 (together with the pSTAT3:pSTAT1 ratio) appears to be a more accurate predictor of ACPA-negative RA than IL-6 before the disease is fully clinically manifest (figure 3), potentially incriminating factors other than IL-6 that affect the STAT3 pathway (such as membrane-bound gp130 expression, which we did not measure in our study). Understanding such processes within the clinical context of early RA will inform efforts to refine targeted RA treatments of the future.32 33

Despite being carefully planned and conducted over a 2-year period, our study was limited by relatively few patients with UA. Considering this alongside the heterogeneity of EA, this affected statistical power. Moreover, it is conceivable that the utility of our putative biomarker is critically dependent on one or other of the technical parameter(s) that were carefully controlled at our unit—including (but not limited to) the UA definition, non-exposure to DMARDs among participants, the time of blood draw or the decision to stain fresh blood (eg, rather than frozen peripheral blood mononuclear cells). Replication of our findings will therefore be the final arbiter of both their validity and their clinical utility.

Key questions raised by our work include the cellular source of excess IL-6 in incipient RA, its influence on downstream effector CD4+ T cell function and the relative contribution of classical versus trans IL-6 signalling to the process. Of further interest will be the extent to which these steps are regulated by genetic determinants.34 35 Finally, the apparent dominance of IL-6-driven CD4+ T cell STAT3 activation in ACPA-negative, over ACPA-positive, RA deserves attention (figure 4B,D). In fact, our data overall suggest that IL-6-driven STAT3 activation is relevant to the pathogenesis of both serotypes, but its relative importance in ACPA-negative disease may be greater. Other mechanisms (including the production of autoantibodies...
themselves) may play a balancing role in ACPA-positive RA induction, but further study will be needed to explore this hypothesis.

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Contributors AEA contributed intellectually to the design and analysis of the study and carried out the majority of the laboratory work, as well as contributing to analyses and manuscript drafting. MAKS also contributed substantially to the design and execution of laboratory work and its analysis. AGP conceived and designed the study, carried out some of the laboratory work, contributed to its analysis and wrote the manuscript. JPD made an important contribution to laboratory work and analysis. CR and BH carried out and recorded detailed assessments of patients, contributing to the analysis of clinical data, with valuable assistance from PMB. K-ALC provided important intellectual and analytical input to the revised manuscript. RT and JDI contributed to the study’s conception, design, interpretation and manuscript drafting. All authors critically appraised and approved the final manuscript.

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Competing interests None.

Ethics approval County Durham and Darlington Regional Ethics Committee, UK.

Provenance and peer review Not commissioned; externally peer reviewed.

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IL-6 driven STAT3 signalling in circulating CD4+ lymphocytes is a marker for early anti-citrullinated peptide antibody-negative rheumatoid arthritis.

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Online Supplementary Data
Prediction Rule.

**Method for modifying Leiden prediction rule** (as described in *Reference 17* of main document).

Binary logistic regression was carried out amongst 32 UA patients in the described cohort whose outcome diagnoses were known, with diagnostic outcome (RA versus non-RA) as the dependent variable (see also main document text and *Supplementary Table S4* for demographic details of this cohort). For each individual in the cohort the Leiden prediction score was calculated according to available baseline clinical and serological parameters, and instructions outlined in *Reference 17*. In addition, the ratio of constitutive pSTAT3/pSTAT1 (MFI) in circulating CD4+ T-cells was calculated for each individual. These 2 variables were entered into the regression model as independent variables, and the results are depicted below.

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<th>B</th>
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<th>p-value</th>
<th>95% CI (B)</th>
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<td>Leiden Score</td>
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<td>0.3</td>
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<td>pSTAT3/pSTAT1</td>
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*B*=regression coefficient; *SE*=standard error; *CI*= confidence interval.

Based on the above, and utilising the respective regression coefficients, we reasoned that for an individual UA patient the probability of RA development was related to the two covariates via the expression:

\[ (-0.6[\text{Leiden Score}]) + (-2.97[\text{CD4+ pSTAT3:pSTAT1}]). \]

In order to simplify the calculation for general use, we rounded regression coefficients to the nearest integer and removed negative charges. Since the revised metric was designed primarily for use amongst ACPA-negative UA patients (and all but one of the patients in our cohort were indeed ACPA negative), we then modified the Leiden prediction rule by stipulating that ACPA status was no longer considered, but that a value of 3 times the constitutive CD4+ T cell pSTAT3:pSTAT1 ratio was added to the accumulating score instead. Hence, for an individual, the modified metric is calculated as shown overleaf:
1. What is the age? Multiply by 0.2: 

2. What is the sex? In case female 1 point 

3. What is the distribution of involved joints?
   - In case small joints hands and feet 0.5 point
   - In case symmetric 0.5 point
   - In case upper extremities 1 point
   Or: In case upper and lower extremities 1.5 point

4. What is the length of the morning stiffness (minutes)?
   - In case 30-59 min 0.5 point
   - In case ≥60 min 1 point

5. What is the number of tender joints (out of 68 joints)?
   - In case 4-10 0.5 point
   - In case 11 or higher 1 point

6. What is the number of swollen joints (out of 66 joints)?
   - In case 4-10 0.5 point
   - In case 11 or higher 1 point

7. What is the C-reactive protein level (mg/L)?
   - In case 5-50 0.5 point
   - In case 51 or higher 1.5 point

8. Is the rheumatoid factor positive?
   - If yes 1 point

9. What is the CD4+ T cell pSTAT3:pSTAT1 ratio?
   Multiply by 3: 

Total score: _______
<table>
<thead>
<tr>
<th></th>
<th>ACPA-RA (n=18)</th>
<th>ACPA+RA (n=24)</th>
<th>Non-RA Inflam. (n=44)</th>
<th>OA / non-inflam. (n=66)</th>
<th>UA (n=35)</th>
<th>p (Inflam. diagnoses(^a))</th>
<th>p (All Diagnoses(^b))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>68 (30-88)</td>
<td>58 (27-81)</td>
<td>55 (18-91)</td>
<td>51 (27-86)</td>
<td>52 (19-79)</td>
<td>0.016</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>% Female</strong></td>
<td>72</td>
<td>71</td>
<td>64</td>
<td>74</td>
<td>71</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Symptom duration (weeks)</strong></td>
<td>12 (4 - &gt;52)</td>
<td>16 (4 - &gt;52)</td>
<td>9 (2 - &gt;52)</td>
<td>18 (3 - &gt;52)</td>
<td>12</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td><strong>ESR (seconds)</strong></td>
<td>23 (1-71)</td>
<td>22 (4-86)</td>
<td>13 (1-113)</td>
<td>8 (1-100)</td>
<td>15</td>
<td>ns</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>CRP (g/l)</strong></td>
<td>10 (&lt;5-91)</td>
<td>10 (&lt;5-56)</td>
<td>8 (&lt;5-189)</td>
<td>&lt;5 (&lt;5-49)</td>
<td>9</td>
<td>ns</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>%ACP A+</strong></td>
<td>0</td>
<td>100</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>%RF+</strong></td>
<td>33</td>
<td>75</td>
<td>9</td>
<td>9</td>
<td>17</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>DAS28</strong></td>
<td>5.15 (2.31-7.16)</td>
<td>5.00 (1.59-7.07)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>ns</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table S1.** Clinical and serological characteristics of Newcastle early arthritis cohort stratified by baseline diagnosis. Except where indicated, median and range is given. \(^a\)Kruskal Wallis ANOVA analysis confined to 3 groups with confirmed inflammatory diagnoses at inception: ACPA- RA, ACPA+ RA and non-RA IA. \(^b\)Kruskal Wallis ANOVA, 5 groups, including OA / non-inflammatory arthralgia and undifferentiated arthritis (UA).
Table S2

<table>
<thead>
<tr>
<th></th>
<th>Untreated RA (n=22)</th>
<th>Treated RA (n=18)</th>
<th>Healthy (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brisbane cohort, n (%)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4 (18)</td>
<td>18 (100)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>15 (68.2)</td>
<td>10 (55.6)</td>
<td>7 (54)</td>
</tr>
<tr>
<td>Age, years; mean (SD)</td>
<td>57 (46-71)</td>
<td>56 (41-71)</td>
<td>31 (23-40)</td>
</tr>
<tr>
<td>Symptom duration, weeks</td>
<td>16 (8-29)</td>
<td>88 (48-192)</td>
<td>n/a</td>
</tr>
<tr>
<td>RF positive, n (%)</td>
<td>14 (63.6)</td>
<td>16 (88.8)</td>
<td>ND</td>
</tr>
<tr>
<td>CCP positive, n (%)</td>
<td>14 (63.6)</td>
<td>16 (88.8)</td>
<td>ND</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>3 (0-7)</td>
<td>0 (0-2)</td>
<td>n/a</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>7 (2-14)</td>
<td>0 (0-5.5)</td>
<td>n/a</td>
</tr>
<tr>
<td>ESR, mm/h</td>
<td>25 (11.8-55.5)</td>
<td>16 (9.5-28.5)</td>
<td>ND</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>10 (5.8-22.5)</td>
<td>3.5 (2-13)</td>
<td>ND</td>
</tr>
<tr>
<td>DAS28&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.35 (2.76-6.17)</td>
<td>3.09 (2-4)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Treatment** (% of cohort)

- Methotrexate | 15 (83%)
- Hydroxychloroquine | 14 (78%)
- Sulphasalazine | 9 (50%)
- Azathiaprine | 1 (5%)
- Low dose prednisolone | 1 (5%)

**B**

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Newcastle (n=7)</th>
<th>Brisbane (n=4)</th>
<th>Total (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, n (%)</td>
<td>5 (71)</td>
<td>2 (50)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>Age, years; mean (SD)</td>
<td>64 (53-75)</td>
<td>57 (46-68)</td>
<td>61 (50-72)</td>
</tr>
<tr>
<td>Symptom duration, weeks</td>
<td>20 (8-52)</td>
<td>13 (12-42)</td>
<td>18 (8-52)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.73 (2.01-7.15)</td>
<td>5.11 (2.11-6.7)</td>
<td>4.73 (2.01-7.15)</td>
</tr>
</tbody>
</table>

Table S2A. Clinical characteristics of early / established RA patients and controls from Newcastle / Brisbane as per Figure 1E. <sup>A</sup>The majority (18/22; 82%) DMARD-naïve early arthritis patients were drawn from the Newcastle cohort, and all other patients from the Brisbane cohort. <sup>B</sup>There was a significant difference in DAS28 between untreated and treated RA patients (p<0.05, Mann-Whitney U test).

Table S2B. Baseline clinical characteristics of inception RA cohort for which data depicted in Figure 1F, and showing respective contribution of patients drawn from Newcastle and Brisbane cohorts (the same 4 Brisbane patients contributed to the untreated RA group in Table 2A). Phosflow measurements were made in fresh blood in each case prior to, and 3 months following, initiation of DMARD therapy. DMARD therapy included methotrexate in all cases; all Brisbane patients received combination therapy with hydroxychloroquine and sulphasalazine (see reference 23, main document); Newcastle patients received a bolus of steroid (80mg intramcinolone).

Except where indicated, median values are presented (interquartile range). ND: not done; n/a: not applicable.
Table S3

| Clinical characteristics of 4 patients commenced on tocilizumab therapy (see text). RTX: rituximab; MTX: methotrexate; HXQ: hydroxychloroquine. |
|---|---|---|---|---|
| RA Patients | 1 | 2 | 3 | 4 |
| Age (Yrs) | 71 | 52 | 80 | 40 |
| Sex | F | M | F | F |
| Disease Dur* (Yrs) | 17 | 13 | 14 | 5 |
| DMARDs | Concurrent | none | MTX | none | MTX, HXQ |
| Prior | RTX, MTX | RTX | Gold | RTX |
Multiple Regression Analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstandardised coefficients:</th>
<th>Standardised coefficients:</th>
<th>p-value</th>
<th>95% CI (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE(B)</td>
<td>β</td>
<td></td>
</tr>
<tr>
<td>Log_{10}[IL-6]</td>
<td>101.7</td>
<td>27.1</td>
<td>0.441</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log_{10}[CRP]</td>
<td>0.90</td>
<td>0.34</td>
<td>0.182</td>
<td>0.01</td>
</tr>
<tr>
<td>Log_{10}[TNF]</td>
<td>5.60</td>
<td>35.81</td>
<td>0.011</td>
<td>0.88</td>
</tr>
<tr>
<td>Age</td>
<td>0.61</td>
<td>0.48</td>
<td>0.083</td>
<td>0.21</td>
</tr>
<tr>
<td>Constant</td>
<td>97.00</td>
<td>27.07</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstandardised coefficients:</th>
<th>Standardised coefficients:</th>
<th>p-value</th>
<th>95% CI (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE(B)</td>
<td>β</td>
<td></td>
</tr>
<tr>
<td>Log_{10}[IL-6]</td>
<td>162.055</td>
<td>19.476</td>
<td>0.558</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log_{10}[CRP]</td>
<td>1.004</td>
<td>0.380</td>
<td>0.161</td>
<td>0.009</td>
</tr>
<tr>
<td>Log_{10}[TNF]</td>
<td>21.118</td>
<td>40.491</td>
<td>0.032</td>
<td>0.603</td>
</tr>
<tr>
<td>Age</td>
<td>0.522</td>
<td>0.548</td>
<td>0.056</td>
<td>0.342</td>
</tr>
<tr>
<td>Constant</td>
<td>91.247</td>
<td>30.733</td>
<td>-</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Table S4 A and B.** Results of standard linear regression analysis to identify variables independently associated with CD4+ T cell pSTAT-3 amongst 187 EA clinic patients. The dependent variable was pSTAT3 (median fluorescence intensity) amongst total circulating CD4+ T cells (Table S4 A) or naïve (CD45RA+ CD62L+) CD4+ T cells (Table S4 B). SE (B): standard error for B; CI: confidence interval. Where necessary variables were log10 transformed to satisfy normality conditions. See main article text.
**Table S5**

<table>
<thead>
<tr>
<th></th>
<th>UA* – RA (n=12)</th>
<th>UA* – Non-RA (n=20)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>55 (35-79)</td>
<td>48 (19-79)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Female: n (%)</strong></td>
<td>8 (67)</td>
<td>15 (75)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Symptom duration.</strong></td>
<td>12 (3-52)</td>
<td>10 (3-52)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Swollen joint count</strong></td>
<td>3 (0-18)</td>
<td>2.5 (0-15)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>ESR</strong></td>
<td>18 (4-65)</td>
<td>14 (1-78)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td>11 (5-53)</td>
<td>8 (&lt;5-76)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>ACPA+ : n (%)</strong></td>
<td>1 (10)</td>
<td>0 (0)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>RF+ : n (%)</strong></td>
<td>4 (33)</td>
<td>1 (5)</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Diagnosis; n (%)**

|          | RA 12 | PsA 6 | S-LIA 7 | ReA 3 | NIA 4 |

**Table S5.** Baseline characteristics and outcome diagnoses for 32/35 patients with undifferentiated arthritis (UA) patients in the cohort for whom all information is available; median follow-up since inception = 20 months (range 11-25); *All patients, classified here with reference to 2010 RA criteria (Ref 15), were also determined UA with reference to pre-existing 1987 criteria (Ref 21). †Mann Whitney-U test. N.b. diagnoses for additional 3 patients in cohort remains UA; median follow-up for this group was shorter at 12 (9-21) months. RA: rheumatoid arthritis; PsA: psoriatic arthritis; S-LIA: self-limiting inflammatory arthritis; ReA: reactive arthritis; NIA: non-inflammatory arthralgia / OA.
Table S6

<table>
<thead>
<tr>
<th>A</th>
<th>Mean AUC</th>
<th>Median AUC</th>
<th>SD AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leiden score</td>
<td>0.67</td>
<td>0.68</td>
<td>0.11</td>
</tr>
<tr>
<td>pSTAT3: pSTAT1</td>
<td>0.78</td>
<td>0.79</td>
<td>0.09</td>
</tr>
<tr>
<td>“Composite”</td>
<td>0.84</td>
<td>0.85</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Table S6A. Summaries of AUC across 1000 bootstrap samples for 3 parameters compared in Figure 5D, main document (see text). The mean values are identical to those derived in the primary analysis. “Composite” refers to composite risk metric derived from Leiden score modified to incorporate pSTAT3:pSTAT1 ratio (see text); AUC: area under curve; SD: standard deviation.*
<table>
<thead>
<tr>
<th>Predicted diagnosis</th>
<th>Actual outcome diagnosis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RA</td>
<td>Non-RA</td>
</tr>
<tr>
<td>RA</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Non-RA</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table S7.** A. Contingency table cross-tabulating predicted diagnosis (based on calculated modified Leiden score, and employing an optimum score cut-off of 9.5, above which progression to RA is predicted) versus actual outcome diagnosis, for 32 UA patients in this study. B. Diagnostic evaluation statistics based on contingency table presented in A. CI: confidence interval; +LR: positive likelihood ratio; -LR: negative likelihood ratio; PPV: positive predictive value; NPV: negative predictive value.

<table>
<thead>
<tr>
<th>Value</th>
<th>(95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence§</td>
<td>0.38 (0.21 - 0.56)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.75 (0.50 - 0.88)</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.90 (0.75 - 0.98)</td>
</tr>
<tr>
<td>+LR</td>
<td>7.5 (1.98 - 42.5)</td>
</tr>
<tr>
<td>-LR</td>
<td>0.28 (0.12 – 0.67)</td>
</tr>
<tr>
<td>PPV§</td>
<td>0.82 (0.54 – 0.96)</td>
</tr>
<tr>
<td>NPV§</td>
<td>0.86 (0.71 – 0.93)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>0.84 (0.65 – 0.94)</td>
</tr>
</tbody>
</table>

§Prevalence value equates to proportion of UA patients in current sample (n=32) who actually progressed to RA, or prior probability of progression to RA. Calculations for PPV and NPV are valid where the value for prevalence is generalizable to the population (i.e. it is assumed that the rate of UA-RA progression of 0.38 is representative of the UA population in general).
Figure S1. A-C. Individual plots depicted in Figure 2C of main article. D-E. Individual plots depicted in Figure 2D of main article. F-H. Individual plots depicted in Figure 2E of main article.
Figure S2. A-D. Lack of correlation between constitutive CD4+ T cell pSTAT3 or pSTAT1 with either sIL-6R or sgp130. Spearman’s correlation coefficient (Rho) for each bivariate analysis; ns: not significant. Paired data available for 187 patients (sIL-6R) and 88 patients (sgp130).
Figure S3

**Figure S3 A-D.** No differences are seen between diagnostic groups with respect to constitutive pSTAT3 (A, B) or pSTAT1 (C, D) in CD8+ T-cells (left panels; 184 patients) or CD19 B-cells (right panels; data available for 71 patients). **E-F.** Similar results to those presented in Figure 4 were obtained when RA patients were stratified according to whether they were RF and ACPA “double-seronegative” versus seropositive for either RF or ACPA; exemplar data shown with respect to constitutive pSTAT3 (E) and pSTAT3 fold-induction (F) in CD4+ T cells; compare with Figures 4B and C of main article. * and ** indicate p<0.05, and <0.001 (Dunn’s post-hoc pairwise analysis following non-parametric ANOVA).
Figure S4. A: Representative flow cytometry histograms depicting constitutive and IL-6 induced pSTAT1 in the CD4+ T cell-gated population of whole blood from exemplar non-RA IA disease control (shaded plots) and ACPA-negative RA patient (non-shaded plots; dotted line denotes fluorescence-minus-one control). B: ANOVA (Kruskall-Wallis) reveals no relationship between constitutive pSTAT1 and diagnostic outcome in circulating CD4+ T cells of early arthritis patients. MFI: median fluorescence intensity.
Figure S5. No differences are seen between diagnostic groups with respect to serum concentrations of (A) soluble IL-6 receptor (184 individuals) or (B) soluble gp130 (data available for 88 individuals.)
Figure S6. Surface IL-6R (A) and intracellular pSTAT1 (B) in circulating CD4+ T cells of patients presenting with undifferentiated arthritis are each comparable between those who evolve into classifiable RA and those with alternative diagnoses at follow-up; see text. No significant differences are seen between comparator groups (Mann-Whitney U test).