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Quantifying circulating Th17 cells by qPCR: potential as diagnostic biomarker for rheumatoid arthritis

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Abstract

Objective. The diagnosis of RA patients remains a challenge, especially in ACPA-negative disease. Novel T-cell subsets, particularly Th17 may be useful, although data on Th17 frequency using flow cytometry in RA are conflicting. We investigated whether a novel epigenetic qPCR assay for the quantification of Th17 could differentiate patients with RA from those with symptoms evolving towards an alternative diagnosis.

Methods. We used a qPCR assay measuring the extent of the methylation at a key position in the IL-17 and CD4 genes. Assays were performed on whole blood from 49 healthy controls (HC) and 165 early arthritis clinic patients. Flow cytometry was further used to detect the expression of CXCR4 on Th17 cells.

Results. In 75 inflammatory arthritis patients who progressed to RA, the qPCR assays showed significantly fewer Th17 cells compared with 90 patients who did not ($P<0.0001$). Regression models demonstrated a high predictive value for RA development (75.8% correct prediction), and particularly for the ACPA-negative group ($n=125$) where Th17 and swollen joint count (SJC) were the only predictors (73% correct prediction). The chemokine receptor CXCR4 had significantly higher expression on Th17 from early RA patients ($n=11$) compared with HC ($n=15$).

Conclusion. The results of the epigenetic qPCR assay showed that low levels of Th17 cells were predictive of developing RA, particularly in the ACPA-negative patients. This could have value for insights into pathogenesis and management. The results suggest the recruitment of Th17 to the inflammatory disease site, consistent with high CXCR4 expression.

Key words: Th17 cells, rheumatoid arthritis, diagnostic biomarker

Rheumatology key messages

- Th17 quantification using quantitative PCR predicts progression to RA in early inflammatory arthritis.
- This quantitative PCR assay also predicts progression to RA in ACPA-negative patients.
- This quantitative PCR assay also predicts response to MTX in early RA.

Introduction

Although the exact immuno-pathological mechanism of RA is not fully understood, numerous investigations have demonstrated abnormalities in CD4+ T lymphocytes. Such abnormalities were shown to range from thymic development of naïve cells [1, 2], cell differentiation [1], activation and polarization [1, 3, 4], signalling [5] to early senescence [2, 6–9] and others (for review see [4]).Also, many genetic risk factors associated with RA are involved in T-cell functions [10, 11]. However, while the role of T cells is thought to be critical at a very early stage of disease development, no specific (common) antigen has been demonstrated to drive RA.

Over the past decade, there has been a considerable amount of interest in the interplay between immune suppression (regulatory T cells, Treg) and polarization, particularly with the discovery of Th17 cells. Polarization and suppression are achieved by epigenetic structural changes in key transcription factors (T-bet/Th1, GATA3/Th2, RORgt/Th17 and FoxP3/Treg). Although Th2 seems intact in RA [12], Th1 polarization has long been known to

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be perturbed notably with lack of T-bet engagement and unstable commitment [13–17]. On the other hand, the plasticity between human Treg/T helper cells may explain why Treg are not capable of suppressing responses at the disease site, while still being useful to predict disease progression in at-risk individuals [18], their frequency also being reduced in early, treatment naïve RA [19]. Abnormal polarization/suppression could be a mechanism by which immune regulation fails, resulting in preferential development of Th1/Th17 cells in RA [20–22].

High levels of CD4+Th17 cells and IL-17 have been observed in the joints of RA patients [21]. However, there have been discrepancies in the reports of Th17 cell frequency in blood of RA patients [21, 23–26], most likely related to different phenotypes or strategies used to define and quantify Th17 cells. To date, these conflicting data have limited the value of flow cytometry for Th17 cell frequency evaluation. To circumvent these methodological issues, here we used an epigenetic assay that was developed for the quantification of DNA-methylation in IL-17 gene. The assay follows the concept described in [27–29], where regulatory elements of genes displaying specific CpG demethylation are detected only in cells where the gene is activated/imprinted, whereas in all other cell types, these CpGs are fully methylated). Here, the lack of methylation in certain CpGs in a specific region of the IL-17A gene promoter is occurring only in differentiated/polarized cells, here the Th17 subset (CpG methylation heat map of the IL-17A gene promoter in different cell types in Supplementary Fig. S1, available at *Rheumatology* online) while these CpGs are fully methylated in other cells types (any other T cell subsets including Treg, as well as in B cell, NK cells, monocytes etc…). The qPCR is designed to detect this specific lack of CpG methylation in Th17 cells. Normalized to an assay that similarly measures demethylation in the CD4 gene to count CD4+T cells, the assay allows the frequency of Th17 cells to be measured as a percentage (%) of CD4+T cells. Being based on cell type specific CpG demethylation, the assay will not detect demethylation in Treg for example, despite the fact that they can express IL-17.

In this study, we used this assay to model the value of methylation-sensitive qPCR on the IL-17 gene in patients attending an early arthritis clinic without a definitive (RA/non-RA) diagnosis. We then used flow cytometry and a surrogate cell surface marker panel for Th17 cells to further phenotype these cells in early RA.

Methods

Patients

The Leeds Inflammatory Arthritis Disease Continuum (IACON, REC approval: 09/H1307/98), is a longitudinal register, comprising over 2000 patients of which 172 with early inflammatory arthritis symptoms (DMARDs naïve, <12 months) were selected (Table 1). Patients were followed for 2 years until fulfilling the EULAR 2010 classification criteria for RA ($n = 75$) or non-RA ($n = 97$), including 20 psoriatic arthritis and three ankylosing spondylitis, 12 other diagnoses (including gout, reactive arthritis and CTD), 37 persistent undifferentiated arthritis (UA) and 18 with self-resolving symptoms (including some inflammatory osteoarthritis). Forty-nine healthy controls (HC) were included. All patients and volunteers provided informed consent.

Table 1 Demographic and clinical parameters

	RA ($n = 75$)	Non RA ($n = 97$)	*P*
Age	53 (23–85)	45 (20–73)	<0.0001
Sex (M/F)	23/53	27/69	0.444
Symptom duration	6 (0–24)	7 (1–24)	0.511
RF (pos/neg)	30/44[a]	13/81[b]	<0.0001
ACPA (pos/neg)	36/39	5/91[a]	<0.0001
TJC	5 (0–26)	2 (0–28)	0.021
SJC	2 (0–20)	0 (0–20)	0.001
CRP(mg/L)	7 (<5–134)	<5 (<5–58)	0.007
DAS(4)28-CRP	3.6 (1–7.5)	2.7 (1.2–6.3)	0.003
HAQ	7 (0–22)	5 (0–20)	0.115
Th17 cells (%)	0.65 (0.16–1.80)	1.04 (0.33–2.73)	<0.0001

Data are reported as median and range unless stated otherwise. [a]missing data for 1 patient. [b]missing data for 2 patients. TJC: tender joint count; SJC: swollen joint count.

In a second study, similar early drug naive RA patients were studied for remission outcome at 6 months, nine patients received MTX+anti-TNF and were matched with 10 patients receiving MTX-only. Clinical outcome was previously published [30]. DAS28(CRP)<2.6 was used to classify response. DNA (extracted from frozen PBMC) was available for some of these patients at week 0 ($n = 15$), week 14 ($n = 12$) and week 22 ($n = 12$).

In a third analysis, 57 of the patients used above for classification were studied for outcome at 6 months, after receiving first line synthetic-DMARDs treatment in accordance with NICE guidelines. Forty-nine were treated with MTX in a standardized fashion, escalating dose over 8 weeks if DAS28(CRP)<2.6 remission was not achieved and adding other s-DMARDs (sulfasalazine or hydroxychloroquine) towards this aim. Eight were treated in a similar fashion but starting with sulfasalazine or hydroxychloroquine. DAS28(CRP)<2.6 at 6 months was used as remission outcome.

Th17 cell quantification

DNA was extracted using the Qiagen DNeasy Blood Kit (Crawley, UK) according to manufacturer's instructions from 200 μL of whole blood. Th17 cell enumeration was performed by Epiontis (Berlin, Germany) using the qPCR based DNA methylation analysis as principally proposed in [27, 31]. Briefly, following bisulphite conversion of genomic DNA, a qPCR assay specific for the de-methylated *IL17A* gene was performed [32]. Details of the assay are

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described in Supplementary Material, section Details of the epigenetic qPCR assay and Supplementary Fig. S1, available at *Rheumatology* online. A second qPCR specific assay was performed for the CD4 gene to serve as normalization for the number of CD4+T cells in a given sample. The percentage of Th17 CD4+T cells was then calculated: Percentage of a particular cell-type = [Copy Equivalents determined by qPCR targeting the cell-specific gene (e.g. Th17)]/[Copy Equivalents determined for the *CD4* assay] [28].

Polarization assay

Following a PBMC separation, a CD4+ depletion strategy (EasySep human CD4+ T cell enrichment cocktail and EasySep^{TM} D magnetic particles, Stemcell Technologies, Cambridge, UK) was used to purify human CD4+ T cell according to manufacturer's instruction. Isolated $CD4^+$ T cells were re-suspended in serum-free X-VIVO15 medium (Lonza, Belgium). Subsequently, 2 ml of cells (at 1 million cells/ml) were seeded in a 12-well microplate. Activation of CD4+T cells was performed using anti-CD3/CD28 beads (Miltenyi Biotect, Germany) at 50 beads/cells. A cytokine cocktail containing IL-1beta (12.5ng/ml), IL-23 (20 ng/ml) and TGF-beta1 (5 ng/ml) was used for Th17 polarization in addition to activating beads. Cells were placed at 37°C (5% CO_2). After 5 days, phorbol myristate acetate (1 μg/ml) and ionomycin (1 μg/ml) were added for 3 h followed by Brefeldin A (1 μl/ml) for two more hours to stimulate cytokine production and allow intracellular detection. Cells were extensively washed with PBS, pelleted and re-suspended for staining. After cell surface staining for different markers (see below, cell surface staining procedures), the intracellular staining was performed using the IntraPrep Kit (Beckman Coulter, France) for intracellular cytokines according to the manufacturer's instructions with two intracellular antibodies: IL-17-PE (Clone SCPL1362, BD, Pharmingen, UK) and IFN-γ-FITC (Clone B27, BD, Pharmingen, UK).

Cell surface staining for flow cytometry

1 ml of peripheral EDTA whole blood from HCs and RA patients was processed for red cell lysis as previously described [19]. For the detection of cell surface markers, 30 μl of cells were incubated with varying combination of antibodies at 4°C for 30 min. Cells were then washed with 200 μl FACS buffer (0.1% bovine serum albumin in PBS with 0.01% sodium azide) and analysed by flow cytometry. Antibodies used were: anti-CD4 (Clone RPA-T4, BD), anti-CD3 (Clone RPA-T8, BD Pharmingen, UK), anti-CD161 (BD Pharmingen, UK), anti-CCR6 (Clone 11Ag, BD Pharmingen), anti-CXCR3 (Clone IC6/CXCR3, BD Pharmingen, UK) and anti-CXCR4 (Clone 12G5, BD). All analyses were performed on LSRII 4 laser flow cytometer (BD Biosciences). Data analysis was done by using FACSDiva software (BD Biosciences).

Statistical analysis

Data was described using median (range) or number (%) and compared between RA and non-RA using Man Whitney-U or χ^2. Logistic regression was performed to confirm that the Th17 cell frequency was associated with RA classification independently of potential confounders shown to have potential associations by unadjusted odd ratios (OR). The adjusted model then used parameters with significant individual association. A ROC analysis was performed to determine a cut-off for ~80% specificity for the prediction of the RA outcome. Analyses were conducted using SPSS 21.1.

Results

Th17 cells quantification in clinical groups

The Th17 qPCR assay was used to measure the frequency of Th17 cells in the blood of 172 patients attending the Leeds Early Arthritis Clinic. Expected demographic (Table 1, age) and clinical parameters (swollen joint count (SJC), CRP, DAS) were also associated with RA on univariate analysis as well as autoantibodies (ACPA and RF).

Th17 cell frequency was similar in healthy controls (HC), non-persistent arthritis (NP) and persistent UA (Fig. 1A), and also showed no significant difference in patients who developed alternative diagnoses (OT) or psoriatic arthritis/ankylosing spondylitis (PsA/AS), despite being lower than in HC for the later. However, in inflammatory arthritis patients progressing to RA (UA>RA) over 24 months and in RA patients (EULAR 2010 classification) at recruitment, Th17 cell frequencies were significantly reduced compared with HC and every other group ($P < 0.01$). Positivity for ACPA (and/or RF) was not associated with more pronounced differences in Th17 cells ($P = 0.166$ in the overall cohort and $P = 0.355$ in the RA subgroup).

When directly comparing 75 RA with 90 non-RA patients, the frequency of Th17 cells was significantly reduced in RA patients (Fig. 1B, $P < 0.0001$).

Regression model

We proceeded to a multivariate regression analysis of several parameters associated with RA diagnosis in this group of 172 patients (from Table 1). First, unadjusted odd ratio (OR) confirmed the association with RA for five parameters in this cohort (Table 2): age, ACPA, SJC, CRP and Th17. DAS28 was also associated ($P = 0.002$). Secondly, we performed two adjusted models, the first one with clinical parameters only and a second including Th17. ORs in the clinical model with these four parameters (Table 2), confirmed the dominant contribution of ACPA (OR = 13.53). ORs in the combined regression model with five parameters placed Th17 frequencies as the best predictor of RA ($OR = 1.32e^7$). Despite this very high OR, the Th17/clinical model performed better than the clinical-only model, but with a modest ~2.5% increase in correct prediction (from 73.3% to 75.8%).

In the ACPA-negative patients currently with limited diagnostic biomarker available (Table 2, $n = 130$), the two initial models were repeated. The clinical-only model performed poorly as no parameter remained significant. In contrast, the Th17/clinical model performed relatively

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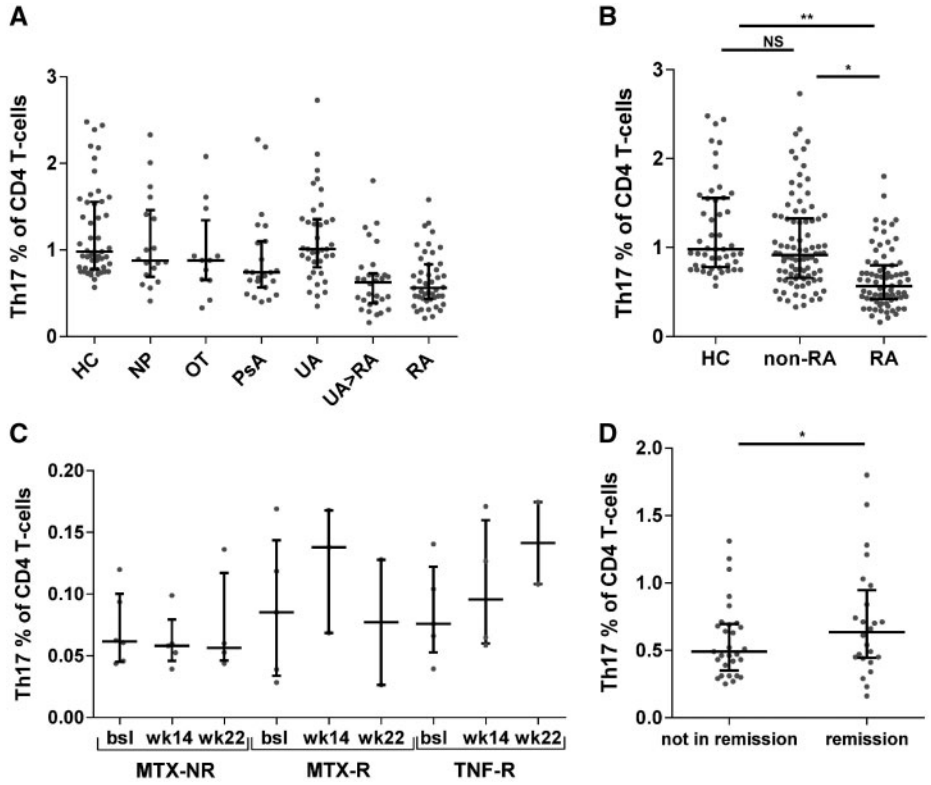
Fig. 1 Th17 cell frequency (%) in patients from an early arthritis clinic



A) The Th17 assay was performed on whole blood extracted from healthy controls (HC, $n = 49$), non-persisting symptoms (NP, $n = 18$), persistent undifferentiated arthritis (UA, $n = 37$), other inflammatory arthritis (OT, $n = 12$, including reactive arthritis, gout and CTD), psoriatic arthritis/ankylosing spondylitis (PsA/AS, $n = 23$), IA patients progressing to RA (UA->RA, $n = 23$) and newly diagnosed RA patients at recruitment (RA, $n = 52$). **B)** Th17 cell frequencies (%) in HC ($n = 49$), non-RA ($n = 90$) and RA ($n = 75$). **C)** Th17 frequency variations (in PBMCs) in three groups of drug naïve, early RA receiving first-time methotrexate (MTX) alone ($n = 10$, responders R, and $n = 5$ non-responders NR) or MTX in combination with anti-TNF ($n = 6$, all responders, R). **D)** Th17 frequency (%) in 57 early RA, drug naïve received MTX. Response is evaluated at 6 months using DAS < 2.6 for remission or not (* $P < 0.05$). Scatter plots are presented with median (line) and interquartile range (whiskers).

well (73% correct) with Th17 frequency offering high OR for RA (OR = 3.65e^6, $P < 0.0001$) and SJC still contributing to the prediction, while age and CRP no longer did.

Specificity/sensitivity comparison

We used ROC analysis to compare the performance of Th17 and the clinical variables associated with RA in the regression model. Using a cut-off for Th17 cell frequency determined by the ROC analysis (Fig. 2, $n = 172$, numerical value 0.66%, aiming for <80% specificity), RA in the whole cohort was associated with lower Th17 cells [area under the curve (AUC) = 0.300 in opposite direction to the clinical variables as predictive when low, $P < 0.0001$] with a sensitivity/specificity of 52%/80% and a positive predictive value/negative predictive value (PPV/NPV) of 66%/68%. In comparison, in this cohort, clinical parameters had lower sensitivity and similar specificity/PPV/NPV (as detailed in Fig. 2) compared with Th17 cells, while ACPA performed very closely to Th17 (AUC = 0.705, $P < 0.0001$), but with a lower sensitivity (48%) than Th17 cells (52%).

In the ACPA-negative patients ($n = 130$), the Th17 (cut-off 0.120, aiming for <80% specificity) had an AUC = 0.282 ($P < 0.0001$) while other parameters had more limited values (AUC < 0.700). The specificity (86%) and PPV (90%) were good and the NPV, if poor, was still better than for the clinical parameters (CRP and SJC).

Association between Th17 frequencies and response to first line treatement in early RA

Using 16 patients with available DNA from a previous study [30], 10 patients had received MTX and five achieved remission (R) at 6 months, while five patients were on anti-TNF+MTX and all achieved remission. Despite the small number (hence relative significance and large error bars), similar Th17 frequencies were observed at baseline in patients achieving R whether on MTX alone or on anti-TNF+MTX (Fig. 1C), in contrast to

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Table 2 Regression models

Parameters	Overall cohort $n = 172$			ACPA-negative cohort $n = 130$		
	Univariate Unadjusted OR (95%CI) *P*	Clinical & Th17 Adjusted OR (95%CI) *P*	Clinical only Adjusted OR (95%CI) *P*	Univariate Unadjusted OR (95%CI) *P*	Clinical & Th17 Adjusted OR (95%CI) *P*	Clinical only Adjusted OR (95%CI) *P*
Age (years)	0.948 (0.925, 0.971) <0.0001	0.978 (0.947, 1.008) NS	0.968 (0.940, 0.999) 0.032	0.945 (0.927, 0.982) 0.001	0.977 (0.945, 1.009) NS	0.970 (0.941, 1.001) NS
ACPA (yes/no)	16.8 (6.13, 46.1) <0.0001	14.5 (4.7, 44.2) <0.0001	13.53 (4.6, 39.3) <0.0001	NA	NA	NA
TJC	0.968 (0.924, 1.014) NS	NA	NA	0.953 (0.905, 1.001) NS	NA	NA
SJC	0.831 (0.746, 0.925) 0.001	0.845 (0.7484, 0.956) 0.007	0.860 (0.767, 0.985) 0.028	0.826 (0.732, 0.931) 0.002	0.883 (0.766, 0.981) 0.023	0.885 (0.781, 1.002) NS
CRP (mg/L)	0.696 (0.944, 0.993) 0.013	0.981 (0.952, 1.011) NS	0.981 (0.955, 1.008) NS	0.972 (0.946, 0.999) 0.032	0.984 (0.955, 1.014) NS	0.986 (0.956, 1.010) NS
DAS28(CRP)	0.665 (0.516, 0.856) 0.002	NA	NA	0.582 (0.421, 0.802) 0.001	NA	NA
Th17 (% of CD4+T-cells)	$1.13e^{6}$ (310, 4.17 e^{9}) <0.0001	$1.32e^{7}$ (128, 1.36 e^{12}) 0.005	NA	7.65 e^{6} (142, 4.1 e^{11}) 0.004	3.65 e^{6} (16, 8.33 e^{11}) 0.016	NA
% correct	NA	75.8%	73.3%	NA	73.0%	NA

TJC: tender joint count; SJC: swollen joint count; NS: non-significant; NA: non-applicable; OR: odds ratio.

NR to MTX showing fewer circulating Th17 at baseline ($P > 0.05$). In R (both MTX alone and anti-TNF+MTX), Th17 increased at week 14, suggesting re-circulation of Th17, although only transiently in the MTX-R, but persistently in the anti-TNF+MTX at week 22. In NR, no clear changes were observed at week 14 or week 22.

Analysing 57 patients with early RA treated with s-DMARDs according to NICE guidelines (Fig. 1D), 33 achieved remission after 6 months. Th17 cells were more frequent at baseline ($P = 0.028$) in these patients compared to the 24 who did not achieve remission.

Th17 cell phenotyping by flow cytometry

We performed different experiments to evaluate Th17 cell frequencies by flow cytometry to confirm our data. We aimed to use a panel of cell surface markers reported to be able to identify Th17 cells: CD161, CCR6 and CXCR3 [33, 34], without the need for intracellular cytokine staining. We first compared resting, activated (CD3/CD28 beads) and newly polarized CD4+T cells towards Th17 for their production of IL-17A and IFN-gamma with respect to these three markers. We observed 0.1% of IL-17-producing cells (Fig. 3A, IL17+ cells, square gate) under resting condition, 0.2% upon activation and 1.2% under Th17 polarization conditions. This suggests that upon activation, 2-fold more pre-committed Th17 cells could be detected in a healthy person, while 12-fold more cells were differentiating from naïve CD4+T cells under polarization conditions. We observed that IL-17+ cells were CD161+/CCR6+/CXCR3- (Fig. 3B, black histograms against total CD4 T cells, grey histograms) while IFN+ cells were CD161+/CCR6-/CXCR3+ (data not displayed). This allowed us to define a surrogate phenotype for Th17 cells based only on these three cell surface markers.

Using this surrogate phenotype (Fig. 4A), quantification of circulating Th17 cells confirmed significant difference between RA and HC with lower frequencies in RA (Fig. 4B, RA $n = 11$ and HC $n = 15$, $P = 0.011$). This difference was, however, small and of limited value on an individual patient basis.

To explain our results further, we examined the potential for recruitment of Th17 cells from the circulation into the joints. The expression of CXCL12 (SDF1) is high in active disease [35] but rarely detected in remission [36]. We therefore analysed the expression of CXCR4, the chemokine receptor targeting lymphocytes to the site of inflammation in RA via CXCL12 [33]. Using the CD161+/CCR6+/CXCR3- surrogate phenotype to define Th17 cells (Fig. 4A, CXCR4 panel), RA patients express significantly more CXCR4 than HC (Fig. 4C, RA median MFI 875 *vs* median MFI 600 in HC, $P = 0.002$).

Discusssion

Th17 cells have been implicated in RA pathogenesis [21, 22] and in its development from very early stages [37, 38]. Here, we demonstrate the potential of Th17 quantification as a diagnostic biomarker for RA in an early inflammatory arthritis population. Our data showed lower Th17 cells in

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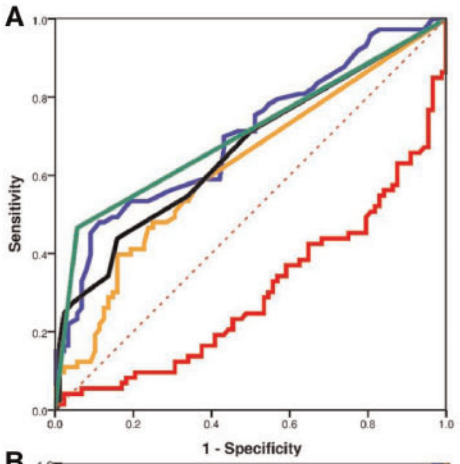
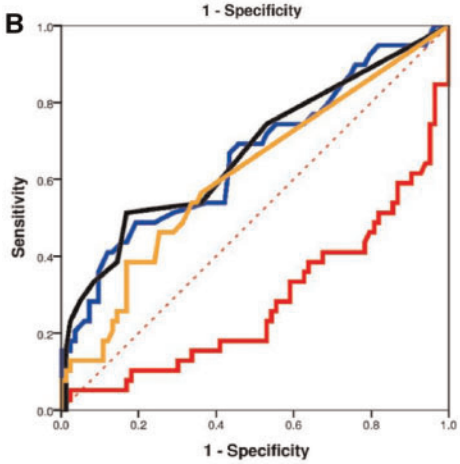
Fig. 2 Diagnostic value (ROC analysis) of potential predictors of RA



Variables	AUC (95%CI)	*p*-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
TH17	0.300 (0.218-0.381)	0.000	52	80	66	68
ACPA	0.705 (0.621-0.788)	0.000	48	94	88	70
SJC	0.663 (0.578-0.748)	0.000	34	86	66	63
CRP	0.623 (0.535-0.710)	0.007	41	82	65	63
Age	0.700 (0.618-0.781)	0.000	35	93	79	65



Variables	AUC (95%CI)	*p*-value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
TH17	0.282 (0.180-0.385)	0.000	35	86	90	27
SJC	0.637 (0.563-0.779)	0.002	24	38	67	9
CRP	0.612 (0.503-0.722)	0.046	26	52	61	20
Age	0.661 (0.553-0.769)	0.004	23	57	48	30

A) ROC analysis plot for five parameters in the whole cohort ($n = 172$). The four clinical parameters were predictive when increased, hence a ROC curve above the middle line (dotted red line). In contrast, Th17 was predictive when low, with a ROC curve under the middle line. **B)** ROC analysis in ACPA negative patients ($n = 130$). Th17, red; ACPA, green; SJC, black; CRP, orange; age, blue. ACPA: anti citrullinated peptides antibodies; SJC: swollen joint count; CRP: C-reactive protein.

patients progressing to RA compared with early IA patients evolving to alternative diagnoses, validated using a regression model and further confirmed by flow cytometry. Positivity for ACPA (and/or RF) was not associated with more pronounced differences in Th17 cells in this cohorts, despite previous suggestions [21, 39]. Modelling suggested a certain added value for using the Th17 qPCR in ACPA-negative patients, while it was only modestly improving diagnosis prediction in the overall cohort. Our data could be explained by the recruitment of Th17 cells from the circulating pool to the inflammatory disease site. Therefore, we strengthened this hypothesis by showing higher expression of the CXCR4 chemokine receptor on Th17 cells in early RA compared with HC, suggesting that Th17 cells may be preferentially migrating to joints where the CXCL12 (SDF1) ligand is highly expressed in active/relapsing disease but not in remission (as we have shown previously by immunohistochemistry in synovial biopsies [36]).

Elevated levels of IL-17 have been observed in the synovial fluid of RA patients where IL-17 promotes inflammation and joint damage [40]. Although over 20 studies have attempted to evaluate the frequency of Th17 cells in the blood of RA patients, results were inconclusive due to the use of different methodologies (recently reviewed in [41]). Our data therefore reinforced the concept of a role for Th17 in early disease. However, it is also clear that disease stages (at-risk *vs* early and long-lasting RA) yield different results in terms of Th17-circulating frequencies [22] (also reviewed in [41]) and this is likely to reflect different levels of implication for this cell subset in developing, early and established disease but also possible interferences of different treatment regimes in established RA. Nonetheless, association between number of circulating Th17 cells (and regulatory T cells) in active *vs* remitting disease was reported in long-lasting disease [21]. Our flow cytometry results also suggested a reduction in the proportions of circulating Th17 (using the surrogate

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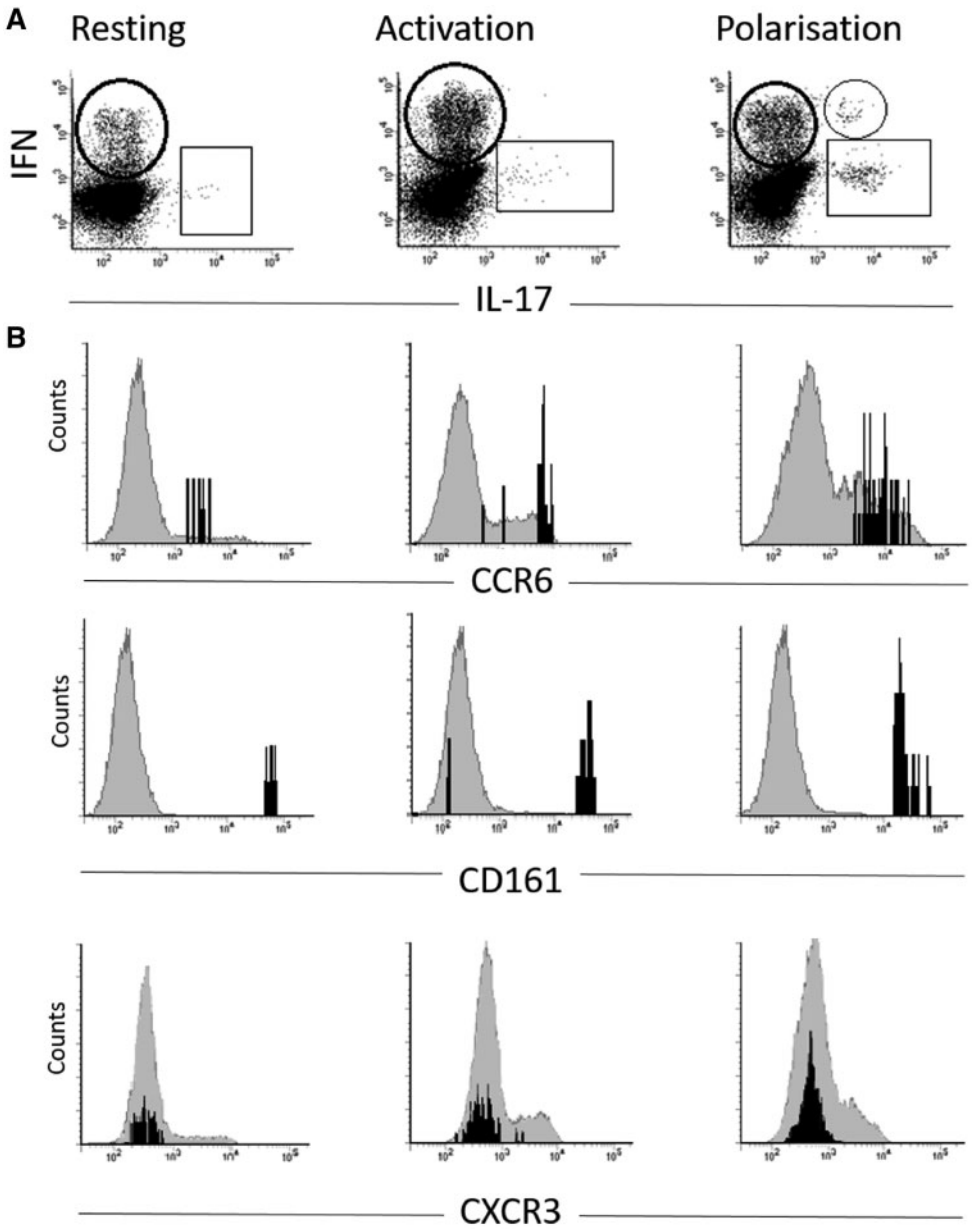
FIG. 3 Representative analysis of IL-17 and IFN-gamma expression in resting activated and polarised CD4+T cells



A) CD4+ T cells were first gated (not shown), then the expression of IL-17 and IFN-gamma was used to gate Th17 and Th1 cells respectively. **B**) Surface expression for CD161, CCR6 and CXCR3 on gated CD4+/Th17 subsets (black histograms) showing positive expression for CD161 and CCR6 but negative for CXCR3, compared with the rest of CD4+T cells population (grey histograms).

phenotype) compared with HC in early RA as also reported in [21]. Although we only analysed 11 RA patients, and HC were not the ideal comparator group, our flow cytometry data yielded similar results as the qPCR assay. The lack of sensitivity and the discrepancies in published flow cytometry results may be related to the fact that they detect active Th17 cells (i.e. expressing IL-17). In HC, there is no reason for Th17 to be active so flow cytometry may actually under-evaluate Th17 frequencies, while in disease, Th17 may indeed be activated and as such driven to the site of inflammation, therefore leaving the blood, thus reducing the frequency of detectable cells. On the other hand, the qPCR assay is detecting all epigenetically committed Th17 cells. It is not relying on IL-17 expression but on whether cells have committed to the Th17 phenotype. Therefore, it is detecting both resting

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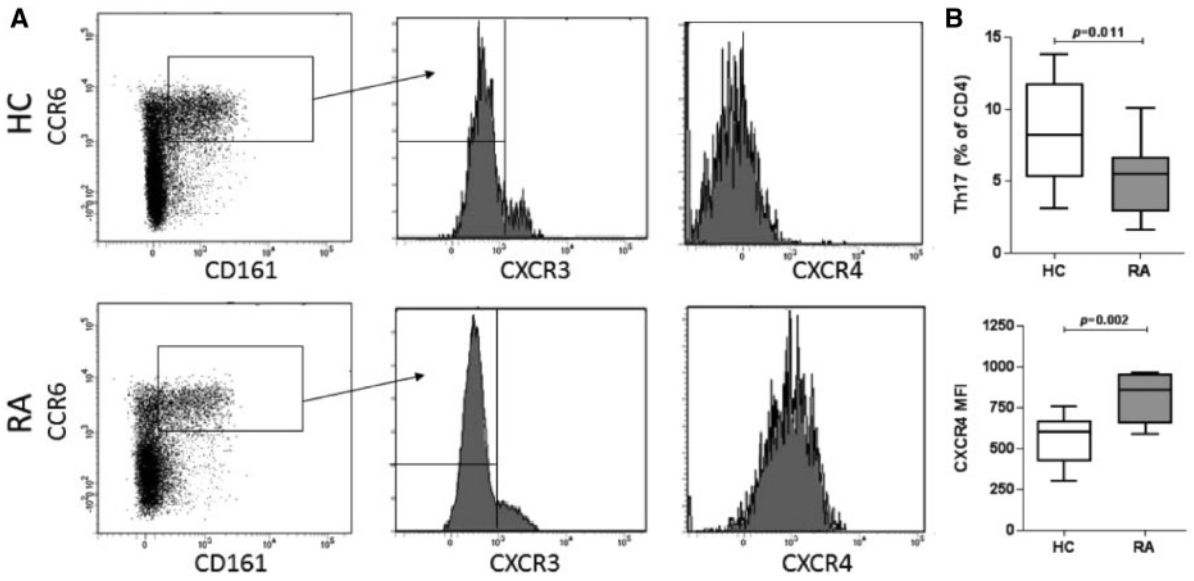
FIG. 4 Flow cytometric analysis of circulating Th17 cells in early RA patients ($n = 19$) and HC ($n = 15$)



A) Expression of CD161 and CCR6 on pre-gated CD4+ T cells (first plot), then used as gate (CD161+/CCR6+ cells) to restrict the population to negative CXCR3 cells (second plot); levels of expression for CXCR4 (MFI being recorded) on Th17 cells identified with the triple CD161+/CCR6+/CXCR3- surrogate phenotye. **B)** Lower Th17 cell (CD161+/CCR6+/CXCR3-) frequencies (as % of CD4+T cells) are observed in RA patients ($n = 19$, $P = 0.011$) compared with HC ($n = 15$). **C)** Higher expression of CXCR4 (MFI) in RA ($n = 19$, $P = 0.002$) compared with HC ($n = 15$). Boxplots are presented with median (line) and interquartile range (box) and extremes (whiskers).

and activated cells and may provide more accurate insight into the actual number of circulating Th17 in long-lasting RA, also helping towards understanding the lack of responses to anti-IL-17 therapy in established RA [42] while still able to predict (although needing further validation) response to MTX in early disease, as was also suggested by Arroyo-Villa *et al.*[21].

We observed a higher expression of the trafficking receptor CXCR4 on the surface of CD161+/CCR6+/CXCR3- Th17 cells in early RA patients compared with HC ($P = 0.002$). Of note, the expression of CXCR4 on total CD4+T cell RA patients was not significantly higher than in HC (data not shown). There is no report directly implicating CXCR4 as a trafficking receptor on Th17 but previous reports have suggested that Th17 cells express a wide profile of such receptors (CCR2, CCR4, CCR5, CCR6), but not all (negative for CXCR3 or CXCR5) [43], which was used to refine a surrogate phenotype with respect to Th1, Th2 and even Treg. These receptors are also associated with migration to inflamed tissues and the differential expression of CXCR4 on RA Th17 is a novel finding. CXCR4 expression (on T cells and other cell types) is induced in many diseases by a variety of stimuli including pro-inflammatory cytokines (IL-1beta), TGF-beta1, prostaglandin E2, hypoxia or neurotrophic factors [44–48]. It is therefore tempting to speculate that systemic inflammation in early RA may result in an increased expression of CXCR4 on the Th17 subset, though this remains to be specifically demonstrated. On the other hand, CXCR4 has been recognized as a potential therapeutic target, notably in cancer and for HIV infection [49] and was examined in an animal model of arthritis [50]. Conceivably, blocking CXCR4 may prevent Th17 cells from moving to the inflamed tissue sites. CCR6 is another important trafficking receptor in RA as its only known ligand, CCL20, was shown to be contributing to RA pathology by triggering self-destructive immune reactions in the joints [51].

Altogether these data also confirm that epigenetic alterations are involved early in the disease process at the time of clinical symptoms development and predict an evolution towards RA. Our data also suggest a further role for Th17 cell quantification along the IA continuum possibly from pre-clinical, at risk stages. Such epigenetic qPCR assays (for several other genes) are routinely used for immune monitoring in HIV infection, vaccinations, cancers, transplantation, aging and immunosuppression [31, 52–55]. This particular Th17 assay has been used to monitor Th17 cell frequency in asthma [55] and is intended for immunological monitoring in clinical trials notably when using an anti-IL-17 drug (such as secukinumab). Therefore, it offers a validated tool to classify RA early (particularly for sero-negative patients) and may also be of further use across the IA continuum for predicting response to conventional and biological therapy.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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