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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 rheumatoid arthritis (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 to 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 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 to 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

Key messages :

1- Th17 quantification using qPCR predicts progression to RA in early IA.
2- This assay also predicts progression to RA in ACPA-negative patients.
3- This assay also predicts response to MTX in early RA

INTRODUCTION:

Although the exact immuno-pathological mechanism of rheumatoid arthritis (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 polarisation [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 polarisation, particularly with the discovery of Th17 cells. Polarisation 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 polarisation has long been known to 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 polarisation/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/polarised cells, here the Th17 subset (CpG methylation heat map of the IL-17A gene promoter in different cell types in 'Supplementary Figure 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. Normalised 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 3 ankylosing spondylitis, 12 other diagnoses (including gout, reactive arthritis and connective tissue diseases (CTD)), 37 persistent UA and 18 with self-resolving symptoms (including some inflammatory osteoarthritis). 49 healthy controls (HC) were included.

In a second study, similar early drug naive RA patients were studied for remission outcome at 6 months, 9 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 patient used above for classification, were studied for outcome at 6 months, after receiving 1st line synthetic-DMARDs treatment in accordance with NICE guidelines. Forty-nine were treated with MTX in a standardised 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 use as remission outcome. All patients and volunteers provided informed consent.

Th17 cell quantification

DNA was extracted using the Qiagen DNeasy Blood Kit according to manufacturer's instructions from 200 uL 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 bisulfite conversion of genomic DNA, a qPCR assay specific for the de-methylated IL17A gene was performed [32]. Details of the assay are described in supplementary material and Figure 1 available at Rheumatology online. A second specific qPCR 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) 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, 2ml of cells (at 1 million cells/ml) were seeded in 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 (20ng/ml) and TGF-beta1 (5ng/ml) was used for Th17 polarisation in addition to activating beads. Cells were placed at 37°C (5% CO_2). After 5 days, phorbol myristate acetate (PMA) (1µg/ml) and ionomycin (1µg/ml) were added for 3 hours followed by Brefeldin A (1µl/ml) for 2 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) and IFN-γ-FITC (Clone B27, BD).

Cell surface staining for flow cytometry

1 ml of peripheral EDTA whole blood from HCs and RA patients was process 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 4ºC for 30 minutes. 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), anti-CD161 (BD Pharmingen), anti-CCR6 (Clone 11Ag, BD Pharmingen), anti-CXCR3 (Clone IC6/CXCR3, BD Pharmingen), 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 Chi-square. 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 (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 to HC and every other groups ($p<0.01$). 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 5 parameters in this cohort (Table 2): age, ACPA, SJC, CRP and Th17. DAS28 was also associated ($p=0.002$). Secondly, we performed 2 adjusted models, the first one with clinical parameters only and a second including Th17. ORs in the clinical model with these 4 parameters (Table 2), confirmed the dominant contribution of ACPA (OR=13.53). ORs in the combined regression model with 5 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 2 initial models were repeated. The clinical-only model performed poorly as no parameter remained significant. In contrast the Th17/clinical model performed relatively 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 (Figure 2, n=172, numerical value 0.066%, aiming for <80% specificity), RA in the whole cohort was associated with lower Th17 cells (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 PPV/NPV of 66%/68%. In comparison, in this cohort, clinical parameters had lower sensitivity and similar specificity/PPV/NPV (as detailed in Figure 2) compared to 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 1st line treatement in early RA.

Using 16 patient with available DNA from previous study [30], 10 patients had received MTX and 5 achieved remission (R) at 6 months, while 5 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 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 week14 or 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 compare to the 24 who did not.

Th17 cell phenotyping by flow cytometry

Towards confirming our data, we performed different experiments to evaluate Th17 cell frequencies by flow cytometry. 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 polarised CD4+T-cells towards Th17 for their production of IL-17A and IFN-gamma with respect to these 3 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 polarisation 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 3 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 patients 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 versus 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 patients progressing to RA compared to 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 these 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 to 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 versus 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 versus 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 phenotype) compared to 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 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 to 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 results in an increased expression of CXCR4 on the Th17 subset although this remain to be specifically demonstrated. On the other hand, CXCR4 has been recognised 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.

References

1 PONCHEL F, MORGAN AW, BINGHAM SJ, et al. Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. Blood 2002;100(13):4550-6.

2 DOUEK DC, BETTS MR, HILL BJ, et al. Evidence for increased T cell turnover and decreased thymic output in HIV infection. J. Immunol. 2001;167(11):6663-8.

3 MCINNES IB, SCHETT G. The pathogenesis of rheumatoid arthritis. New England Journal of Medicine 2011;365(23):2205-19.

4 PONCHEL F, VITAL E, KINGSBURY SR, et al. CD4+ T-cell subsets in rheumatoid arthritis. International Journal of Clinical Rheumatology 2012;7(1):37-53.

5 ISOMÄKI P, PANESAR M, ANNENKOV A, et al. Prolonged Exposure of T Cells to TNF Down-Regulates TCRζ and Expression of the TCR/CD3 Complex at the Cell Surface. The Journal of Immunology 2001;166(9):5495-507.

6 MACKALL CL, GRESS RE. Thymic aging and T-cell regeneration. Immunol. Rev. 1997;160:91-102.

7 KOETZ K, SPICKSCHEN K, GORONZY JJ, et al. Premature aging in the immune system of patients with rheumatoid arthritis. In: Arthritis and Rheumatism. Abstract 41.

8 PAWŁOWSKA J, SMOLEŃSKA Ż, ZDROJEWSKI Z, et al. Changes in proliferation kinetics of T cells: a new predictive cellular biomarkers for early rheumatoid arthritis? Journal of clinical immunology 2012;32(5):991-9.

9 PAWŁOWSKA J, SMOLEŃSKA Ż, DACA A, et al. Older age of rheumatoid arthritis onset is associated with higher activation status of peripheral blood CD4+ T cells and disease activity. Clinical & Experimental Immunology 2011;163(2):157-64.

10 GOULIELMOS GN, ZERVOU MI, MYRTHIANOU E, et al. Genetic data: The new challenge of personalized medicine, insights for rheumatoid arthritis patients. Gene 2016;583(2):90-101.

11 EYRE S, BOWES J, DIOGO D, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. Nat Genet 2012;44(12):1336-40.

12 VAN ROON JAG, GLAUDEMANS CA, BIJLSMA JWJ, et al. Differentiation of naive CD4(+) T cells towards T helper 2 cells is not impaired in rheumatoid arthritis patients. Arthritis Res. Ther. 2003;5(5):R269-R76.

13 KAWASHIMA M, MIOSSEC P. mRNA quantification of T-bet, GATA-3, IFN-γ, and IL-4 shows a defective Th1 immune response in the peripheral blood from rheumatoid arthritis patients: link with disease activity. Journal of clinical immunology 2005;25(3):209-14.

14 SKAPENKO A, LEIPE J, LIPSKY PE, et al. The role of the T cell in autoimmune inflammation. Arthritis research and therapy 2005;7(2):S4.

15 KAWASHIMA M, MIOSSEC P. Defect of Th1 immune response of whole blood cells from active patients with rheumatoid arthritis (RA). Arthritis Res Ther 2003;5(Suppl 1):1-.

16 PONCHEL F, BROWN A, FIELD S, et al. T-bet expression in RA patients with early, DMARDs naive disease is low and correlates with low levels of IL-7 and T-cell dysfunctions. Arthritis and Rheumatism 2004;50(9):S525-S.

17 CHURCHMAN SM, EL-JAWHARI JJ, BURSKA AN, et al. Modulation of peripheral T-cell function by interleukin-7 in rheumatoid arthritis. Arthritis Res. Ther. 2014;16(6):1.

18 HUNT L, HENSOR E, NAM J, et al. T-cell subsets: An immunological biomarker to predict progression to clinical arthritis in ACPA positive individuals. Annals of the Rheumatic Diseases 2016;75:1884-9.

19 PONCHEL F, GOËB V, PARMAR R, et al. An immunological biomarker to predict MTX response in early RA. Annals of the rheumatic diseases 2014;73:2047-53.

20 ANNUNZIATO F, COSMI L, SANTARLASCI V, et al. Phenotypic and functional features of human Th17 cells. The Journal of experimental medicine 2007;204(8):1849-61.

21 ARROYO-VILLA I, BAUTISTA-CARO M-B, BALSA A, et al. Frequency of Th17 CD4+ T Cells in Early Rheumatoid Arthritis: A Marker of Anti-CCP Seropositivity. PLoS ONE 2012;7(8):e42189.

22 CHALAN P, KROESEN B-J, VAN DER GEEST KS, et al. Circulating CD4+ CD161+ T lymphocytes are increased in seropositive arthralgia patients but decreased in patients with newly diagnosed rheumatoid arthritis. PloS one 2013;8(11):e79370.

23 LEIPE J, GRUNKE M, DECHANT C, et al. Role of Th17 cells in human autoimmune arthritis. Arthritis & Rheumatism 2010;62(10):2876-85.

24 GULLICK NJ, EVANS HG, CHURCH LD, et al. Linking power Doppler ultrasound to the presence of Th17 cells in the rheumatoid arthritis joint. PLoS One 2010;5(9):e12516.

25 EGGLETON P, BREMER E, TARR JM, et al. Frequency of Th17 CD20+ cells in the peripheral blood of rheumatoid arthritis patients is higher compared to healthy subjects. Arthritis Res. Ther. 2011;13(6):1-15.

26 YAMADA H, NAKASHIMA Y, OKAZAKI K, et al. Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. Annals of the rheumatic diseases 2008;67(9):1299-304.

27 WIECZOREK G, ASEMISSEN A, MODEL F, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Research 2009;69(2):599-608.

28 BARON U, WERNER J, SCHILDKNECHT K, et al. Epigenetic immune cell counting in human blood samples for immunodiagnostics. Science Translational Medicine 2018;10(452):eaan3508.

29 TÜRBACHOVA I, SCHWACHULA T, VASCONCELOS I, et al. The cellular ratio of immune tolerance (immunoCRIT) is a definite marker for aggressiveness of solid tumors and may explain tumor dissemination patterns. Epigenetics 2013;8(11):1226-35.

30 QUINN MA, CONAGHAN PG, O'CONNOR PJ, et al. Very early treatment with infliximab in addition to methotrexate in early, poor ‐ prognosis rheumatoid arthritis reduces magnetic

resonance imaging evidence of synovitis and damage, with sustained benefit after infliximab withdrawal: results from a twelve‐month randomized, double‐blind, placebo‐controlled trial. Arthritis & Rheumatology 2005;52(1):27-35.

31 SEHOULI J, LODDENKEMPER C, CORNU T, et al. Epigenetic quantification of tumor-infiltrating T-lymphocytes. Epigenetics 2011;6(2):236-46.

32 OLEK A, OSWALD J, WALTER J. A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic acids research 1996;24(24):5064-6.

33 ANNUNZIATO F, COSMI L, LIOTTA F, et al. Defining the human T helper 17 cell phenotype. Trends in immunology 2012;33(10):505-12.

34 GOSSELIN A, MONTEIRO P, CHOMONT N, et al. Peripheral blood CCR4+ CCR6+ and CXCR3+ CCR6+ CD4+ T cells are highly permissive to HIV-1 infection. The Journal of Immunology 2009:ji_0903058.

35 KANBE K, CHIBA J, INOUE Y, et al. SDF-1 and CXCR4 in synovium are associated with disease activity and bone and joint destruction in patients with rheumatoid arthritis treated with golimumab. Modern rheumatology 2016;26(1):46-50.

36 BURGOYNE CH, FIELD SL, BROWN AK, et al. Abnormal T cell differentiation persists in patients with rheumatoid arthritis in clinical remission and predicts relapse. Annals of the Rheumatic Diseases 2008;67(6):750-7.

37 CASCÃO R, MOURA RA, PERPÉTUO I, et al. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. Arthritis Res. Ther. 2010;12(5):R196.

38 LUBBERTS E. Th17 cytokines and arthritis. In: Seminars in immunopathology. Springer. Abstract 32.

39 PAULISSEN SM, VAN HAMBURG JP, DAVELAAR N, et al. CCR6+ Th cell populations distinguish ACPA positive from ACPA negative rheumatoid arthritis. Arthritis Res. Ther. 2015;17(1):344.

40 SHEN H, GOODALL JC, HILL GASTON J. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis & Rheumatism 2009;60(6):1647-56.

41 ALUNNO A, MANETTI M, CATERBI S, et al. Altered immunoregulation in rheumatoid arthritis: the role of regulatory T cells and proinflammatory Th17 cells and therapeutic implications. Mediators of inflammation 2015;2015.

42 GENOVESE MC, DUREZ P, RICHARDS HB, et al. Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a phase II, dose-finding, double-blind, randomised, placebo controlled study. Annals of the Rheumatic Diseases 2013;72(6):863-9.

43 LIM HW, LEE J, HILLSAMER P, et al. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. The Journal of Immunology 2008;180(1):122-9.

44 OBERMAJER N, MUTHUSWAMY R, ODUNSI KO, et al. PGE2-dependent CXCL12 production and CXCR4 expression control the accumulation of human MDSCs in ovarian cancer environment. Cancer research 2011:canres. 2449.011.

45 WANG J, WANG H, CAI J, et al. Artemin regulates CXCR4 expression to induce migration and invasion in pancreatic cancer cells through activation of NF-κB signaling. Experimental cell research 2018;365(1):12-23.

46 EDDLESTON J, CHRISTIANSEN SC, ZURAW BL. Functional expression of the CXC chemokine receptor CXCR4 by human bronchial epithelial cells: regulation by proinflammatory mediators. The Journal of Immunology 2002;169(11):6445-51.

47 BUCKLEY CD, AMFT N, BRADFIELD PF, et al. Persistent induction of the chemokine receptor CXCR4 by TGF-β1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. The Journal of Immunology 2000;165(6):3423-9.

48 GUO M, CAI C, ZHAO G, et al. Hypoxia promotes migration and induces CXCR4 expression via HIF-1α activation in human osteosarcoma. PLOS one 2014;9(3):e90518.

49 PATRUSSI L, T BALDARI C. The CXCL12/CXCR4 axis as a therapeutic target in cancer and HIV-1 infection. Current medicinal chemistry 2011;18(4):497-512.

50 ZHANG L, KAJIWARA H, KUBOYAMA N, et al. Reduction of CXCR4 expression in rheumatoid arthritis rat joints by low level diode laser irradiation. Laser therapy 2011;20(1):53-8.

51 HIROTA K, YOSHITOMI H, HASHIMOTO M, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. Journal of Experimental Medicine 2007;204(12):2803-12.

52 SCHWARZER A, WOLF B, FISHER JL, et al. Regulatory T-cells and associated pathways in metastatic renal cell carcinoma (mRCC) patients undergoing DC-vaccination and cytokine-therapy. PLoS One 2012;7(10):e46600.

53 DELEMARRE EM, VAN DEN BROEK T, MIJNHEER G, et al. Autologous stem cell transplantation aids autoimmune patients by functional renewal and TCR diversification of regulatory T cells. Blood 2016;127(1):91-101.

54 STERVBO U, BOZZETTI C, BARON U, et al. Effects of aging on human leukocytes (part II): immunophenotyping of adaptive immune B and T cell subsets. Age 2015;37(5):1-13.

55 SINGH A, YAMAMOTO M, RUAN J, et al. Th17/Treg ratio derived using DNA methylation analysis is associated with the late phase asthmatic response. Allergy, Asthma & Clinical Immunology 2014;10(1):1.

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Figure Legends

Figure 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 3 groups of drug naïve, early RA receiving 1st 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 month using DAS<2.6 for remission or not (* $p<0.05$). Scatter plots are presented with median (line) and interquartile range (whiskers).

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

A) ROC analysis plot for 5 parameters in the whole cohort (n=172). The 4 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.

Figure 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 define 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 to the rest of CD4+T-cells population (grey histograms).

Figure 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 to HC (n=15).

C) Higher expression of CXCR4 (mean fluorecence intensity MFI) in RA (n=19, p=0.002) compared to HC (n=15).

Boxplots represented median (line) and interquartile range (box) andd extrems (whiskers).