Plasma interleukin-23 and circulating IL-17A⁺IFNγ⁺ ex-Th17 cells predict opposing outcomes of anti-TNF therapy in rheumatoid arthritis

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Abstract

Objectives: TNF-α inhibitors are widely used in rheumatoid arthritis (RA) with varying success. Response to TNF-α inhibition may reflect the evolution of rheumatoid inflammation through fluctuating stages of TNF-α dependence. Our aim was to assess plasma concentrations of Th17-related cytokines and the presence of circulating effector T-cells to identify predictors of response to TNF-α inhibitors.

Methods: Ninety-three people with RA were seen prior to and 4–6 months after commencing etanercept or adalimumab. Plasma concentrations of Th17-related cytokines, circulating effector T-cells, their production of relevant transcription factors and intracellular cytokines were measured at baseline. EULAR response criteria were used to define poor (ΔDAS28 ≤ 1.2 and/or DAS28 > 3.2) and good (ΔDAS28 > 1.2 and DAS28 ≤ 3.2) responders. Multivariate logistic regression was used to identify predictors of response.

Results: Participants with plasma IL-23 present at baseline were more likely to be poor responders [15/20 (75%) of IL-23⁺ versus 36/73 (49.3%) of IL-23⁻; \( p = 0.041 \)]. While frequencies of Th1, Th17, ex-Th17 and Treg cell populations were similar between good and poor responders to anti-TNF therapy, IL-17A⁺IFNγ⁺ ex-Th17 cells were more prevalent in good responders (0.83% of ex-Th17 cells) compared to poor responders (0.24% of ex-Th17 cells), \( p = 0.023 \). Both plasma IL-23 cytokine status (\( OR = 0.17 \) (95% CI 0.04–0.73)) and IL-17A⁺IFNγ⁺ ex-Th17 cell frequency (\( OR = 1.64 \) (95% CI 1.06 to 2.54)) were independently associated with a good response to anti-TNF therapy. Receiver operator characteristic (ROC) analysis, including both parameters, demonstrated an area under the ROC curve (AUC) of 0.70 (95% CI 0.60–0.82; \( p = 0.001 \)).

Conclusions: Plasma IL-23 and circulating IL-17A⁺IFNγ⁺ ex-Th17 cells are independently associated with response to anti-TNF therapy. In combination, plasma IL-23 and circulating IL-17A⁺IFNγ⁺ ex-Th17 cells provide additive value to the prediction of response to anti-TNF therapy in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial joint inflammation that, without adequate treatment, leads to destruction of joint cartilage and bone. RA pathology is heterogeneous, arising from a spectrum of genetic, molecular and cellular mechanisms that varyingly control the presentation and perpetuation of the disease. Consequently,
treatment and its efficacy are similarly heterogeneous and unpredictable [1, 2]. Sub-types of RA inflammation may be distinguished by histologic features and signature expression profiles of synovial tissue — knowledge which may hold promise for guiding treatment options [2–6].

The advent of biological disease-modifying antirheumatic drugs (bDMARDs), including anti-tumour necrosis factor (TNF) therapy, has revolutionised the treatment of RA and made remission a realistic goal for many people with RA. TNF-α is a pivotal pro-inflammatory cytokine in RA that stimulates a variety of cell types in the joint space to promote inflammation and cartilage degradation [7, 8], while also contributing to disease chronicity [9]. The importance of TNF-α signalling in driving RA pathogenesis is highlighted by the potential for anti-TNF therapies to arrest radiographic damage [10]. However, approximately 30–40% of people with RA do not respond to anti-TNF therapy when used as the first-line bDMARD [1, 7].

Evidence from RA synovial tissue analysis suggests myeloid-dominant inflammation may be more amenable to anti-TNF therapy than other classes of biologic therapies, such as those targeting interleukin (IL)-6 or CD20 [4, 11]. Furthermore, recent evidence implicates dysregulation of the T-helper (Th)17 cell plasticity within the inflamed joint as an important contributor to the onset, chronicity and progression of RA [12–14]. TNF-α has been shown to co-mediate both the differentiation of Th17 cells [15] and their progression to the more pathogenic, pro-inflammatory ex-Th17 (also known as Th17.1/non-classic Th1) phenotype, capable themselves of producing much higher amounts of TNF-α than their Th17 precursor [16, 17]. Some studies have reported circulating Th17 cells and high pre-treatment levels of their prototypic cytokine, IL-17A, are associated with poor response to anti-TNF therapy [18–20]. However, there is a collective lack of consensus between various studies investigating the predictive value of circulating biomarkers, either protein or cellular, in RA therapy [21].

The high cost of anti-TNF therapies, the possibility of further disease progression in non-responders and the potential risk of adverse reactions to therapy necessitate an approach to more accurately predict the outcome of anti-TNF therapy and dictate the most effective course of treatment. Hence, our objective was to explore whether screening plasma levels of Th17-related cytokines and peripheral T-cell populations in RA patients immediately prior to commencing anti-TNF therapy could identify a blood-based biomarker profile that associates specifically with clinical improvement in disease activity after the initial phase of treatment.

Materials and methods

Participants

Ninety-three biologic-naïve people with RA as defined by the American College of Rheumatology (ACR) classification criteria [22], commencing anti-TNF therapy (etanercept n = 20 or adalimumab n = 73), were recruited from three public hospitals. The study was approved by the University of Otago Ethics Human Health Committee (13/040). All participants provided written informed consent. Choice of therapy was at the discretion of the treating clinician in conjunction with patient preference. The criteria for anti-TNF therapy in New Zealand require people to have had RA for at least 6 months; to have radiographic evidence of erosions or be anti-cyclic citrullinated peptide antibody (ACPA) positive; to have failed therapy with (i) methotrexate alone, (ii) methotrexate in combination with salazopyrin and hydroxychloroquine, and (iii) leflunomide or cyclosporine; to have ≥4 large or 20 total active joints; and to have a C-reactive protein (CRP) > 15mg/L unless they have received concomitant prednisone at doses > 5mg per day for the previous 3 months.

Participants were assessed immediately prior to commencing anti-TNF therapy (baseline) and 4 to 6 months after commencing anti-TNF therapy (follow-up). Demographic details and clinical history were recorded by participant questionnaire and review of the medical record. Disease activity was assessed at each visit using standard clinical parameters: 28 tender and 28 swollen joint counts, CRP and patient’s global assessment of disease activity clinical parameters: 28 tender and 28 swollen joint counts, CRP and patient’s global assessment of disease activity.

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Determining response to treatment

Good response to treatment with anti-TNF therapy was defined by European League Against Rheumatism (EULAR) response criteria: low disease activity at the follow-up visit (DAS28 ≤3.2) and a change in DAS28 score greater than 1.2 (∆DAS28 >1.2) between baseline and follow-up visits [23]. Participants with follow-up DAS28 >3.2 and/or ∆DAS28 ≤1.2 were classified as poor responders to therapy.

Circulating cytokine measurement

Plasma concentrations of Th17-related cytokines were determined using the 15-plex magnetic bead-based Bio-Plex Pro™ Human Th17 Cytokine Panel kits (171AA001M) run on a calibrated Bioplex 200 System (Bio-Rad Laboratories, Inc.) and analysed using...
Milliplex Analyst 5.1 analysis software (VigeneTech, Inc.) as previously described [24].

Cytokines detected in ≤ 50% of samples at baseline were dichotomised (IL-4, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, interferon (IFN)-γ and sCD40L), and the remaining cytokines (IL-1β, IL-6, IL-10, TNF-α, IL-31 and IL-33), detected in > 50% of participants, were treated as continuous variables. A measure above the lower limit of quantitation (LLOQ) was considered positive and samples below the LLOQ for each individual assay were assigned a value of zero.

**Flow cytometry**

Peripheral blood mononuclear cells (PBMC) were immediately isolated from EDTA-treated blood samples using density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). To facilitate batch processing, the isolated PBMC were stored in liquid nitrogen at 1 × 10^7 cells/mL in 90% foetal calf serum (FCS)/10% di-methyl sulphoxide solution, following a controlled rate freezing step. Upon thawing at 37 °C, cells were washed in PBS/2%FCS then rested overnight in a 37°C, 5% CO2 humidity chamber at 1

10^6 viable cells/mL in 10% FCS (Gibco), 1% penicillin-streptomycin (Sigma-Aldrich)-supplemented RPMI 1640 media (Gibco). Eighty-four of 93 samples contained sufficiently viable cells (> 80%) for inclusion in the PBMC analysis.

Each sample was divided in two, and each portion incubated in a cocktail of Brilliant Stain Buffer (Becton Dickinson [BD] Horizon), Fc Block (BD Pharmingen), PBS/2%FCS, and fluorescent-labelled antibodies specific for detection of cell surface markers for either Th cells or Treg cells. Cells were stimulated with 125 ng/mL phorb 12-myristate 13-acetate (PMA) and 2.5 ug/mL ionomycin using leucocyte activation cocktail with BD GolgiPlug (BD Pharmingen) and PMA (Sigma-Aldrich) for 6 h at 37°C in a 5% CO2 humidity chamber, (IFN)-γ and sCD40L, and the remaining cytokines (IL-1β, IL-6, IL-10, TNF-α, IL-31 and IL-33), detected in > 50% of participants, were treated as continuous variables. A measure above the lower limit of quantitation (LLOQ) was considered positive and samples below the LLOQ for each individual assay were assigned a value of zero.

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Stained and washed cells were resuspended and analysed on a BD LSRFortessa flow cytometer with FACSDiva software (BD Bioscience). An average of 42,817 events were acquired for analysis within the singlet/live/lymphocyte/CD3+/CD4+ gate and software application settings were used to maintain consistent placement of the negative populations between multiple runs.

**Immunophenotyping**

Effector T-cell populations were identified from within the circulating CD3+/CD4+ lymphocyte population, derived from single, live events, using FlowJo software (version 10.6.1, BD). Fluorescence-labelled antibody panels (detailed in Additional file 1) were used to stain and identify Th17 cells: CXCR3+/CD161+/CCR4+/CCR6+; ex-Th17 cells: CXCR3+/CD161+/CCR6+/CCR4+/-; and Th1 cells: CXCR3+/CD161−/CCR4−/CCR6−. Treg cells were identified in a parallel sample as a distinct sub-population of CD3+/CD4+/CD25+/CD127lo lymphocytes (see Additional file 2 for gating strategies). Additionally, podoplanin (PDPN) expression, intracellular cytokines IL-17A and IFNγ, and RORγT transcription factor expression were assessed within Th17, ex-Th17 and Th1 cells, while intracellular IL-17A and both RORγT and FOXP3 transcription factor expression were assessed within Treg cells (Additional file 1).

Gating thresholds were determined for both surface markers and intracellular cytokines by comparison with respective fluorescence minus one (FMO) controls (Additional file 2). For FMO controls, positive gates contained 0–0.1% cells. Gating thresholds for intracellular cytokines were further validated by comparisons with unstimulated, stained, control samples. In these unstimulated control samples, a varying percentage of positive cells were detected, reflecting pre-existing intracellular cytokine production. Further validation of IL-17A staining specificity was enabled by determining co-expression of RORγT in stimulated samples (Additional file 2). All FACS data reported is from stimulated and stained cells.

**Statistical analysis**

Based on indices of EULAR response criteria at follow-up, all participants were categorised as either good or poor responders. Between the two response groups, comparisons of participants’ original baseline demographics or clinical variables were performed using Pearson’s chi-square tests, Student’s T tests or Mann-Whitney U tests where appropriate. Statistical significance was defined as a two-tailed p value < 0.05.

For each dichotomously treated cytokine, good or poor response rate was compared between cytokine-present or cytokine-absent groups, using Pearson’s chi-square or Fisher’s exact test. The baseline concentration of cytokines with continuous data treatment was compared between participants showing good or poor response using the Mann-Whitney U test.

For flow cytometry analysis, the median of the frequency of various cell populations or the median fluorescence intensity (MFI) for marker expression was
comparing between participant groups showing good or poor response using Mann-Whitney U tests.

Univariable and multivariable binary logistic regression, using good response to anti-TNF treatment as the outcome, was performed with quartile-transformed double-positive IL-17A+IFNγ+ ex-Th17 cell frequencies and IL-23 as independent variables. To determine the combined effects of IL-23 status and IL-17A+IFNγ+ ex-Th17 cell frequency on the likelihood of a good response, the predicted probabilities from the multivariate logistic regression were used in the receiver operating characteristic curve (ROC) curve analysis.

Data statement
The datasets generated and/or analysed during the current study are the subject of ongoing analysis and will be available from the corresponding author on reasonable request and subject to ethical approval.

Results

Participant demographics
The baseline demographics and clinical variables of the two response groups are summarised in Table 1. Two-thirds of the participants had disease duration less than 10 years, and the mean baseline DAS28 score was 5.07 (SD 1.23), with an overall moderate to high disease activity reflected in a DAS28 score above 3.2 in 92.5% of patients. At the follow-up visit, 45% of participants were deemed good responders, while 55% were poor responders according to established EULAR criteria. Individual baseline demographic variables, disease characteristics or existing pharmaceutical treatment regime were not different between good and poor responders (Table 1). Similarly, within the sub-cohort of 84 participants included in the PBMC analysis, demographic variables, disease characteristics or treatments were not different between good and poor responders (data not shown), and all variables within this group were representative of the overall cohort.

Table 1 Biologic-naive RA patient demographics and clinical variables at baseline

| Baseline measures | EULAR response outcome | P value* |
|-------------------|------------------------|---------|
|                   | Good | Poor |
| Number            | 42   | 51   |
| Female, n (%)     | 29 (69) | 34 (67) | 0.81 |
| Age, mean years ± SD | 56.5 ± 12.8 | 58 ± 13.7 | 0.58 |
| DAS28, mean ± SD  | 5.08 ± 1.08 | 5.07 ± 1.36 | 0.97 |
| DAS28 >3.2, n (%) | 40 (95) | 46 (90) | 0.36 |
| CRP, mg/L median (IQR) | 11.5 (3–21) | 11 (3–28) | 0.86 |
| RF+, n (%)        | 36 (86) | 39 (77) | 0.26 |
| ACPA+ , n (%)     | 32 (80) | 41 (80) | 0.96 |
| Nodules present, n (%) | 16 (39) | 18 (35) | 0.71 |
| Erosions present, n (%) | 39 (93) | 46 (90) | 0.65 |
| Years since diagnosis, median (IQR) | 7.6 (3.2–18.0) | 6.3 (2.6–13.4) | 0.37 |

TNF inhibitor commencedd
- Adalimumab, n (%) 31 (74) 42 (82) 0.32
- Etanercept, n (%) 11 (26) 9 (18) 0.32

Concomitant DMARDS
- Prednisone, n (%) 30 (71) 41 (80) 0.31
- Prednisone dose, mg, median (IQR) 10 (6.0–15.0) 10 (6.0–15.0) 0.89
- Methotrexate, n (%) 25 (60) 24 (47) 0.23
- Leflunomide, n (%) 11 (26) 11 (21) 0.89
- Sulphasalazine, n (%) 2 (5) 5 (10) 0.36
- Hydroxychloroquine (%) 9 (21) 6 (12) 0.21

*Pearson’s chi-square test was used to compare frequency. Student's T test was used to compare mean. The Mann-Whitney U test was used for comparing median

**Two participants had no record of ACPA measurement due to the test not being available at the time of diagnosis

†One participant did not have the presence/absence of nodules confirmed

‡Low numbers within a divided cohort precluded any reliable comparison between adalimumab and etanercept treated participants

§Median dose in prednisone-only participants. Information on prednisone dose was not available for one participant

The presence of plasma IL-23 at baseline is associated with poor response to anti-TNF therapy
Of the 15 select Th17-related cytokines measured (detailed in Additional file 3), a statistically significant association with response to anti-TNF therapy was only seen for plasma IL-23 (p=0.041). Of only those participants where IL-23 cytokine was present at baseline (IL-23+), 75% were poor responders to anti-TNF therapy, compared to only participants lacking IL-23 at baseline (IL-23−), where 49% were poor responders (Fig. 1A).

Higher frequencies of IL-17A+IFNγ+ exTh17 cells are associated with good response to anti-TNF therapy
PBMC isolated from baseline blood samples were subjected to multiplex flow cytometric analysis, where an immunophenotyping strategy based on specific cell surface marker expression was employed to identify and quantify cell sub-populations (Additional file 4). Comparisons were made between good and poor responders. As summarised in Table 2, interrogation of CD3+CD4+ T-lymphocytes showed similar frequencies and ratios of surface-marker-defined Th1, Th17, ex-Th17 and T_reg cell populations between good and poor responders to anti-TNF therapy.
Analysis within T-effector cell populations, focusing on IL-17A and IFNγ production, further characterised the various sub-populations (Table 2 and Additional file 4). Within the ex-TH17 cell population, a higher median frequency of cells co-producing IL-17A and IFNγ (IL-17A⁺IFNγ⁺ ex-Th17 cells) were seen in good responders (0.83% of ex-T H17 cells) compared to poor responders (0.24% of ex-Th17 cells), $p = 0.023$ (Table 2 and Fig. 1B). The various remaining cell sub-populations showed no statistically significant associations with response to anti-TNF therapy (Additional file 4).

The combination of pre-treatment plasma IL-23 cytokine status and IL-17A⁺IFNγ⁺ ex-Th17 cell frequency predicts response to anti-TNF therapy

Having established that baseline presence of IL-23 in plasma and a higher frequency of IL-17A⁺IFNγ⁺ ex-Th17 cells associate with divergent response to anti-TNF (Fig. 1 and Table 2), we next explored the combination of these two variables in the prediction of good response to anti-TNF therapy. Multivariable logistic regression analysis, including both IL-17A⁺IFNγ⁺ ex-Th17 cell frequency and plasma IL-23 cytokine status as co-variates, showed each measure was independently associated with a good response to anti-TNF therapy (Fig. 2A).

Any potential influence from individual clinical characteristics (presence of subcutaneous rheumatoid nodules; RF⁺ and/or ACPA⁺; disease duration; age at baseline) and/or concomitant therapies (prednisone use; any of methotrexate, leflunomide, salazopyrin and/or hydroxychloroquine) was considered in an expanded multivariable logistic regression model. This model maintained the independent association of both frequency of IL-17A⁺IFNγ⁺ ex-Th17 cells [$OR = 1.64$ (95% CI 1.06 to 2.54)] and IL-23⁺ status [$OR = 0.17$ (95% CI 0.04–0.73)] with a good response to anti-TNF therapy (Fig. 2A). Furthermore, the predicted probabilities from multivariate logistic regression that included both parameters.
demonstrated an area under the ROC curve (AUC) of 0.70 (95% CI 0.60–0.82; \( p = 0.001 \)), highlighting the value in combining both IL-23 status and IL-17A+IFNγ cell frequency in the prediction of good response to anti-TNF therapy (Fig. 2B).

### Discussion

This study identified potential biomarkers for the prediction of clinical response to anti-TNF therapy among Th17-related plasma cytokines and circulating effector T-cells in a biologic-naïve RA cohort. Among cytokines, the presence of IL-23 in pre-treatment blood predicted a poor response to anti-TNF therapy. In addition, within baseline PBMC samples, immunophenotyping analysis revealed a small T-cell population, defined by surface marker expression profile as ex-Th17 cells (also recognised as non-classic Th1, or Th17.1 cells), in which both IL-17A and IFNγ were co-expressed. Higher frequencies of this IL-17A+IFNγ+ ex-Th17 cell population at baseline predicted a good response to anti-TNF therapy. Incorporating both baseline plasma IL-23+ cytokine status and IL-17A+IFNγ+ ex-Th17 cell frequency increased the strength of the response prediction model. These current findings provide insight into inflammatory mechanisms in RA, known to be co-mediated by TNF-α, and with potential to influence response to anti-TNF therapy.

Both the source and functional impact of TNF-α require consideration in order to reconcile the involvement of IL-17A+IFNγ+ ex-Th17 cells and IL-23 in response to anti-TNF therapy. In accord with published studies [25, 26], baseline plasma TNF-α levels within our RA cohort gave no indication of response to anti-TNF therapy. This suggests a more subtle impact from TNF-α, incorporating potential variations that are contingent upon the balance between inflammatory processes that establish or maintain RA at any juncture, through early- or late-stage disease. Among alternative possibilities, TNF-α makes a contribution towards the differentiation of IL-17A producing Th17 cells [15] and subsequent synergy with IL-17A further increases the potency of TNF-α [8]. Pathogenic Th17 cells are a feature of RA, distinguished by a pro-inflammatory gene signature that includes elevated TNF transcript in non-responders [27] and is sustained by USF2 signalling pathways [28]. Importantly, TNF-α production by Th17 lineage cells increases as the cells trans-differentiate from classic Th17 cells, through IL-17A+IFNγ+ ex-Th17 intermediaries, towards non-classical IFNγ+ Th1 cell phenotypes, which concomitantly acquire a more pathogenic phenotype [29]. Th17 trans-differentiation appears inherently biased in RA [18, 19] with evidence that the process is TNF-α dependent [17]. Consequently, it has been proposed that TNF inhibitors reduce the shift of Th17 cells towards the ex-Th17/non-classic Th1 cells [30]. A recent report revealing anti-TNF-induced alterations in the blood transcriptome,

### Table 2

|                | Good responders (n=39) | Poor responders (n=45) | \( P \) value |
|----------------|------------------------|------------------------|--------------|
| **Lymphocytes** |                        |                        |              |
| CD3+CD4+       | 53.5 (44.8–61.6)       | 52.6 (43.7–61.1)       | 0.968        |
| **CD3+CD4-**   |                        |                        |              |
| γTh1           | 8.36 (4.87–13.00)      | 9.34 (5.83–13.70)      | 0.667        |
| Treg           | 4.49 (3.70–5.44)       | 4.43 (3.42–5.22)       | 0.647        |
| Th17           | 1.32 (0.73–1.94)       | 1.37 (0.54–2.09)       | 0.904        |
| ex-Th17        | 0.64 (0.32–1.16)       | 0.48 (0.22–1.26)       | 0.451        |
| **ex-Th17**    |                        |                        |              |
| γIFNγ+         | 8.73 (6.06–12.20)      | 11.0 (7.14–14.90)      | 0.131        |
| IL-17A+IFNγ+   | 0.83 (0.19–1.60)       | 0.24 (0.00–1.16)       | \( 0.023^* \) |
| IL-17A-IFNγ+   | 7.08 (5.15–10.41)      | 9.18 (6.23–14.28)      | 0.115        |
| IL-17A+        | 2.39 (0.95–3.93)       | 1.82 (0.94–4.51)       | 0.771        |
| **Cell ratios**|                        |                        |              |
| Th1:Th17       | 6.0 (4.4–12.8)         | 7.3 (3.7–18.9)         | 0.69         |
| Th1:ex-Th17    | 12.1 (7.3–24.5)        | 16.9 (9.1–32.1)        | 0.422        |
| Th17:ex-Th17   | 1.8 (1.4–4.7)          | 1.9 (1.1–5.2)          | 0.854        |
| Treg:Th17      | 3.7 (2.5–5.3)          | 4.1 (2.1–6.0)          | 0.897        |

*Frequency values for indicated PBMC cellular sub-types are median percentages with IQR in parenthesis of the indicated parent cell populations (shown in bold). Comparisons of each cellular sub-type between patients demonstrating a good or poor response to anti-TNF therapy are shown (Mann-Whitney U tests) with \( p<0.05 \) considered statistically significant.
### A

**Prediction of Good Response**

| OR (95% CI) | p value |
|-------------|---------|
| **Univariate logistic regression** | | |
| Plasma IL-23+ | 0.32 (0.11 – 0.99) | 0.047* |
| %IL-17a+IFNγ+ ex-Th17 | 1.58 (1.07 – 2.33) | 0.022* |

**Multivariate logistic regression, without confounders**

| OR (95% CI) | p value |
|-------------|---------|
| Plasma IL-23+ | 0.24 (0.07 – 0.86) | 0.029* |
| %IL-17a+IFNγ+ ex-Th17 | 1.66 (1.10 – 2.49) | 0.015* |

**Multivariate logistic regression, with confounders**

| OR (95% CI) | p value |
|-------------|---------|
| Plasma IL23+ | 0.17 (0.04 - 0.73) | 0.017* |
| %IL-17a+IFNγ+ ex-Th17 | 1.64 (1.06 – 2.54) | 0.026* |

### B

![ROC curve analysis](image)

**Fig. 2** Predicting good response to anti-TNF therapy: independent and combined strength of baseline plasma IL-23+ status and frequency of circulating IL-17A+IFNγ+ ex-Th17 cell sub-population. **A** Uni- and multivariate logistic regression models with baseline variables for the prediction of good response after an initial 4–6 months of anti-TNF treatment. Values for IL-17A+IFNγ+ ex-Th17 cell frequency were quartile-transformed; plasma IL-23+ defined as cytokine present above detectable levels. *Significant p values ≤ 0.05. **B** ROC curve analysis to determine the combined effects of IL-23 status and IL-17A+IFNγ+ ex-Th17 cell frequency on the likelihood of a good response to anti-TNF therapy. Predicted probabilities from the multivariate logistic regression analysis were used. *Odds ratios of this model are adjusted for additional confounders including concomitant cDMARD use (any of methotrexate, leflunomide, salazopyrin and/or hydroxychloroquine); presence of subcutaneous rheumatoid nodules; RF+ and/or ACPA+; prednisone use; disease duration; and age at baseline.
including reduced expression of the CD39/ENTPD1 gene [31], which is highly expressed in IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells, supports this speculation, as does our finding that IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells are Elevated in the peripheral blood of participants who respond to anti-TNF therapy. Finally, although the derivation of the IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells during Th17 transdifferentiation provides a fitting explanation, recent evidence demonstrates increased plasticity of Th17.1/ non-classic IFN\(\gamma\)^+ Th1 cells [32] fostering the opposite conversion towards the Th17 phenotype, including IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells. Our data do not allow discrimination of the exact route involved.

We found that the presence of IL-23 in baseline plasma predicted poor response to anti-TNF therapy. Among IL-23\(^+\) participants that responded poorly, there was no evidence that at baseline the presence of IL-23 per se or measured plasma levels were associated with increased disease activity, nor the extent or features of the effector cells considered (data not shown). Subsequently, IL-23 status was variably affected by anti-TNF therapy, suggesting the in vivo pathogenic mechanisms that involve IL-23 and reduce clinical efficacy are mainly independent of TNF-\(\alpha\). One implication is that IL-23 contributes towards alternative, immune-driven process(es), as shown by the importance of monocyte-derived IL-23 for synovial IL-17A expression [33] and the necessity for IL-23 in development of the pathogenic Th17 cells seen in RA [27]. While it is clear that most of the actions of IL-23 are mediated through IL-17, the disparate predictions for anti-TNF therapy outcome from the presence of IL-23 compared to IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells indicate a potential disconnect in the IL-23/IL-17 axis. The presence of IL-23 might “simply” reflect the outcome of a pathological process, as exemplified by the IL-1p\(\beta\) -dependent induction of IL-23 p19 in fibroblast-like synoviocytes [34]. More likely, IL-23 is involved in multiple, independent but parallel processes as indicated by activation of the IL-23 pathway, which is strongly associated with synovial ectopic lymphoid neogenesis (ELN) in human RA, yet is seemingly independent of the IL-17A expression necessary for ELN development [6, 35]. Conceivably, the situation might also reflect a temporal effect from IL-23, consistent with evidence suggesting the IL-23/Th17 cell-cytokine(s) axis may not have a constant influence throughout the disease course in RA [36–38]. That IL-23 and IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells are associated with different response outcomes to anti-TNF therapy suggests we are dealing with separate groups of patients with subtle but key differences in their disease course. The variable contribution from IL-23 appears to be at least one distinguishing feature that dictates response to anti-TNF therapy.

This study has a number of limitations. While cytokines like TNF-\(\alpha\), IL-1\(\beta\) and IL-6 were prevalent, others were only detected in a small number of participants at baseline, which may reduce statistical power to identify associations. We have made no adjustment for multiple testing of the range of cytokines and cells; therefore, it is possible that these results represent a chance finding and type I error. However, there is biological plausibility to the observed associations, which suggests this is unlikely. There was a wide range of disease duration in our cohort, although two-thirds of participants had disease duration <10 years. This reflects the situation in New Zealand where access to TNF inhibitors is restricted. The cytokine profile in RA may vary over time, and with prior use of DMARDs. For example, methotrexate and leflunomide have been reported to inhibit Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling and alter TNF and IL-17 production [39, 40]. In addition, the effect from bDMARDs is known to extend to an influence on the transcriptome of both monocytes and CD4\(^+\) T cells [41]. Finally, we do not have a replication cohort and it is important that these findings are replicated in other populations.

In conclusion, we demonstrate that the presence of plasma IL-23 and circulating IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells have the potential to predict disparate outcomes for anti-TNF therapy in RA. IL-23 presence predicts poor outcome, suggesting involvement in a TNF-independent inflammatory mechanism. In contrast, a higher frequency of circulating IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells predicts a good outcome from anti-TNF therapy highlighting these cells as a significant component of TNF-mediated inflammation in RA. In combination, plasma IL-23 and circulating IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells provide additive value to the prediction of good response to anti-TNF therapy. Further work is required to understand the connection between these two biomarkers, the disease course in RA and the susceptibility to TNF inhibition. Knowledge of joint synovial inflammation associated with these circulating biomarkers may hold the key to understanding this conundrum.

**Conclusions**

This study showed that plasma IL-23 and circulating IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells are independently associated with response to anti-TNF therapy. Our results suggest the involvement of IL-23 in a TNF-independent inflammatory mechanism in RA, while highlighting IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells as a significant component of rheumatoid inflammation that is reliant on TNFa. In combination, plasma IL-23 and circulating IL-17A\(^+\)IFN\(\gamma\)^+ ex-Th17 cells provide additive value to the prediction of response to anti-TNF therapy in RA.
Abbreviations
ACPA: Anti-citrullinated peptide antibody; ACR: American College of Rheumatology; AUC: Area under the curve; bDMARDs: Biological disease-modifying anti-rheumatic drugs; BD: Becton Dickinson; CD: Cluster of differentiation; CRP: C-reactive protein; CI: Confidence interval; DAS28: Disease activity score-4V-CRP; EDTA: Ethylenediaminetetraacetic acid; ELN: Ectopic lymphoid neogenesis; EULAR: European League Against Rheumatism; FCS: Foetal calf serum; FMO: Fluorescence minus one; IFN: Interferon; IL: Interleukin; JAK: Janus kinase; LLOQ: Lower limit of quantitation; MFI: Median fluorescence intensity; OR: Odds ratio; PBMC: Peripheral blood mononuclear cell; PBS: Phosphate-buffered saline; PDPN: Podoplanin; PMA: Phorbol myristate acetate; RA: Rheumatoid arthritis; ROC: Receiver operating characteristic; RORC: Retinoic acid receptor-related orphan gamma T; sCD40L: Soluble CD40 ligand; STAT: Signal transducer and activator of transcription; Th: T-helper, Treg: T-regulatory; TNF: Tumour necrosis factor.

Supplementary Information
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Additional file 1. Antibodies for T-cell identification.
Additional file 2: Figure S1. Flow cytometry immunophenotyping strategy.
Additional file 3. Detection or concentration of plasma Th17-related cytokines at baseline and EULAR response to anti-TNF therapy.
Additional file 4. PBMC cell populations determined by flow cytometry.

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Authors’ contributions
LKS and PAH designed the study and provided overall supervision of the work. NCF coordinated participant recruitment and data collection. Cytokine assays, FACS analysis and interpretation were performed by MJM. NCF and CF performed statistical analysis. Manuscript was prepared by MJM, NCF, CF, LKS and PAH. The author(s) read and approved the final manuscript.

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Availability of data and materials
Additional raw datasets collected and/or analysed during the current study are available from the corresponding author on reasonable request. Some restrictions may apply pursuant to ongoing analysis.

Declarations
Ethics approval and consent to participate
The study was approved by the University of Otago Ethics Human Health Committee (13/040). All participants provided written informed consent.

Consent for publication
Not applicable

Competing interests
LKS reports speaker fees from AMGEN. The other authors declare that they have no competing interests.

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