Lower peripheral helper T cells in the synovium are associated with a better response to anti-TNF therapy in rheumatoid arthritis

CURRENT STATUS: UNDER REVIEW

Arthritis Research & Therapy  BMC

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DOI: 10.21203/rs.3.rs-21821/v1

SUBJECT AREAS
   Allergy & Immune Disorders   Orthopedics

KEYWORDS
  Rheumatoid arthritis, anti-TNF therapy, synovial membrane, clinical response, deconvolution, peripheral T helper, gene expression, immunofluorescence.
Abstract

**Background:** The mechanisms by which only some rheumatoid arthritis (RA) patients respond favorably to TNF blockade are still poorly characterized. The goal of this study was to identify biological features that explain this differential response using a multilevel transcriptome analysis of the synovial membrane.

**Methods:** Synovial samples from 11 patients starting anti-TNF therapy were obtained by arthroscopy at baseline and week 20. Analysis of the synovial transcriptome was performed at the gene, pathway and cell-type levels. Newly characterized pathogenic cell types in RA, peripheral helper T cells (T<sub>PH</sub>) and CD34-THY1+ fibroblasts, were estimated using a cell-type deconvolution approach. T<sub>PH</sub> association was validated using immunofluorescence. External validation was performed on an independent dataset.

**Results:** After multiple test correction, 16 and 4 genes were differentially expressed at baseline and week 20, respectively. At the pathway level, 86 and 17 biological processes were significantly enriched at baseline and week 20, respectively. Longitudinal expression changes were associated with a drastic decrease of innate immune activity (P<5e-30), and an activation of bone and cartilage regeneration processes (P<5e-10). Cell-type deconvolution revealed a significant association between low T<sub>PH</sub> cells at baseline and a better response (P=0.026). Lower T<sub>PH</sub> cells were maintained in good responders up to week 20 (P=0.032). Immunofluorescent analyses confirmed the accuracy of the cell-type estimation (r^2=0.58, P=0.005) and the association with response. T<sub>PH</sub> association with anti-TNF response was validated in an independent sample of RA patients (P=0.0040).

**Conclusions:** A lower abundance in the synovial membrane of the new pathogenic T cell type associated to RA, peripheral helper T lymphocyte, is associated to a good response to anti-TNF therapy. Major changes in the myeloid cell compartment are also seen in response to therapy. The results of this study could help develop more effective therapies aimed at treating the pathogenic mechanisms in RA that are currently not well targeted by anti-TNF agents.

**Background**

Anti-TNF therapies have been a major breakthrough in the management of Rheumatoid Arthritis (RA)
To date, disease remission has become an attainable goal for many patients [2]. However, 30 to 40% of anti-TNF treated patients will not show a significant clinical improvement [3]. The reasons behind this differential response are still elusive [3]. Clinical and basic studies have attempted to identify key factors, with very limited success [4]. Identifying the determinants of anti-TNF response would be of high value: these could be used to stratify patients, guide drug selection and improve disease management, and could also help to develop more efficacious therapies, either alone or in combination with anti-TNF agents.

The synovial membrane is the target tissue of the chronic inflammation in RA [5] and its analysis should provide highly valuable information on the biological mechanisms underlying the differential response to TNF blocking agents. However, obtaining synovial biopsies is highly invasive and consequently, very few studies have been performed on this key tissue with regards to drug efficacy. Using in-house two-color microarrays, van der Pouw Kraan [6] and Lindberg et al [7] analyzed the baseline transcriptome of RA patients starting infliximab therapy but neither study found differentially expressed genes. The higher technical variability associated with these early analysis platforms could have undermined the power to accurately identify transcript changes [8]. Using a single-color commercial array, Badot et al analyzed the longitudinal change in expression of \( n = 8 \) adalimumab-treated patients [9], and found some expression changes mainly related to cell division genes. However, differential expression was restricted to only 14% of the analyzed transcripts, thereby limiting the global screening capacity to a minor fraction of the total biological variability.

The cellular composition of tissues and the extent of infiltration of immune cell types has proven to be predictive of disease subtypes of complex diseases like cancer [10]. Very recently, studies based on single-cell approaches in the synovial membrane have been able to characterize new pathogenic subsets associated to RA. Using single-cell RNA-seq, Mizoguchi et al [11] have been able to classify RA synovial fibroblasts into three different subsets, with CD34-THY1 + subtype showing a significant expansion in comparison to osteoarthritis patients. Similarly, using mass-cytometry on T cells isolated from the synovium, Rao et al [12] found an expanded population of \( \text{PD-1}^{\text{hi}}\text{CXCR5-CD4}^{+} \) T cells in RA.

Due to their capacity to provide B cell help, this new T cell subset was described as peripheral helper...
T cell ($T_{\text{Ph}}$). The relationship of these new pathogenic cell subsets with the response to anti-TNF therapy in RA has not yet been addressed.

In the present study we have performed a multilevel analysis of the synovial membrane transcriptome to identify critical biological mechanisms associated with the response to anti-TNF therapy in RA. Using a longitudinal cohort of patients starting anti-TNF treatment we identify differentially expressed genes, pathways and cell types associated with the clinical response at week 20. In the latter analysis, using a cell-deconvolution approach we find that a lower $T_{\text{Ph}}$ infiltration is associated with a good response to therapy, and these lower levels are maintained through time. A lower baseline presence of myeloid immune cells is also associated with a favorable response and drastic reduction of this cell compartment is the main biological change observed in responders. The results of this study provide a comprehensive look at the biological mechanisms associated to anti-TNF response in RA.

Methods
Patients and samples
Eleven patients (8 women, 3 men) fulfilling the American College of Rheumatology/European League Against Rheumatism 2010 criteria for RA [13], with a basal mean Disease Activity Score in 28 joints (DAS28) [14] of 5.3 [4.2–6.9]) (median and interquartile range [IQR]), for whom an anti-TNF was prescribed by their rheumatologist ($n = 6$ infliximab, $n = 3$ adalimumab, $n = 2$ etanercept), were enrolled in this study. Response to treatment defined by the EULAR response criteria [15] was determined after 20 weeks of therapy, aggregating moderate and good responders into a single responder group. The study was approved by the local ethics committee and all the patients signed the informed consent. This study was conducted in accordance with the Declaration of Helsinki principles.

Synovial samples were obtained by guided arthroscopy using a 2.7 mm arthroscope (Storz, Tuttlingen, Germany) under local anesthesia. In all patients, 6–8 biopsies were taken from the suprapatellar pouch and medial and lateral gutters with a 3 mm grasping forceps before starting a TNF antagonist. All patients underwent a second arthroscopy with synovial sampling after 20 weeks of
therapy. Synovial biopsies for mRNA analysis were immediately stored in RNALater preserving agent (QIAGEN, USA) and frozen to -80°C until RNA extraction [16],

Microarray Analysis

RNA was extracted from synovial biopsies using the RNA Mini Kit (Qiagen, US) and the integrity was assessed using BioAnalyzer microfluidic gel analysis (Agilent, USA). All samples were of high quality (RNA Integrity Number > 8) and were subsequently analyzed using Sentrix whole genome Beadchips WG6 version 2 (Illumina, US). Briefly, after RNA isolation, biotin-labeled cRNA was prepared using the Ambion Illumina RNA amplification kit (Ambion, US) and Illumina TotalPrep RNA Amplification Kit (Ambion, US). Biotin-labeled cRNA (1.5 µg) was hybridized to WG6 v2 Beadchips and scanned on the 500x Illumina BeadStation. Data collection was performed using BeadStudio 3.1.1.0 software (Illumina, US).

To perform an independent validation of the cell type association with treatment response, publicly available transcriptomic datasets (microarray or RNA-seq) were searched in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). Using the keywords “synovial membrane” and “rheumatoid arthritis” a total of 62 datasets were found to be performed on human samples. From these, after excluding in vitro culture studies and two-colour microarrays, only two datasets, GSE57376 [17] and GSE15602 [9] on anti-TNF treatment were found. The former dataset was discarded since it was only based on 3 RA patients and had no information on clinical response. The latter dataset consisted on the synovial membrane biopsies of n = 11 RA patients (3 Good, 5 Moderate and 3 None EULAR responders) collected at week 12 of adalimumab treatment.

Gene-level and pathway-level association with anti-TNF response

After log2 transformation, the raw gene expression data was quantile-normalized. Batch adjustment was performed using ComBat [18]. Differential gene expression was assessed using the t-test and the resulting P-values corrected for multiple testing using Bonferroni’s method. Biological pathway association was tested using the Gene Ontology (GO) enrichment approach [19] implemented clusterProfiler R package. Genes with a nominally significant differential expression and a fold change > 1.2 were selected as input for this analysis; Bonferroni multiple test correction was applied to
determine the significant GO terms.

**RT-QPCR analysis of associated genes**

Real-Time quantitative PCR analyses of the genes most significantly associated with treatment response as well as marker genes for PD-1\(^{hi}\)CXCR5-CD4 + T\(_{PH}\) cells were performed using Taqman assays (Life Technologies, US). In the former group, FAM-labeled assays were used to measure the expression of *PIK3CD* (Hs00192399_m1), *CX3CL1* (Hs00171086_m1) genes, while *TIMELESS* (Hs01086960_g1) and *CTLA4* (Hs01011591_m1) were used as T\(_{PH}\)-associated markers. *Ribosomal Protein L11* (VIC-labeled assay, Hs00831112_s1) was used as an endogenous control gene, and all assays were performed using the Taqman Universal PCR master mix (Life Technologies, US). Gene expression analysis was performed using the recommended protocol with the Applied Biosystems HT7900 system (ABI, USA) and fold-change expression was calculated from the Ct values of the test gene and the endogenous gene using the \(-\Delta\Delta\text{Ct}\) method. Correlation between microarray gene expression values and RT-PCR values was calculated using Pearson’s product moment correlation.

**Deconvolution analysis of pathogenic cell subsets**

In order to estimate the enrichment of relevant T cell and fibroblast subsets in the synovial membrane samples, we adapted the SPEC (Subset Prediction from Enrichment Correlation) method [20]. Briefly, this computational method uses gene signatures characteristic of specific cell types to estimate their contribution to the observed gene expression in a complex tissue like blood. The ranking of the cell-type signature genes within the global ranked list of genes is then used to compute the enrichment score with an adaptation of the Gene Set Enrichment Method [21]. The enrichment score corresponds to the maximum deviation from zero of a running sum statistic calculated by running down the ranked list of genes. This approach has proven to have a high accuracy in determining cell subsets within PBMC RNA analysis. Here, this deconvolution method was adapted to estimate fibroblast and T cell subtypes from synovial membrane transcriptomic data. To estimate the enrichment of the fibroblast types, we determined the top set of genes overexpressed in each subset. For this objective we used the microarray gene expression data generated from the three isolated cell types by Mizoguchi et al [11] and available at the GEO ID GSE107105 (supplementary Table 4). In the case of PD-1\(^{hi}\)CXCR5-CD
+ T cells, we used the set of $n = 54$ significantly overexpressed genes in $T_{PH}$ reported in the original discovery study [12] (supplementary Table 5).

**Immunofluorescence analysis of $T_{PH}$ in RA synovial samples**

Immunofluorescence analysis of the $T_{PH}$ lymphocytes was performed following the same methodology as described in the original discovery study [12]. Briefly, OCT frozen sections (7 µm) of the synovial membrane biopsies of the 11 patients treated with anti-TNF therapy and at both time points (baseline and week 20) were fixed with ice-cold acetone. Quenching of endogenous peroxidase was performed using Peroxidase-Blocking Solution (ref: S2023, Dako-Agilent). Blocking of unspecific unions was done using 5% goat normal serum (Life technologies, 16210064) and 2.5% of bovine serum albumin (Sigma-Aldrich, 10735078001). Primary antibodies for PD-1 (NAT105, ref: 760–4895, Roche), CD4 (SP35, ref: 790–4423, Roche), CXCR5 (51505, ref: MAB190, R&D systems) were used RTU for PD-1 and CD4 and 1:50 for CXCR5 to detect the presence of $PD-1^{hi}CXCR5^{-}CD4^{-}$ cells. As secondary antibodies, Alexa Fluor 488 Goat-anti Mouse IgG1 (ref: A21121, ThermoFisher), Alexa Fluor Plus 647 goat anti Rabbit IgG (ref: A32733, ThermoFisher) and Biotinilated goat Anti-Mouse IgG2b (ref: ab98701, Abcam) plus Streptavidin Texas Red ® (NEL721001EA, PerkinElmer) were used, respectively. Tissue samples were stained with DAPI (D9542, sigma) and mounted with Fluorescence Mounting Medium (S3023, Dako-Agilent). Specificity of the primary antibodies were determined by using rabbit and mouse IgG isotype controls (ref: ab27478 and ab37355, Abcam) and by omission of the primary antibody.

Digital scanned fluorescent images were acquired using a NanoZoomer-2.0 HT C9600 scanner (Hamamatsu, Photonics, France) with the 20X objective and coupled to a mercury lamp unit L11600-05 and using NDP.scan2.5 software U10074-03 (Hamamatsu, Photonics, France). All images were visualized with the gamma correction set at 1.0 and the sharpen filter enabled in the image controls panel of the NDP.view 2 U12388-01 software (Hamamatsu, Photonics, France). Image analysis was performed using the QuPath software [22]. Cell detection was performed by using the cell detection algorithm based on the DAPI nuclei fluorescence and subsequently, cells were segmented between positive and negative according to the membranous labeling by the three different markers (Alexa
488 for PD-1, Texas Red® for CXCR5 and Alexa 647 for CD4). For each marker, isotype controls as well as negative samples (i.e. samples were the primary antibodies were omitted) were used to set the threshold values for each marker.

Results
From the 11 RA patients, 8 responded to anti-TNF treatment and 3 patients did not show a significant clinical response at week 20 of therapy. The clinical and demographic data of the longitudinal patient cohort are detailed in Table 1.

**Demographics**

| Category                  | Value       |
|---------------------------|-------------|
| Women, n (%)              | 8 (72.7)    |
| Age average, years        | 60.3 (7.1)  |
| Disease duration, years    | 17.0 (10.2) |
| Follow-up, years          | 2.6 (1.9)   |
| DAS28 baseline            | 5.3 (1.6)   |
| DAS28 week 20             | 4.2 (1.7)   |
| RF positive, n (%)        | 7 (64%)     |
| Anti-CCP positive, n (%)  | 10 (82%)    |

**EULAR response**

| Category                  | Value       |
|---------------------------|-------------|
| Good and Moderate n (%)   | 8 (72.7%)   |
| No Response n (%)         | 3 (27.3%)   |

*Mean (Standard Deviation). Anti-CCP: anti-cyclic citrullinated peptide antibodies; RF: rheumatoid factor.

Table 1

Clinical and demographical characteristics of the RA patient cohort

**Single-gene and pathway association with response**

At week 0, \( n = 16 \) genes were differentially expressed between responders and non-responders (Table 2), from which 11 were overexpressed in responders and 5 in non-responders. At week 20, \( n = 4 \) genes were differentially expressed after Bonferroni multiple test correction (Table 2), one of which
was overexpressed in responders (*PDE4B*, \(P = 1.09 \times 10^{-9}\)) and the remaining 3 overexpressed in non-responders. At the nominal level, 7 out of the 11 differential genes in the synovial membrane at week 0, continued to be differential at week 20 between responders and non-responders (\(P < 0.05\), same direction of change, Supplementary Table 1). The remaining 5 genes, while not reaching statistical significance, preserved the same direction of change as in found in the baseline (supplementary Fig. 1). Conversely, none of the four differentially expressed genes at week 20 were already differential at week 0 (Supplementary Table 1). This result suggests that the biological features that lend a patient unresponsive to TNF blocking therapy prevail throughout anti-TNF treatment. Using RTPCR, we validated the mRNA expression measurements of the gene most differentially expressed at baseline in responders (i.e. *CX3CL1*, correlation \(P\)-value \(= 2.11\times 10^{-11}\)) and the gene most highly expressed in non-responders (*PIK3CD*, correlation \(P\)-value \(= 2.42\times 10^{-10}\)) (Supplementary Fig. 2).

**Genes significantly overexpressed in non-responders, week 0**

| Gene Symbol | Accession      | Definition                                      | Fold Change | P-value  |
|-------------|---------------|-------------------------------------------------|-------------|----------|
| *PIK3CD*    | NM_005026.2   | Phosphoinositide-3-kinase, catalytic, delta polypeptide | -1.56       | 7.11E-18 |
| *REEP4*     | NM_025232.2   | Receptor accessory protein 4                     | -1.50       | 7.14E-17 |
| *HCLS1*     | NM_005335.3   | Hematopoietic cell-specific Lyn substrate 1      | -1.63       | 4.38E-09 |
| *GCDH*      | NM_013976.2   | Glutaryl-Coenzyme A dehydrogenase                | -1.50       | 3.2E-07  |
| *ADA*       | NM_000022.2   | Adenosine deaminase                              | -1.73       |         |
| Gene symbol | Entrez Gene ID | Description | Fold Change |
|-------------|----------------|-------------|-------------|
| NT5DC2      | NM_022908.1    | 5'-nucleotidase domain containing 2 | -1.86       |
| SERPINH1    | NM_001235.2    | Serpin peptidase inhibitor, clade H, member 1 | -1.66       |

**Genes significantly overexpressed in responders, week 0**

| Gene symbol | Entrez Gene ID | Description | Fold Change |
|-------------|----------------|-------------|-------------|
| CX3CL1*     | NM_002996.3    | chemokine (C-X3-C motif) ligand 1 | 1.94        |
| PLS3        | NM_005032.3    | plastin 3 (T isoform) | 1.71        |
| DIXDC1      | NM_033425.1    | DIX domain containing 1 | 1.90        |
| TMOD1       | NM_003275.1    | tropomodulin 1 | 2.10        |
| PCOLCE2     | NM_013363.2    | Procollagen C-endopeptidase enhancer 2 | 2.45        |
| PPP1R3C     | NM_005398.3    | protein phosphatase 1, regulatory (inhibitor) | 2.13        |
| CRTAP       | NM_006371.3    | cartilage associated protein | 1.58        |
| PRELP       | NM_002725.3    | proline/arginine-rich end leucine-rich repeat | 2.02        |
| DKFZP686A01247 | NM_014988.1 | hypothetical protein | 2.30        |
| UNQ689      | NM_212557.1    | RSTI689 | 5.09        |
### Genes significantly overexpressed in non-responders, week 20

| Gene | Accession | Description | Fold Change |
|------|-----------|-------------|-------------|
| OR2A9P | NR_002157.1 | olfactory receptor, family 2, subfamily A, member 9 | 2.01 |
| RPS6KA2 | NM_001006932.1 | ribosomal protein S6 kinase, 90kDa, polypeptide 2 | -1.53 |
| LIPA | NM_000235.2 | lipase A, lysosomal acid, cholesterol esterase (Wolman disease) | -1.50 |
| ALPL | NM_000478.2 | alkaline phosphatase, liver/bone/kidney | -2.77 |

### Genes significantly overexpressed in responders, week 20

| Gene | Accession | Description | Fold Change |
|------|-----------|-------------|-------------|
| PDE4B | NM_002600.2 | phosphodiesterase 4B, cAMP-specific | 1.57 |

The list of genes that are significant after Bonferroni correction and that show an >1.5 absolute fold change between the two groups are shown. Fold change is calculated contrasting the mean expression in the anti-TNF responder group to the mean expression in the non-responder. *Top differentially expressed genes validated using RT-QPCR.

**Table 2**
List of genes differentially expressed between anti-TNF responders and non-responders

At the pathway level, at week 0 n = 16 GO terms were significantly enriched in responders, and n = 70
GO terms were enriched in non-responders (Table 3 and Supplementary Table 2). At week 20, n = 17 GO terms were significantly enriched, all due to genes overexpressed in non-responders to anti-TNF (Table 3 and Supplementary Table 3). From these, three gene ontologies were also significantly enriched at baseline in non-responders: GO:0043299|leukocyte degranulation (adjusted $P_{wk0}=9.8e-4$, adjusted $P_{wk20}=3.97e-11$), GO:0002275|myeloid cell activation involved in immune response (adjusted $P_{wk0}=3.1e-4$, adjusted $P_{wk20}=3.6e-10$), and GO:0002444|myeloid leukocyte mediated immunity (adjusted $P_{wk0}=6.98e-3$, adjusted $P_{wk20}=6.36e-10$). This result suggests that the innate immune system composition in the synovial membrane conditions the response to anti-TNF therapy, with non-responders bearing a higher infiltrate of myeloid lineage cells (i.e. neutrophils and macrophages). Comparing the longitudinal changes in biological pathways in the responder group of patients, we found a profound modification with n = 149 GO terms significantly enriched from week 0 to week 20 (Table 3 and Supplementary Table 4). Most of the changes are due to the inactivation of pathways (n = 123 GO terms, 82.6%), but also several biological processes are activated due to anti-TNF efficacy (n = 26 GO terms, 17.4%). In the former group we found that the top associated biological changes in responders correspond to the same three GO terms that are consistently different when comparing responders vs non-responders at the two time points:

GO:0043299|leukocyte degranulation (adjusted $P = 3.26e-32$), GO:0002275|myeloid cell activation involved in immune response (adjusted $P = 1.14e-31$), and GO:0002444|myeloid leukocyte mediated immunity (adjusted $P = 2.99e-30$). This result corroborates that TNF efficacy is mainly mediated by the drastic inactivation of the innate immune system. Of relevance, the pathways that become activated due to TNF response include biological processes strongly related to the production of the specific components of the joint stromal tissue, including GO:0001501|skeletal system development (adjusted $P = 2.11e-10$), GO:0001503|ossification (adjusted $P = 4.04e-8$), GO:0001649|osteoblast differentiation (adjusted $P = 2.61e-4$), GO:0051216|cartilage development (adjusted $P = 8.42e-4$) and GO:0002063|chondrocyte development (adjusted $P = 8.99e-4$). This biological evidence shows that the effective clearance of inflammation is paralleled by the activation joint tissue reconstruction
Table 3  
Top associated Gene Ontologies with anti-TNF response at baseline and week 20, and with the longitudinal change.

| ID         | Description                              | Count | P-value     | Gene Group          |
|------------|------------------------------------------|-------|-------------|---------------------|
| GO:0044772 | mitotic cell cycle phase transition      | 75    | 3.64E-17    | Non-Response        |
| GO:0044770 | cell cycle phase transition              | 77    | 1.07E-16    | Non-Response        |
| GO:0140014 | mitotic nuclear division                 | 47    | 2.39E-13    | Non-Response        |
| GO:0000280 | nuclear division                         | 56    | 3.70E-12    | Non-Response        |
| GO:0051301 | cell division                            | 70    | 1.81E-11    | Non-Response        |
| GO:0007346 | regulation of mitotic cell cycle         | 71    | 2.04E-11    | Non-Response        |
| GO:0048285 | organelle fission                        | 57    | 4.90E-11    | Non-Response        |
| GO:0000070 | mitotic sister fission chromatid segregation | 31  | 5.86E-11    | Non-Response        |
| GO:0000819 | sister chromatid segregation             | 33    | 3.24E-10    | Non-Response        |

| ID         | Description                              | Count | P-value     | Gene Group          |
|------------|------------------------------------------|-------|-------------|---------------------|
| GO:0043299 | leukocyte degranulation                   | 40    | 3.37E-11    | Non-Response        |
| GO:0042119 | neutrophil activation                    | 38    | 1.07E-10    | Non-Response        |
| GO:0036230 | granulocyte activation                   | 38    | 1.46E-10    | Non-Response        |
| GO:0002275 | myeloid cell activation involved in immune response | 39  | 3.64E-10    | Non-Response        |
| GO:0002444 | myeloid leukocyte mediated immunity       | 39    | 6.36E-10    | Non-Response        |
| GO:0002283 | neutrophil activation involved in immune response | 65  | 1.18E-26    | DownRegulated       |
| GO:0002446 | neutrophil mediated immunity             | 65    | 5.17E-26    | DownRegulated       |
| GO:0043312 | neutrophil degranulation                 | 64    | 5.65E-26    | DownRegulated       |
| GO:0030198 | extracellular matrix organization        | 53    | 4.20E-16    | UpRegulated         |
| GO:0043062 | extracellular structure organization      | 53    | 4.01E-13    | UpRegulated         |
| GO:0009617 | response to bacterium                    | 50    | 1.64E-11    | DownRegulated       |
| GO:0002764 | immune response-regulating signaling pathway | 45  | 2.69E-11    | DownRegulated       |
Cell type deconvolution of RA pathological cell subtypes

Using single-cell characterization technologies, recent studies have identified distinct cell subsets within RA synovium. In particular, two new subtypes, peripheral helper CD4+ T cells and THY1+ synovial fibroblasts have been found to be key pathological types. Based on the genes that are characteristic of each subtype and using a deconvolution approach we estimated the relative proportion of each cell type. We found that, at baseline, response to anti-TNF therapy was associated to having lower numbers of T_{PH} cells (P = 0.021, Fig. 1A). We did not find evidence that THY1+ fibroblasts were associated with the response to anti-TNF therapy. Instead, we found that the baseline proportions of both CD34 + THY1- and CD34-THY1- (i.e. double negative) fibroblasts were associated with the response at week 20 (P = 0.027 and P = 0.021, respectively, Supplementary Fig. 4). At the time of response (week 20), we found that all three associated cell types (CD34 + and CD34-THY1- fibroblasts and T_{PH}) showed the same trend of association as identified at baseline although the difference was not statistically significant (P > 0.05, supplementary Fig. 5).

To perform technical validation of the observed gene expression associated to T_{PH} cells, we performed RTPCR analysis of T_{PH}-marker genes CTLA4 and TIMELESS. We found a highly significant correlation between the microarray and the RTPCR gene expression quantification (Pearson correlation coefficients r = 0.89 P = 3.6e-8 and r = 0.65 P = 0.0009 for CTLA4 and TIMELESS, respectively) (Supplementary Fig. 3).

Immunofluorescence analysis of T_{PH} cells

In order to corroborate the association between T_{PH} enrichment and anti-TNF response, we performed...
immunofluorescence analysis on the synovial membrane biopsies from the same set of patients (Fig. 2). All samples were positive for all markers which presented a membranous pattern. From them, CXCR5 expression was minimal in comparison with PD-1 and CD4. Anti-TNF non-responder patients presented higher groups of PD-1$^{hi}$CXCR5-CD4$^{+}$ T cells. We found a significant correlation between the proportions of PD-1$^{hi}$CXCR5-CD4$^{+}$ T cells estimated through our approach and the actual abundance of this pathogenic T cell subtype in the RA synovia ($r^2 = 0.58$, $P = 0.0051$, Supplementary Fig. 6).

Having confirmed the association of $T_{PH}$ cells with the response to anti-TNF therapy, we applied the cell-type deconvolution method to an external microarray dataset of RA patients treated with anti-TNF therapy [9]. In this transcriptional data of patients at week 12 of therapy ($n = 3$ Good, 5 Moderate and 3 None), we found a highly similar $T_{PH}$ profile to that found in our group of patients at week 20 ($P = 0.029$ and $P = 0.00019$, respectively) (Fig. 3). In both cases, good responders show a markedly lower frequency of the pathogenic T cell subset ($P = 0.00035$ and $P = 0.0040$, t-test comparing $T_{PH}$ cell levels in good vs moderate and none patients in our patient cohort and the external RA patient cohort, respectively).

Discussion
The reasons behind the variable efficacy of anti-TNF agents in RA have been elusive. Using single-gene, pathway and cellular deconvolution analyses on longitudinal transcriptomic data from the synovial membrane of RA patients treated with anti-TNF therapy, we have found key biological determinants of the response to this drug. At the gene level, baseline overexpression of chemokine CX3CL1 and downregulation of leukocyte signaling kinase PIK3CD were significantly associated with a better response. At the pathway level, anti-TNF non-responders had a higher activation of myeloid cell immune response processes at baseline. A drastic downregulation of these same innate immunological processes was strongly associated to the efficacy of the drug at week 20. At the cellular level, lower $T_{PH}$ cells were associated to a good response to anti-TNF agents, both at baseline and at week 20. The results from this study provide a multilevel description of the biological determinants of anti-TNF response in RA.
Recent studies based on single-cell analysis techniques have been able to characterize new pathogenic cell subtypes associated to RA. Using single-cell mass cytometry, PD-1\textsuperscript{hi}CXCR5-CD4 + T (T\textsubscript{PH}) cells were found to be significantly expanded in the synovial membrane of seropositive RA patients. Using the genes characterizing this T cell subset, we were able to estimate the presence of a significantly lower number of T\textsubscript{PH} cells in responders to anti-TNF therapy. This difference was clearly maintained up to week 20 in the good responder group of patients and validated in an independent cohort of patients. The transcriptional similarity between PD-1\textsuperscript{hi}CXCR5-CD4 + T and follicular helper T (T\textsubscript{FH}) and the \textit{in vitro} capacity to induce differentiation of memory B cells, suggests a peripheral helper phenotype. Our results suggest that an important part of this T\textsubscript{PH} pathogenic activity in RA is associated to mechanisms that are independent of TNF. T\textsubscript{PH} may act by recruiting T\textsubscript{FH} and B cells, promoting local autoantibody production that may not be reflected in the serum and leading to pathogenic immune complex fixation. But also, activated B cells can polarize into different subtypes and produce an array of proinflammatory cytokines other than TNF [23], including interferon gamma, IL-6 and IL-17. The latter cytokine, for example, promotes the accumulation of neutrophils in the synovial fluid, where they produce large amounts of cartilage-degrading enzymes and reactive oxygen species [24]. This way, in patients with higher levels of T\textsubscript{PH}, the inflammation will have alternative mechanisms to progress despite the reduction of TNF levels by anti-TNF agents.

Our results also suggest that the stromal component of the synovial membrane participates in the favorable response to anti-TNF therapy. Single cell analysis of the synovial membrane identified three different synovial fibroblast types [11]: CD34-THY1-, CD34-THY1 + and CD34+. CD34-THY1 + fibroblasts have shown to be expanded compared to osteoarthritis synovium and therefore have been appointed as the principal pathogenic subset. In our cell type analysis, we did not find evidence for enrichment of CD34-THY1 + fibroblasts with anti-TNF response. Instead, we found a significant increase of CD34 + and CD34-THY1- fibroblasts. Functional evidence suggests that CD34 + fibroblasts have a leading role in the recruitment of monocytes into the inflamed synovial, producing large amounts of IL6, CXCL2 and CCL2 in response to TNF stimulation. In comparison, CD34-THY1-
fibroblasts produce higher levels of matrix metalloproteinases MMP1 and MMP3. According to our results, both fibroblast types would represent more TNF-sensitive stromal cells, in which reduction of local levels of this cytokine by therapy would be translated into a less proinflammatory and matrix-degrading activity and, ultimately, into a higher likelihood for clinical response.

At the single gene level, we have found two genes, *CX3CL1* and *PIK3CD*, showing distinctively high expression levels at baseline in responders and non-responders, respectively. From these, only *CX3CL1* is still overexpressed in the synovial membrane of responders at week 20. *CX3CL1*, also known as fractalkine, is a chemokine that can be present as membrane-bound or in soluble forms, and is highly expressed in endothelial cells activated by TNF [25]. Interaction of membrane-bound fractalkine with its receptor CX3CR1, mediates the capture of circulating immune cells and infiltration into the inflamed tissue. In particular, CX3CR1\textsuperscript{hi} monocytes that patrol endothelium have been shown rapidly accumulate in sites after activation of the endothelium [26]. Accordingly, our results suggest that patients having a vascular tissue richer in CX3CL1 expression will have a more beneficial impact of TNF blocking.

*PIK3CD* is highly expressed in the synovial membrane of patients that will not respond to therapy. Together with *PIK3CA* and *PIK3CB*, *PIK3CD* conforms the class I phosphoinositide 3-kinases, a group of enzymes that phosphorylate inositol lipids and participate in different signaling processes [27]. While *PIK3CA* and *PIK3CB* are expressed in many different tissues, *PIK3CD* is limited to the cells of the immune system including T and B lymphocytes [28], neutrophils [29] and macrophages [30]. More recently, *PIK3CD* has also been found to be highly expressed in RA synovial fibroblasts [31]. In vitro studies have shown that TNF is a major inducer of *PIK3CD* expression in synovial fibroblasts while it does not activate this gene in leukocytes [32]. In these studies, the expression of *PIK3CD* in RA synovial fibroblasts was shown to induce cell growth by sensitizing fibroblasts to platelet-derived growth factor (PDGF) [31]. Based on this evidence, non-responders would have a stromal component more prone to PDGF-derived hyperplasia. For this group of patients, combination with drugs interfering with PDGF signaling might be an efficacious approach to rescue non-responder patients. Experimental and clinical evidence from of the therapeutic potential of interfering with the PDGF
pathway in RA clearly supports this possibility [33].

Pathway-level analysis of the response to anti-TNF therapy revealed an important contribution of the myeloid cell load. Our longitudinal data shows that a high myeloid cell activity not only conditions to a worse response but, also, that it is the biological process that is more highly altered by the drug’s efficacy. The existence of two patient groups in RA with different myeloid leukocyte activation is in high accordance with recent findings using single cell RNA-seq analysis. Analyzing the synovial membrane cell composition of a relatively large patient cohort of RA and osteoarthritis individuals, Zhang et al [34] found identified two subtypes of patients in RA defined as leukocyte-poor RA and leukocyte-rich RA. Our study extends these findings into the therapeutic domain. Despite not showing distinguishable clinical activities, non-responder patients have a significantly higher myeloid activity, which is also one of the top-ranking biological processes detected in the leukocyte-rich RA patients.

Finding less invasive biomarkers like blood analytes that correlate with the two patient subtypes would therefore be a useful means to stratify patients for anti-TNF therapy.

Conclusions

In the present study we have performed a multilevel analysis of the RA synovial membrane transcriptome in relation to the response to anti-TNF therapy. Using a cell-type deconvolution approach we have found that a newly characterized pathogenic T cell subtype in RA, peripheral T helper cell, is associated to the lack of response to TNF blocking. Our longitudinal analysis, shows that good response at week 20 is preserved through sustained low $T_{PH}$ cell numbers. On the pathway-level, response to anti-TNF treatment is strongly linked to an efficacious clearance of myeloid leukocyte activation. On the single-gene level, two genes previously associated with RA pathology, $CX3CL1$ and $PIK3CD$, were found also to be markers for anti-TNF response, implicating variability in the immune cell recruitment and fibroblastic growth capacities of the synovial membrane. The results from this study show new biological features of anti-TNF response in RA and could be useful starting points to allow patient stratification and also develop new therapeutic approaches, alone or in combination with TNF blockers, to achieve good responses in a larger number of patients.

List Of Abbreviations
EULAR: European Leagues Against Rheumatism

GO: gene ontology

mRNA: messenger RNA

RA: rheumatoid arthritis

RT-PCR: real-time polymerase chain reaction

TNF: tumor necrosis factor alpha

$T_{PH}$: peripheral helper T cells

Declarations

**Ethics approval and consent to participate**

The study was approved by the Hospital Clinic de Barcelona ethics committee and all the patients signed the informed consent. This study was conducted in accordance with the Declaration of Helsinki principles.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets generated and analysed during the current study are available at the NCBI Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/gds) with accession numbers GSE140036, GSE47726 and GSE15602.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This study was supported by of the Fondo de Investigación Sanitaria del Instituto de Salud Carlos III, grant numbers: P11/1890, PI040710 and PI12/01362. The funding source had no role in the collection, analysis or interpretation of the data, or in the writing of the manuscript.

**Authors' contributions**

AJ: Study design, Methodology, Statistical Analysis, Writing; GA: Resources, Investigation, Data Curation, Writing; RC: Resources, Data Curation; RS: Resources, Investigation; JR: Resources,
Investigation; SM: Resources, Methodology, Writing; JDC: Study design, Methodology, Writing. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Prof. Joachim Schultze and Svenja Debey-Pascher (University of Bonn, Germany) for their technical assistance in the transcriptome analysis, as well as Dr Neus Prats and Dr Mónica Aguilera (Histopathology Facility, Institute for Research in Biomedicine, Spain) for their generous assistance in the synovial immunofluorescence analyses.

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Figures
The TPH enrichment in the synovial membrane of RA patients at baseline therapy is associated with the clinical response. A. Boxplot displaying the PD-1hiCXCR5-CD4+ cell enrichment levels estimated using the cell-type deconvolution approach on the microarray transcriptional data from 11 patients starting anti-TNF therapy. The estimated enrichment scores for TPH cells are significantly associated with the EULAR response at week 20 (P=0.021). B. Boxplot displaying the PD-1hiCXCR5-CD4+ cell percentage as directly quantified using immunofluorescence on synovial biopsies of the same set of patients at baseline. As seen in the cell-type deconvolution approach, lower TPH cells are associated with better responses to anti-TNF agents (P=0.025). In both cases, moderate and good EULAR responders show distinctively lower proportions of this pathogenic T cell subset associated with RA.
Figure 2

Representative immunofluorescence of TPH in synovial tissues of a good responder (A, C, E, G) and a non-responder (B, D, F, H) at week 20. (C, D) Show positive cells showing PD-1 in green, (E, F) Show positive CXCR5 labelling in orange and (G, H) Show positive CD4 cells in red labeling. (A) and (B) show the complete immunofluorescence image of the responder and non-responder where it can be clearly seen that the response anti-TNF therapy is associated with lower TPH cells in the synovial membrane.
Figure 3

TPH enrichment analysis in the synovial membrane of the external dataset reproduces the patterns observed in our patient cohort. A. Boxplot displaying the PD-1hiCXCR5-CD4+ cell percentage as directly quantified using immunofluorescence on synovial biopsies of at week 20 of anti-TNF therapy. B. Boxplot displaying the PD-1hiCXCR5-CD4+ cell enrichment levels estimated using the cell-type deconvolution approach on the external microarray transcriptional data from 11 patients at week 12 of anti-TNF therapy. In both cases, EULAR good responders show distinctively lower TPH cell levels than moderate or none responders (P=3.5e-4 and P=4e-3 for our patient cohort and the external RA patient cohort, respectively).

Supplementary Files
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