ARTHRITIS & RHEUMATOLOGY
Vol. 66, No. 1, January 2014, pp 15–23
DOI 10.1002/art.38202

Global Molecular Effects of Tocilizumab Therapy in Rheumatoid Arthritis Synovium

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Objective. **To investigate the global molecular effects of tocilizumab (TCZ) in comparison with methotrexate (MTX) treatment in synovial biopsy tissue obtained from patients with previously untreated rheumatoid arthritis (RA) before therapy (T0) and 12 weeks after the initiation of therapy (T12), and to compare the results with previous gene expression data obtained in synovial biopsy tissue from adalimumab (ADA)– and rituximab (RTX)–treated patients with RA.**

Methods. **Paired synovial biopsy samples were obtained at T0 and T12 from the affected knee of TCZ-treated RA patients and MTX-treated RA patients. Gene expression studies were performed using GeneChip Human Genome U133 Plus 2.0 microarrays, and confirmatory quantitative real-time reverse transcription–polymerase chain reaction experiments were performed on selected transcripts. The effects of TCZ and MTX on synovial cell populations and histologic characteristics were assessed by immunohistochemistry.**

Results. **Gene expression studies showed that blockade of the interleukin-6 receptor (IL-6R) gene (*IL6R*) using TCZ induced a significant decrease in the expression of numerous chemokine and T cell activation genes in the RA synovium. These effects strongly correlated with the molecular effects of MTX and RTX therapy on RA synovial tissue, but differed from the molecular changes induced by ADA (decreased expression of genes involved in cell proliferation).**

Conclusion. **The molecular similarities between the effects of TCZ, RTX, and MTX therapies in the RA synovium indicate that B cell– and *IL-6*–dependent pathways play synergistic roles in the pathogenesis of the disease, in particular through activation of T cell responses. Moreover, these results open perspectives for the individualization of therapeutic decisions, based on a better knowledge of the synovial molecular effects of each type of RA therapy.**

Tocilizumab (TCZ) is a humanized anti–IL-6 receptor (anti–IL-6R) monoclonal antibody that inhibits all IL-6R– and soluble IL-6R (sIL-6R)–mediated signals. TCZ, either as monotherapy or in combination with methotrexate (MTX), is labeled for the treatment of RA patients who have an insufficient response to MTX or other disease-modifying antirheumatic drugs (DMARDs) (1–3).

In this study, we investigated the global molecular effects of TCZ therapy in synovial biopsy samples collected prospectively from DMARD-naive RA patients at baseline (T0) and 12 weeks after the initiation of TCZ therapy (T12). In parallel, synovial biopsy samples were harvested from DMARD-naive RA patients before and 12 weeks after the initiation of MTX therapy. We previously identified specific molecular pathways that were associated with response to treatment with adalimumab (ADA) and rituximab (RTX) in RA patients (4,5). In the present study, we sought to determine whether the molecular changes induced in the synovium by treatment with TCZ might overlap with the molecular effects of these other treatments. We found that TCZ significantly down-regulated numerous chemokine genes and genes involved in T cell activation, and

Supported by a grant from Roche Belgium. Dr. Ducreux's work was supported in part by a UCB/Université catholique de Louvain Chaire sur les Rhumatismes Systémiques Fellowship.

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Submitted for publication May 7, 2013; accepted in revised form September 17, 2013.

induced the expression of genes associated with healing processes. Strikingly, these effects overlapped with the molecular effects of RTX and MTX therapy in RA synovial biopsy tissue, but differed from the molecular changes induced by tumor necrosis factor (TNF) blockade with ADA.

PATIENTS AND METHODS

Patients and synovial biopsy samples. Thirty patients with untreated RA (most of whom had a disease duration of <1 year) were included in the study. All patients met the American College of Rheumatology 1987 revised classification criteria for RA (6). The demographic and clinical characteristics of the patients are summarized in Table 1. None of the patients had been previously treated with MTX (or any other DMARD) at the time of inclusion, and 3 of the patients were being treated with low-dose glucocorticoids (prednisone ≤7.5 mg/day). All patients had a painful or swollen knee joint at inclusion.

Patients were randomized into 1 of 2 treatment groups. Seventeen patients were randomized to receive TCZ intravenously at a dosage of 8 mg/kg/month over 6 months, and 13 were randomized to receive MTX that was administered orally at an initial dosage of 15 mg/week, increasing up to a dosage of 20 mg/week. Disease activity at baseline and at 6 months was evaluated with the Disease Activity Score in 28 joints using the C-reactive protein level (DAS28-CRP) (7) and the Simplified Disease Activity Index (SDAI; comprising 5 variables) (8). Response to therapy was assessed using the European League Against Rheumatism (EULAR) response criteria (9) and the SDAI-defined remission status at 6 months.

Synovial biopsy samples from 27 patients (16 in the TCZ-treated group, 11 in the MTX-treated group) were obtained using needle mini-arthroscopy of the affected knee at T0 and T12. For each procedure, 4–8 synovial samples were kept overnight at 4°C in an RNA stabilization solution (RNALater; Ambion) and then stored at −80°C for later RNA extraction. The same amount of tissue was snap-frozen in liquid nitrogen and kept at −80°C for immunostaining experiments on frozen sections. The remaining material was fixed in 10% formaldehyde and embedded in paraffin for conventional optical evaluation and immunostaining of selected markers. All experiments (RNA extraction, histology, immunohistochemistry) were performed on at least 4 biopsy samples harvested during every procedure in order to correct for variations related to the potential heterogeneous distribution of synovial inflammation (10,11). The study was approved by the ethics committee of the Université catholique de Louvain, and informed consent was obtained from all patients.

Table 1. Baseline demographic and clinical characteristics of the patients with rheumatoid arthritis treated with methotrexate (MTX) or tocilizumab (TCZ)*

	MTX-treated group (n = 13)	TCZ-treated group (n = 17)
Female, %	76.9	76.5
Age, mean ± SD years	56.9 ± 10.4	49.4 ± 13.9
Disease duration, mean ± SD years	0.49 ± 1.30	0.99 ± 2.30
RF positivity, %	77	81
Anti-CCP positivity, %	61	70
Swollen joint count, mean ± SD	5.9 ± 4.8	6.7 ± 4.8
Tender joint count, mean ± SD	10.2 ± 10.4	8.4 ± 6.6
Serum CRP, mean ± SD mg/liter	24 ± 20	19 ± 24
HAQ score, mean ± SD	1.4 ± 0.7	0.9 ± 0.6
DAS28-CRP, mean ± SD	4.7 ± 1.3	4.4 ± 1.0
SDAI score, mean ± SD	19.5 ± 14.5	17.8 ± 9.3
Radiographic erosions, %	38	35

* RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; CRP = C-reactive protein; HAQ = Health Assessment Questionnaire; DAS28-CRP = Disease Activity Score in 28 joints with CRP level; SDAI = Simplified Disease Activity Index.

Microarray hybridization. Total RNA was extracted from the synovial biopsy samples using a NucleoSpin RNA II extraction kit (Macherey-Nagel), including DNase treatment of the samples. At least 2 μg total RNA was extracted for further processing from 12 paired samples (obtained at T0 and T12) in the TCZ-treated group, and from 8 paired samples (obtained at T0 and T12) in the MTX-treated group. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA nanochips (Agilent Technologies). All samples had an RNA integrity number of >6.5.

Complementary RNA (cRNA) was synthetized and biotin-labeled according to a standard Affymetrix procedure (GeneChip 3′ IVT Express kit), as previously described (12). Briefly, total RNA was first reverse transcribed into single-stranded complementary DNA (cDNA) using T7 oligo(dT) primer. The second-strand cDNA was then synthesized using DNA polymerase and RNase H to simultaneously degradate the RNA. The double-stranded cDNA served as a template for the in vitro transcription reaction, which was performed for 16 hours in the presence of a biotinylated ribonucleotide analog mix. At the end of the procedure, biotinylated cRNA was purified and fragmented to prepare the target for hybridization to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix).

The GeneChip Human Genome U133 Plus 2.0 arrays were hybridized overnight at 45°C with 10 μg fragmented biotinylated cRNA. The slides were then washed and stained using a EukGE-WS2v5 fluidics protocol on a GeneChip Fluidics Station 450, before being scanned on a GeneChip Scanner 3000 (Affymetrix). For the initial normalization and analysis steps, data were retrieved on Affymetrix GeneChip Operating Software. The frequency of positive genes (genes with a flag present) was between 45% and 55% on each slide. After scaling of all probe sets to a value of 100, the amplification scale was reported to be inferior to 3.0 for all slides. The signals yielded by the poly(A) RNA, hybridization, and housekeeping controls were indicative of the good quality of the amplification and hybridization procedures. The Affymetrix .CEL and .CHP files were deposited in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO accession number GSE45867.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) experiments. Quantitative RT-PCR evaluation of *CXCL13* (also known as B lymphocyte chemoattractant), *CCL2*, *CCL8*, *IL6*, *IL7*, and

EGFL6 expression was performed in synovial biopsy samples obtained at T0 and T12 from 10–12 TCZ-treated patients and 5–8 MTX-treated patients. The cDNA was synthesized from 500 ng total RNA using RevertAid Moloney murine leukemia virus reverse transcriptase (Fermentas) and oligo(dT) primers. Analysis by qRT-PCR was performed on a LightCycler 480 real-time PCR system (Roche Applied Science) using SYBR Green detection mix (Eurogentec). For each sample, 20 ng cDNA was loaded in triplicate with 1× SYBR Green mix and 0.2 μM primers (Eurogentec). The melting curves obtained after each PCR amplification reaction confirmed the specificity of the SYBR Green assays. Normalized expression values for the target genes in the studied samples were obtained using standard curves.

The following primers were used: for *ACTB* (β-actin) (GenBank accession no. NM_001101.3), 5′-TCACCCACACTGTGCCCATCTACGA-3′ (forward) and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′ (reverse); for *CCL2* (GenBank accession no. NM_002982.3), 5′-CCTCCAGCATGAAAGTCTCTG-3′ (forward) and 5′-TCACAGCTTCTTTGGGACACT-3′ (reverse); for *CCL8* (GenBank accession no. NM_005623.2), 5′-ATCCAGAGGCTGGAGAGCTAC-3′ (forward) and 5′-AGGCTCATGGCTTCAGATTTT-3′ (reverse); for *CXCL13* (GenBank accession no. NM_006419.2), 5′-CCTCTCTCCAGTCCAAGGTG-3′ (forward) and 5′-TGAGGGTCCACACACACAAT-3′ (reverse); for *EGFL6* (GenBank accession no. NM_001167890.1), 5′-GACCACGAGTGAGGATGAAAA-3′ (forward) and 5′-GGACATAAGCCTGAAACAAGC-3′ (reverse); for *IL6* (GenBank accession no. NM_000600.3), 5′-CAGACAGCCACTCACCTCTTC-3′ (forward) and 5′-TCTTTTTCAGCCATCTTTGGA-3′ (reverse); and for *IL7* (GenBank accession no. NM_000880.3), 5′-AAGCCCAACCAACAAAGAGTT-3′ (forward) and 5′-TTCTTGGAGGATGCAGCTAAA-3′ (reverse). Conventional PCR amplification (40 cycles) and agarose gel electrophoresis of the amplicons showed the presence of a single band using these primers.

Histopathologic and immunohistochemical analyses of paraffin-embedded sections. Fresh synovial biopsy tissue samples were fixed overnight in 10% formalin buffer at pH 7.0 and embedded in paraffin for histologic and immunohistochemical analyses. Fifteen samples were available for analysis in the TCZ group, and 9 were available in the MTX group. Serial histologic sections were stained with hematoxylin and eosin. The following parameters were evaluated: synovial hyperplasia, lymphoplasmacytic cell infiltrates, fibrinoid necrosis, and vascular hyperplasia. Immunolabeling experiments were performed using a standard protocol, as previously described (5). The following antibodies were used: anti-CD3 (Neomarkers), anti-CD20 (Biocare Medical), anti-CD68, and anti-CD138 (both from DakoCytomation).

Evaluation of all optical and immunohistochemical variables was performed using a semiquantitative score on a 0–3 scale, where 0 indicates absence of the feature and 3 represents the highest level. A specific score was assigned for the extent of hyperplasia of the synovial lining layer, where 0 indicates 1 or 2 affected cell layers, 1 indicates 3 or 4 affected cell layers, 2 indicates 5 or 6 affected cell layers, and 3 indicates at least 7 affected cell layers.

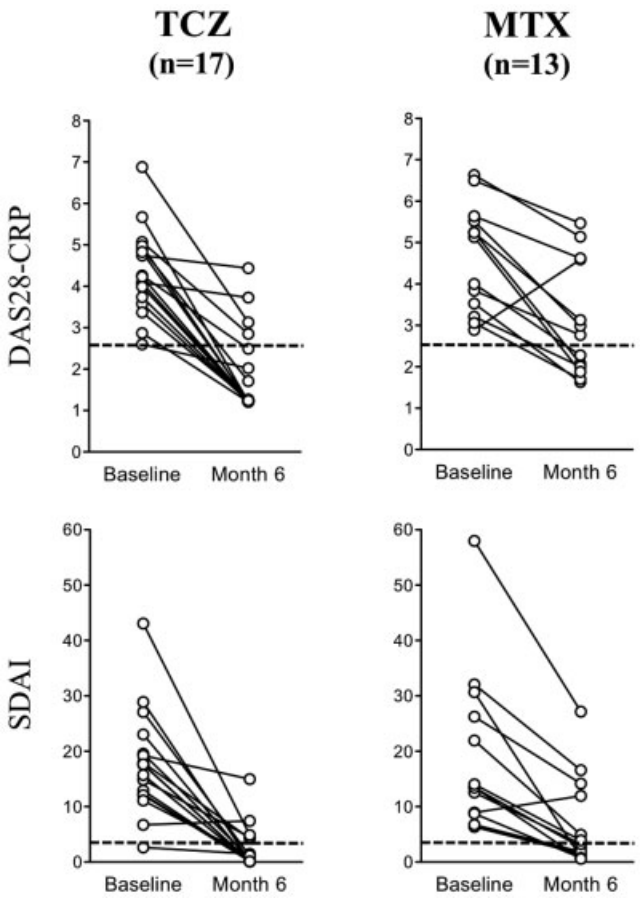

Figure 1. Evolution of the Disease Activity Score in 28 joints using the C-reactive protein level (DAS28-CRP) and Simplified Disease Activity Index (SDAI) in patients with rheumatoid arthritis, at baseline and 6 months after the initiation of therapy with tocilizumab (TCZ) or methotrexate (MTX). The broken line indicates the cutoff value for clinical remission according to each disease activity measure (DAS28-CRP score ≤2.6; SDAI score ≤3.3).

Statistical analysis. Statistical analyses of the microarray data were performed using GeneSpring software (Agilent Technologies). Fluorescence intensity data were normalized using robust multiarray analysis (13). Differences in gene expression were assessed using Student's *t*-test. Pathway-enrichment analyses were performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery), an application that interrogates functional annotation databases (Gene Ontology annotations, KEGG pathways, Biocarta, and InterPro) and finds overrepresented biologic themes within a group of genes (http://david.abcc.ncifcrf.gov/) (14).

Comparative analyses of the effects of therapies were performed using data sets previously generated by our group. Thus, the GSE15602 data set comprises the Human Genome U133 Plus 2.0 GeneChip data obtained in 8 MTX-resistant RA patients before and 12 weeks after the initiation of ADA therapy. The GSE24742 data set comprises the data from 12

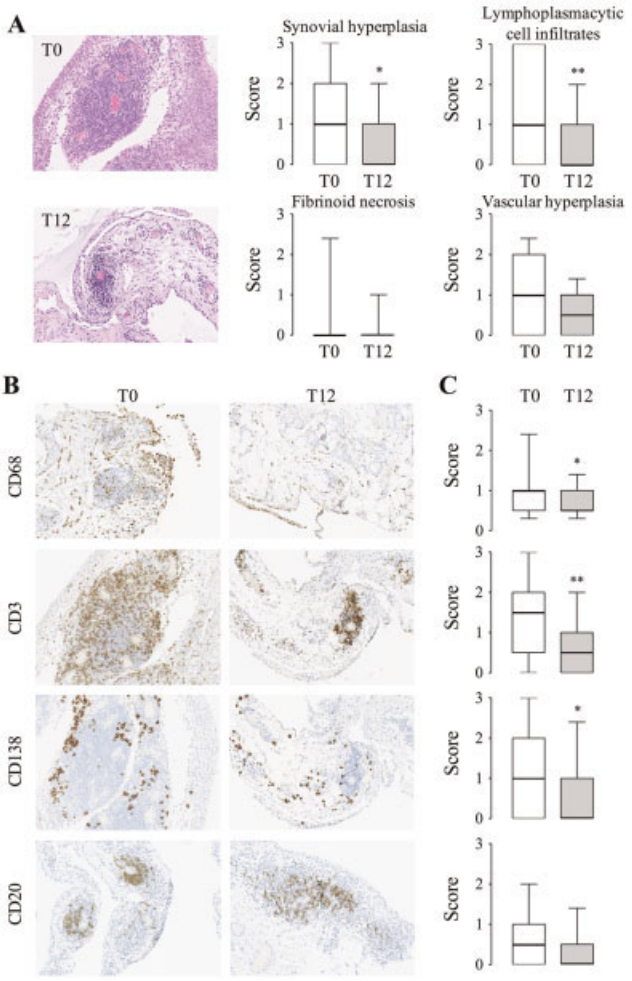

Figure 2. Effect of tocilizumab (TCZ) on histologic characteristics of the synovial tissue and distribution of cell populations in synovial biopsy samples obtained from patients with previously untreated rheumatoid arthritis (RA) prior to therapy (T0) and 12 weeks after the initiation of therapy (T12). **A**, Immunohistologic findings in representative synovial tissue samples obtained from an RA patient at T0 and T12 after TCZ therapy (original magnification × 200) (left), and semiquantitative evaluation of immunostained synovial tissue sections from RA patients at T0 and T12 to assess the levels of synovial hyperplasia, lymphaplasmacytic cell infiltrates, fibrinoid necrosis, and vascular hyperplasia (right). **B** and **C**, Immunohistologic findings for CD68, CD3, CD138, and CD20 markers in representative synovial tissue samples from an RA patient at T0 and T12 after TCZ therapy (original magnification × 200) **(B)**, and semiquantitative evaluation of immunostained synovial tissue sections from RA patients at T0 and T12 to assess the staining intensity of each marker **(C)**. Results of semiquantitative evaluations were obtained in 15 paired samples (obtained at T0 and T12). Values are shown as box plots. Each box represents the 25th and 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. $* = P < 0.05$; $** = P < 0.005$ versus T0, by Wilcoxon's matched pairs signed rank test.

RA patients resistant to TNF blockade, obtained before and 12 weeks after the initiation of RTX therapy. For each probe set, the effect of therapy was calculated as the mean $\log_2$-transformed intensity value at T12 minus the mean $\log_2$-transformed intensity value at T0. These computations were performed using either all probe sets from the microarray slides or a subgroup of probe sets that are more relevant to the pathogenesis of RA. The latter list of probe sets was generated using data from the GSE36700 data set (submitted previously by our group), which reports gene expression profiles in synovial biopsy samples from untreated patients with RA or patients with osteoarthritis (OA); these data were compared between RA and OA samples using Student's *t*-tests ($P < 0.05$ after Bonferroni correction for multiple comparisons) (see Supplementary Table 1A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). Finally, comparative analyses were also performed on probe sets included in specific pathways that were found to be specifically up- or down-regulated by the therapies (see Supplementary Table 1B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). Statistical comparisons of the other experimental data were performed using GraphPad Prism software for Mac (version 5.0).

RESULTS

Disease activity was prospectively evaluated in the RA patients at baseline and 6 months after the initiation of therapy based on the DAS28-CRP and SDAI score evaluations (15). As shown in Figure 1, 12 of 17 TCZ-treated patients and 7 of 13 MTX-treated patients achieved remission (defined as an SDAI score ≤3.3) at 6 months. Using the DAS28-CRP definition of remission (a DAS28-CRP score ≤2.6), 13 of 17 TCZ-treated patients and 6 of 13 MTX-treated patients achieved remission at 6 months.

We first evaluated the effects of TCZ therapy compared with MTX therapy on synovial cell populations and the immunohistochemical characteristics of the synovial biopsy tissue at T0 and T12. Semiquantitative evaluations and paired comparisons of the synovial biopsy samples indicated that treatment of RA patients with TCZ induced a significant decrease in the extent of synovial hyperplasia and in the number of infiltrating lymphoplasmacytic cells (Figure 2A). In addition, TCZ induced a significant decrease in the numbers of synovial CD68+, CD3+, and CD138+ cells between T0 and T12 (Figures 2B and C). In contrast, treatment with MTX did not induce significant changes in any of these variables (results not shown).

Next, the effects of TCZ on synovial gene expression profiles were investigated using high-density oligonucleotide-spotted microarrays. Comparison of the re-

sults by Student's paired *t*-tests indicated that 6,683 of 54,675 probe sets displayed a significant difference in gene expression in the synovial tissue samples between T0 and T12; of these, 3,413 genes were down-regulated and 3,270 were up-regulated (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). Pathway-enrichment analyses, performed using DAVID bioinformatics resources, indicated that the genes that were down-regulated by treatment with TCZ clustered in 2 specific pathways: cytokines and chemokines (e.g., *IL6*, *IL22*, *CCL8*, *CCL11*, *CCL13*, *CCL19*, *CCL20*), and T cell activation (e.g., *IL23A*, *IL7*, *RAG2*, *ZAP70*) (Figures 3A and B and Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). In contrast, genes that were up-regulated by TCZ were significantly enriched in transcripts associated with healing processes (*COL2A1*, *COL6A1*, *COL15A1*, *COL16A1*, *COL18A1*) (Figure 3C and Supplementary Table 2).

Similar analyses were performed on paired synovial biopsy samples before and 12 weeks after the initiation of MTX therapy. We found that 1,196 probe sets were differentially expressed between T0 and T12 (586 genes were down-regulated and 610 were up-regulated; see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). Pathway-enrichment analyses showed that the down-regulated genes were significantly enriched in T cell activation and immune response pathways (see Supplementary Table 3). Overall, the molecular effects of MTX were similar to the effects of TCZ therapy, but of lower amplitude.

In order to confirm the microarray data, qRT-PCR experiments were performed on the following genes that were either up-regulated or down-regulated by TCZ: *IL6*, *CXCL13*, *CCL8*, *CCL2*, *IL7*, and *EGFL6*. As shown in Figure 3D, results of the qRT-PCR analysis confirmed the differential expression of several selected transcripts between T0 and T12 in RA patients receiving TCZ therapy.

When we restricted these analyses to treatment response according to the EULAR criteria (good or moderate responders) or to patients who achieved clinical remission 6 months after the initiation of therapy, we found that the changes in gene expression were, overall, similar to those observed in the whole population of patients, both in the TCZ-treated group and in the MTX-treated group, and this resulted in the identi-

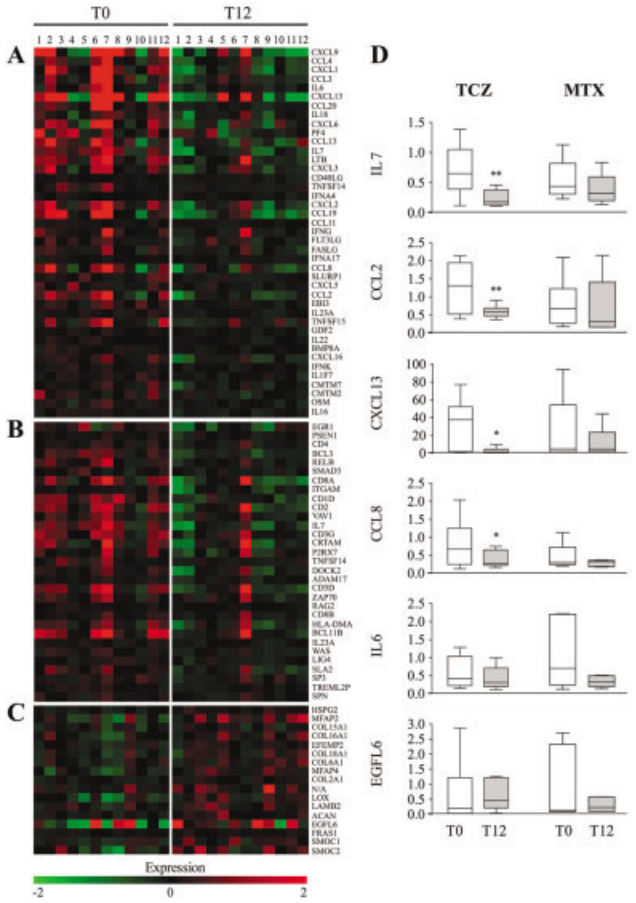

Figure 3. Genes differentially expressed before therapy (T0) and 12 weeks after the initiation of therapy (T12) with tocilizumab (TCZ), as compared with methotrexate (MTX), in synovial biopsy samples from 12 previously untreated patients with rheumatoid arthritis (RA). **A–C**, Student's paired *t*-tests indicated that 6,683 of 54,675 probe sets displayed significant differences in gene expression between T0 and T12. Pathway analyses indicated that a significant proportion of these genes clustered in specific pathways. Down-regulated genes were significantly enriched in cytokines and chemokines **(A)** and T cell activation pathways **(B)**, while up-regulated genes were significantly enriched in transcripts associated with healing processes **(C)**. **D,** Paired synovial biopsy samples obtained from RA patients at T0 and T12 after the initiation of therapy with TCZ or MTX were evaluated by quantitative reverse transcription–polymerase chain reaction for the expression of selected genes. Samples were loaded in triplicate, and results were normalized to the values for the β-actin gene. Results, obtained in 10–12 paired samples in the TCZ-treated group and 5–8 paired samples in the MTX-treated group, are shown as box plots, where each box represents the interquartile range and lines inside the boxes represent the median. Whiskers represent 1.5 times the upper and lower interquartile ranges. $* = P < 0.05$; $** = P < 0.01$ versus T0, by Wilcoxon's matched pairs signed rank test.

fication of the same pathways in pathway-enrichment analyses (results not shown). We also compared changes in gene expression between patients who achieved SDAI clinical remission at 6 months (TCZ n = 7; MTX n = 5) and those who did not (TCZ n = 5; MTX n = 3). T cell activation and immune response pathways were not differentially affected in the TCZ- and MTX-treated patients, regardless of whether any patients achieved clinical remission. In patients who achieved remission at 6 months after the start of TCZ therapy, genes involved in, for example, induction of apoptosis and myeloid cell differentiation were more down-regulated, and genes involved in regulation of Ras protein signal transduction and ubiquitin-dependent protein catabolic processes were more up-regulated (see Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). In the group of MTX-treated patients, genes involved in, for example, cell division were more down-regulated in patients achieving remission at 6 months (see Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract).

Previous studies by our group have demonstrated that ADA therapy and RTX therapy each display distinct molecular effects on global gene expression profiles in the RA synovium (4,5). In particular, ADA induces a significant down-regulation of genes involved in cell proliferation (4), whereas RTX down-regulates transcripts that are significantly enriched in immunoglobulin and T cell activation genes (5). We therefore wanted to compare the effects of these treatments on synovial gene expression profiles, in order to understand whether they share similar molecular effects. These analyses were performed using either all probe sets or a subset of genes more specifically associated with RA (in order to avoid contamination of our analyses by gene expression differences observed in probe sets with very low intensity values). As shown in Figures 4 and 5, we found that MTX, TCZ, and RTX displayed similar molecular effects (albeit of different magnitudes) in the RA synovium, which were distinct from the molecular changes induced by ADA. In particular, the majority of the genes up- or down-regulated by TCZ in the RA synovium were similarly up- or down-regulated by MTX and RTX, whereas this was not the case with ADA. Similar observations were made in all 4 therapeutic groups when the analyses were restricted to EULAR (good and moderate) responders (results not shown).

In a last set of analyses, we investigated whether remission status at 6 months could be predicted at baseline on the basis of the clinical, immunohistochemical, or molecular characteristics of the patients. Disease activity at baseline, both in TCZ-treated patients and in MTX-treated patients, was not associated with remission status at 6 months (results not shown). Similarly, no

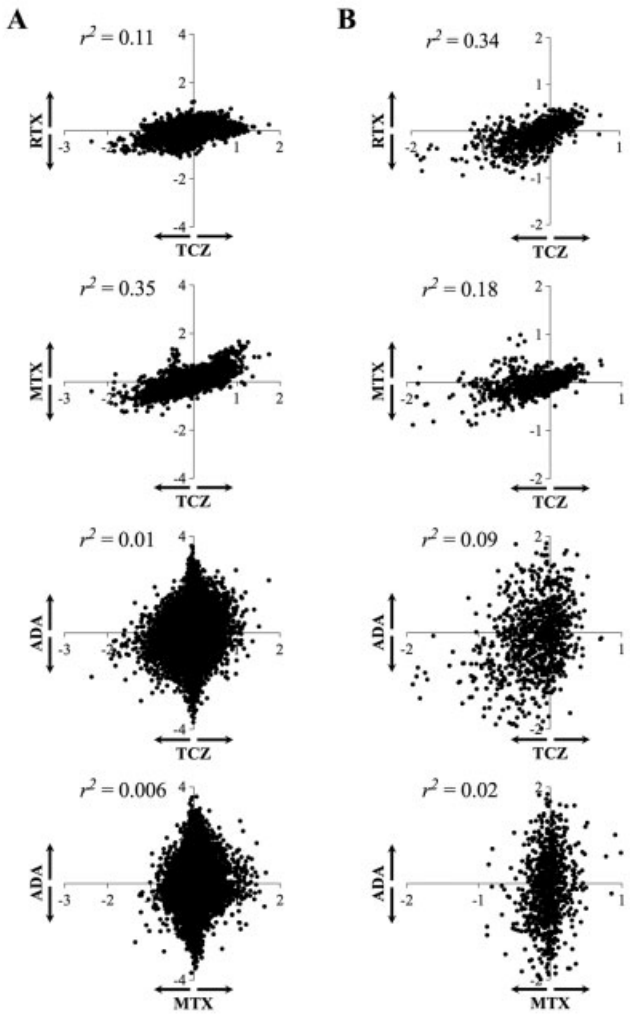

Figure 4. Comparison of the effects of tocilizumab (TCZ), rituximab (RTX), methotrexate (MTX), and adalimumab (ADA) therapies on gene expression profiles in rheumatoid arthritis (RA) synovium. The effects of the 4 therapies are displayed as the change (Δ) in intensity values, calculated as the mean $\log_2$-transformed gene expression at 12 weeks after the initiation of therapy (T12) minus the mean $\log_2$-transformed gene expression before the initiation of therapy (T0) (1 unit corresponds to a 2-fold change in gene expression), for all probe sets **(A)** and for probe sets specific to RA **(B)** (for complete lists of the genes and differences in gene expression, see Supplementary Table 1A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). $P < 0.0001$ for all correlations.

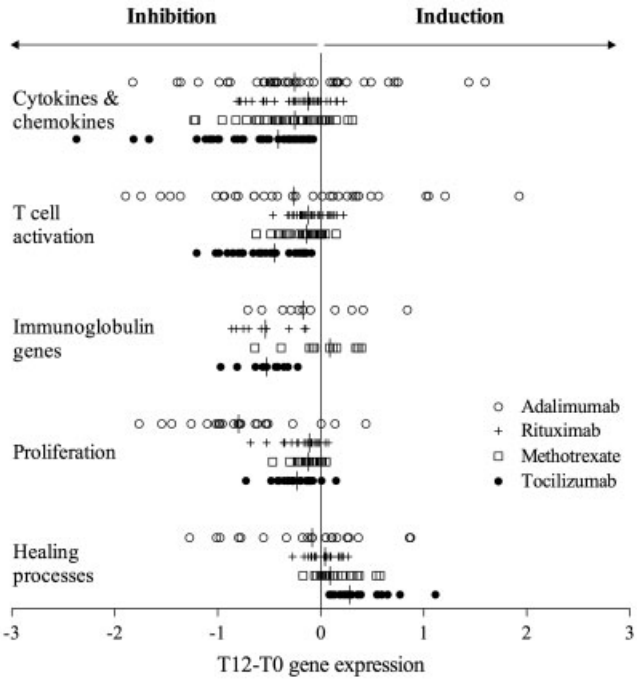

Figure 5. Comparison of the effects of adalimumab, rituximab, methotrexate, and tocilizumab therapies on genes involved in specific pathways in rheumatoid arthritis synovium. The effects of the 4 therapies in specific gene inhibition or gene induction pathways are displayed as the difference in intensity values, calculated as the mean $\log_2$-transformed gene expression at 12 weeks after the initiation of therapy (T12) minus the mean $\log_2$-transformed gene expression before the initiation of therapy (T0), for probe sets belonging to relevant Gene Ontology pathways (for complete lists of the genes and differences in gene expression, see Supplementary Table 1B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract).

difference was found in the baseline immunohistochemical parameters (synovial cell populations and histopathologic characteristics of the synovial tissue) between patients who achieved remission at 6 months and patients who did not, in either the TCZ or the MTX treatment group (results not shown).

In contrast, specific molecular signatures identified at baseline were associated with the SDAI remission status at 6 months in both groups of patients. In the TCZ treatment group, overexpression of genes involved in Ras protein signal transduction and cell cycle pathways was associated with later achievement of SDAI remission (see Supplementary Table 6, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract). In the MTX treatment group, overexpression of transcripts involved in myeloid cell function (HLA–DQ genes, *CD86*, *CCL13*, and *CCL18*) was predictive of the achievement of SDAI remission at 6 months (see Supplementary Table 7, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38202/abstract).

DISCUSSION

We found that IL-6R blockade induces a significant decrease in the expression of chemokine and T cell activation genes in the RA synovium. These molecular changes are paralleled by a significant decrease in synovial macrophages, T cells, and plasma cells. Moreover, we described a strong molecular similarity between the effects of TCZ, MTX, and RTX in RA synovial biopsy tissue. In contrast, the molecular targets of TNF blockade in the RA synovium are different.

The pleiotropic functions of IL-6 are thought to be responsible for many symptoms in RA patients at local and systemic levels (16,17). IL-6 signals through both membrane-bound and soluble receptors (IL-6R and sIL-6R, respectively), increasing the numbers of cell types responsive to the cytokine. Nevertheless, IL-6 was originally described as a T cell activation factor (18,19), and our results indicate that synovial T cell activation is a major target of IL-6R blockade in the RA synovium. More recently, the role of IL-6 in the differentiation of pathogenic Th17 cells was highlighted by numerous reports (20). Although no definite Th17 gene expression signature can be used for comparison, the observed down-regulation of several Th17-associated genes (*IL7*, *IL22*, *IL23*) in our data set suggests that this specific T cell population is affected by TCZ therapy in RA synovial tissue.

Our results also highlight the role of IL-6 in regulating chemokine production in the RA synovium. No fewer than 15 different chemokines were found to be significantly down-regulated by the administration of TCZ therapy in our transcriptomic study. Most of these chemokines are involved in the recruitment of T and B cells into inflammation sites, thereby underscoring the role of IL-6 in the maintenance of an inflammatory environment in the RA synovium.

In previous studies, we found that ADA (a TNF-blocking agent) and RTX (a depleting CD20 antibody) displayed distinct molecular effects in RA synovial biopsy samples collected prospectively before and 3 months after the initiation of therapy (4,5). We therefore used these data in order to compare the molecular effects of TCZ and MTX with the effects of these other agents in RA synovial tissue collected at the same time

points. Strikingly, we found a strong molecular concordance between the effects of MTX, TCZ, and RTX therapies, either when assessing all transcripts present on the microarray slides or when focusing on the transcripts that are known to be specifically up- or down-regulated in RA synovial biopsy tissue (as compared with synovial biopsy tissue from patients with OA). In contrast, administration of ADA was associated with different patterns of molecular changes in the RA synovium.

It is common knowledge that clinical improvement in RA can result from the inhibition of multiple synovial targets. However, our results indicate that several of the therapeutic agents used in routine clinical practice share homogeneous modes of action in the rheumatoid synovium. Thus, both TCZ and RTX predominantly down-regulate genes involved in T cell activation. More than 20 years ago, Field and colleagues found that IL-6 production in the rheumatoid synovium was restricted to macrophage-, T cell–, and B cell–containing cellular aggregates (21). Based on those findings and our own observations, it is tempting to speculate that synovial IL-6–producing cells and B cells play major and complementary roles in the pathogenesis of RA, in particular as antigen-presenting cells that drive the differentiation of pathogenic T cells. In studies of RTX, we also demonstrated, by double immunofluorescence experiments, that IL-17 production by CD3-positive synovial cells was significantly decreased upon treatment with RTX (22). In contrast, our data indicate that T cell–driven responses are not affected by TNF blockade in such a systematic way, an observation that is consistent with a recent meta-analysis demonstrating that TNF blockade preferentially down-regulates fibroblast-associated pathways (cell cycle, cell adhesion, and migration) rather than T cell– and B cell–associated genes (23).

One potential caveat in these comparisons is that the patients used in our synovial gene expression studies were recruited at different stages of the disease. In particular, MTX- and TCZ-treated patients had early disease; ADA-treated patients were MTX resistant, while RTX-treated patients were resistant to both MTX and TNF blockade. Accordingly, gene expression profiles at T0 were significantly different in these 3 groups of patients (results not shown). This is the reason we performed analyses only of the change in gene expression (Δ intensity values) between T0 and T12, a difference that is more likely to reflect the effects of therapy regardless of the stage of the disease at the time of treatment administration. Nevertheless, it is theoretically possible that the global gene expression effects of ADA and RTX would have been different if the synovial samples had been collected from patients at an early stage of RA, as was the case for the samples from the MTX and TCZ treatment groups. In particular, we hypothesize that the molecular effects of these therapies in patients with early RA are of higher magnitude, due to the absence of selection of patients with higher disease severity and decreased response to therapy.

Nevertheless, the observation that MTX, TCZ, and RTX share similar molecular effects in the RA synovium opens novel perspectives in terms of treatment selection in individual cases. The choice between these 3 agents is not predicated on identifying the right target; instead, it has to take into account how markers of response to therapy can direct the selection of the drug with the highest probability of inducing a clinical response. Thus, we found that higher expression of Ras-dependent genes and cell cycle genes in the RA synovium was associated with an increased probability of achieving clinical remission after the administration of TCZ. Similarly, we and others found that the presence of B cell activation genes and markers was associated with a better response to RTX therapy in RA patients (5,24). Further studies in larger groups of patients will be needed to confirm whether these findings can translate into decision trees in individual cases.

Our results also bring new insights to the question as to whether different drugs may have associated effects in RA patients. Association of MTX with biologic agents is standard in RA patients, because MTX therapy has the ability to prevent the development of anti-drug antibody formation. In patients treated with TNF-blocking agents, MTX therapy also results in additional benefits in terms of clinical response to therapy, whereas there is presently no such evidence for the combination of TCZ and MTX therapy in RA (25,26). By unraveling the molecular effects of these therapies in the synovium, our data contribute to the understanding of how drug combinations have the ability to interfere with different molecular targets, thereby supporting the theoretical possibility of additive or synergistic therapeutic effects, as is the case for TNF blockade and MTX therapy.

Taken together, our results bring us closer to a molecular approach in the care of patients with RA, by identifying the synovial targets of TCZ therapy in the RA synovium, and showing how the effects of TCZ administration compare to the molecular effects of other agents. Further work is needed to identify clinically useful biomarkers, in order to guide therapeutic deci-

sions in individual cases, based on molecular mapping of the pathways to be targeted in patients' synovial tissue.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ducreux had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ducreux, Durez, Galant, Van den Eynde, Houssiau, Lauwerys.

Acquisition of data. Ducreux, Durez, Galant, Nzeusseu Toukap, Houssiau, Lauwerys.

Analysis and interpretation of data. Ducreux, Durez, Galant, Van den Eynde, Houssiau, Lauwerys.

ROLE OF THE STUDY SPONSOR

This study was supported by an unrestricted grant from Roche Belgium. Roche Belgium had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Roche Belgium.

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