Chapter 2.2
Pharmacogenomics in rituximab treated rheumatoid arthritis patients

2.2A Pharmacological induction of interferon type I activity following treatment with rituximab determines clinical response in rheumatoid arthritis

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ABSTRACT

Objective. Despite the fact that rituximab depletes B cells in all treated RA patients, not all patients show a favorable clinical response. The goal of this study was to provide insight into pharmacological changes in peripheral blood that are associated with clinical response to rituximab.

Methods. Gene-expression profiling was performed on peripheral blood RNA of 13 RA patients (test group) using Illumina® HumanHT beadchip microarrays. An independent group of 9 patients was used for validation using Taq-man qPCR. Clinical responder status was determined after six months using ΔDAS28 and EULAR response criteria. Significance Analysis of Microarrays, and ontology analysis were used for data analysis and interpretation.

Results. Pharmacogenomic analyses demonstrated marked inter-individual differences in the pharmacological responses at 3 and 6 months after start of therapy with rituximab. Interestingly, only differences in the regulation of type-I IFN-response genes after 3 months correlated with the ΔDAS28-response. Good responders ((ΔDAS28>1.2; n=7) exhibited a selective increase in the expression of type-I IFN-response genes, whereas this activity was not or hardly changed in non-responders (ΔDAS28<1.2; n=6) (p = 0.0040 at a cut-off of 1.1-fold induction ). Similar results were obtained using EULAR-response criteria. These results were validated in an independent cohort of 9 patients (5 non-responders and 4 responders, p = 0.0317).

Conclusions. A good clinical response to rituximab in RA is associated with a selective drug-induced increase in type-I IFN-response activity in RA patients. This finding may provide insight in the biological mechanism underlying the therapeutic response to rituximab.
INTRODUCTION

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints that may cause permanent cartilage and bone destruction. Although the etiology is still unknown it is assumed that the interplay between genetic background, immunological and environmental factors is important in disease pathogenesis.[1]

No curative therapy is currently available, and patients are subjected to a prolonged course of therapy. Because of the increased insight in the role of pro-inflammatory mediators and cellular principles of immune tolerance a number of therapeutical options for RA have been developed and are currently available.[2,3] However, a major challenge in the successful treatment of RA is disease heterogeneity, which is also presented in the diverse effects of treatment responses.[4] One of the treatment strategies is targeting of B cells making use of rituximab, a chimeric monoclonal antibody directed against the B cell marker CD20. This therapy was shown to be highly effective for suppression of disease activity in RA.[5-7] Clinical studies have demonstrated that in analogy to anti-TNF therapies not all patients show a favorable response to rituximab therapy.[8] Despite the fact that rituximab directly depletes specific B-cell populations in all patients treated, the existence of interindividual differences in clinical outcome of rituximab therapy has raised questions regarding the mechanism of action.[9,10] In order to provide insight in the biological basis for the clinical response towards rituximab we evaluated the pharmacological effects of rituximab in relation to the clinical outcome using genome-wide gene expression technology in whole blood of RA patients.

PATIENTS AND METHODS

Study population

Patients with established RA according to the revised American College of Rheumatology criteria for the diagnosis of RA [11] were consecutive recruited from two rheumatology outpatient clinics in The Netherlands (VU University Medical Center and Reade/Jan van Breemen Institute, Amsterdam). Inclusion criteria for this study are according to the guidelines of the Dutch Society for Rheumatology for treatment with rituximab, i.e. active disease status despite previous treatment with methotrexate (MTX) and one other disease modifying antirheumatic drug (DMARD) and despite TNF-blocking agents, unless contraindicated in the opinion of the treating physician. The study was approved by the local medical ethics committees and patients provided written informed consent. Patients had a delay of at least 4 weeks between last dose of TNF-blocker and first injection of rituximab.

Treatment and clinical evaluation
Patients received rituximab 1,000 mg intravenously on days 1 and 15, in combination with clemastine (2 mg intravenously), methylprednisolone (100 mg intravenously) and acetaminophen 1,000 mg orally, as premedication. Every 4 weeks after the first infusion and from 12 weeks on every 3 months patients were assessed for disease activity by the 28 joints Disease Activity Score (DAS28) [12] and blood sampling. The use of concomitant DMARDs, prednisolone or non-steroidal anti-inflammatory drugs (NSAIDs) during the study duration was permitted. Response to treatment was classified according to EULAR response and to change to DAS28.[12]

**Blood sampling and RNA isolation**

For RNA isolation, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix, GmbH, Germany) and stored at -20°C. After overnight thawing at room temperature total RNA was isolated using Bio robot MDX (Qiagen, Benelux b.v., Venlo, The Netherlands) according to the manufacturer’s instructions (PAXgene Blood RNA Mdx kit). Samples were cleaned using Qiagen RNA MinElute (Qiagen, Venlo, The Netherlands). Total RNA concentration was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and RNA purity and integrity was verified using lab-on-chip technology (Agilent 2100 Bioanalyzer, Californie, USA).

**Micro-array analyses**

The Illumina® TotalPrepTM RNA-amplification kit (Ambion, Austin, USA) was used to synthesize biotine-labeled cRNA from 500 ng total RNA. 750 ng biotinylated cRNA was hybridized onto the HumanHT-12 v3 Expression BeadChip (Illumina, San Diego, CA).

Amplification and hybridization were performed at the outsourcing company ServiceXS (Leiden, the Netherlands). Bead summary intensities were log2-transformed and normalized using quantile normalization.[13,14]

**Calculation of IFN type I response scores**

Expression of 6 IFN-response genes (IFI44, IFI44L, HERC5, RSAD2, LY6E and Mx1) was quantitated using the log2-transformed and quantile normalized expression values. IFN type I response scores were calculated as the mean of the expression values of the 6 IFN response genes for every single patient and at every single time-point.
cDNA synthesis and quantitative real time PCR

RNA (0.5 μg) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany). Quantitative realtime PCR was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA, USA). Gene expression levels were determined using Taqman Gene expression assays following manufactures’ guidelines. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies for each gene a standard curve was constructed. Expression levels of target genes were quantified relative to housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Flow Cytometry

To determine the relative amount of peripheral T- and B-lymphocytes, whole blood was stained for 30 min with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC) conjugated monoclonal antibodies directed against lymphocyte subset-associated surface molecules. Four color antibody combinations used were (FITC/PE/PerCP/APC): CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19 (all from BD Biosciences, San Jose, CA). Subsequently, red cells were lysed (Lysing Solution, BD Biosciences) and lymphocyte subsets were analysed by flow cytometry on a standard 4-color Fluorescence Activated Cell Scanner (FACSCalibur, BD Biosciences). Data were analyzed using Cellquest Pro software (BD Biosciences). Care was taken to analyze only viable cellular events based on light scatter properties. All analyses were performed on lymphocytes, based on bright CD45 staining and low sideward scatter.

Statistical analysis

Statistical analysis on microarray data was performed using Significant Analysis of Microarray (SAM) version 3.09.[15] Two class paired analysis using SAM at a false discovery rate (FDR) of less than 5% between pre- and post-therapy data was applied to identify genes that were significantly changed in expression after rituximab therapy. Cluster analysis was used for the categorization of coordinately differentially expressed genes.[16] Treeview was used to visualize differentially expressed genes. Gene Set Enrichment Analysis (GSEA; http://www.broad.mit.edu/gsea) was used for pathway analysis.[17,18] We used gene set permutation to adjust for multiple testing, indicated by a false discovery rate. A minimal gene set size of 15 genes per pathway was applied, and pathways with a p-value of <0.05 and a FDR of <0.05 were considered significant. A total of 282 pathways from Biocarta and KEGG were applied in this analysis. In addition, we incorporated the IFN-response gene set from [19] (genes with at least a 5-fold upregulation in PBMC after treatment with type I IFN). For ontology
analysis of gene sets identified by cluster analysis we used MetaCoreTM Pathway analysis, using the
MetacoreTM Ontology tools, developed by GeneGo (GeneGO, St Joseph, MI, http://www.genego.com/). Data mining in MetacoreTM is based on a manually curated database of human protein-protein, protein-DNA interactions, transcription factors, signaling pathways and metabolic pathways. Calculation of statistical significance are based on p-values which are defined as the probability of a given number of genes from the input list to match a certain number of genes in the functional GeneGO Ontology categories. Differences in gene expression levels of IFN type I response score between patients with a ΔDAS28 >1.2 versus ΔDAS28<1.2 or between EULAR good versus moderate versus non-responders were analyzed using Student’s unpaired t-test or Mann-Whitney U test were appropriate. Correlations between ΔDAS28 and induction of IFN type I response score (ratio t3/t0) were tested using Pearson correlation test.

Table 1. baseline characteristics rituximab treated RA patients

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Test cohort (n=13)</th>
<th>Validation cohort (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>55 ± 11</td>
<td>54 ± 10</td>
</tr>
<tr>
<td>female, %</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>Disease characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA duration, yrs</td>
<td>11 (4 – 21)</td>
<td>5 (3 – 15)</td>
</tr>
<tr>
<td>DAS28-score</td>
<td>5.6 ±1.0</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>ESR, mm/hr</td>
<td>12 (7 – 26)</td>
<td>22 (14 – 35)</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>8 (3 – 16)</td>
<td>18 (7 – 35)</td>
</tr>
<tr>
<td>IgM RF positive, %</td>
<td>70</td>
<td>78</td>
</tr>
<tr>
<td>ACPA positive, %</td>
<td>77</td>
<td>67</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous biologicals, n</td>
<td>2 (1 – 3)</td>
<td>2 (1 – 3)</td>
</tr>
<tr>
<td>&gt; 1 Biological in history, %</td>
<td>54</td>
<td>56</td>
</tr>
<tr>
<td>Previous DMARDs, n</td>
<td>4 (4 – 5)</td>
<td>3 (3 – 4)</td>
</tr>
<tr>
<td>Current prednisolone use, %</td>
<td>77</td>
<td>89</td>
</tr>
<tr>
<td>Prednisolone dosage, mg/day</td>
<td>10 (4 – 14)</td>
<td>10 (6 – 11)</td>
</tr>
<tr>
<td>Current MTX use, %</td>
<td>62</td>
<td>67</td>
</tr>
<tr>
<td>MTX dosage, mg/wk</td>
<td>7.5 (0 – 22.5)</td>
<td>20 (0 – 25)</td>
</tr>
<tr>
<td>Current SSZ use, %</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>Current HCQ use, %</td>
<td>23</td>
<td>0</td>
</tr>
</tbody>
</table>

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; ACPA: anti-cyclic citrullinated protein; DMARD: disease modifying anti-rheumatic drug; MTX: methotrexate; SSZ: Sulphasalazine; HCQ: hydroxychloroquine. Continuous variabels are presented as mean with standard deviation or median with interquartile range when appropriate.
RESULTS

Pharmacological response to rituximab treatment in RA

To determine the pharmacological effects of rituximab we analyzed the changes in peripheral blood gene expression profiles of 13 RA patients at baseline, and 3 and 6 months following the start of treatment. Baseline characteristics are shown in table 1. To search for single genes that were significantly regulated in all patients after three months of treatment with rituximab we applied two-class paired analysis using Statistical Analysis of Microarrays (SAM) at a False Discovery Rate (FDR) of less than 5% between pre- and post- therapy (table 2). The analysis revealed 16 B-cell specific genes that were significantly downregulated in all patients, confirming observations by others of an effective and selective B-cell depletion after three months of therapy.[9;20;21] All patients reached a comparable low level of expression of B-cell related genes, indicative that the pharmacological depletion at 3 months was reached irrespective of the clinical response status. Accordingly, pathway level analysis using Gene Set Enrichment Analysis (GSEA), identified “B-cell-mediated immunity” as the only significantly downregulated pathway (p=0.0020). At six months following therapy only 6 of the B-cell related genes were still significantly down regulated. At both time points, no genes were significantly upregulated. These data are indicative for a gradual rise in B-cell markers from 3 to 6 months following the start of therapy. These findings were confirmed by CD19 based FACS analysis (data not shown). Altogether, this indicates that under the influence of rituximab only changes in B-cell related processes were consistently regulated in all patients.

Table 2. Significantly downregulated genes by peripheral blood cells during rituximab treatment at 3 months (t=3) and 6 months (t=6) after the start of therapy.

<table>
<thead>
<tr>
<th>Downregulated genes</th>
<th>Downregulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>T=0 vs T=3</td>
<td>T=0 vs T=6</td>
</tr>
<tr>
<td>LOC642113</td>
<td>FCRLA</td>
</tr>
<tr>
<td>CD19</td>
<td>CD19</td>
</tr>
<tr>
<td>CD79A</td>
<td>CD79A</td>
</tr>
<tr>
<td>VPREB3</td>
<td>VPREB3</td>
</tr>
<tr>
<td>CD79B</td>
<td>CD79B</td>
</tr>
<tr>
<td>IGLL1</td>
<td></td>
</tr>
<tr>
<td>T=0 vs T = 3</td>
<td>T=0 vs T=6</td>
</tr>
<tr>
<td>FCRLA</td>
<td>LOC652493</td>
</tr>
<tr>
<td>HLA-DOB</td>
<td>LOC647450</td>
</tr>
<tr>
<td>LOC652694</td>
<td>BLK</td>
</tr>
<tr>
<td>LOC90925</td>
<td>CXCR5</td>
</tr>
<tr>
<td>LOC590925</td>
<td>CD72</td>
</tr>
<tr>
<td>LOC653800</td>
<td></td>
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</tbody>
</table>
Variation in the pharmacological response to rituximab between RA patients

Given the heterogeneous nature of RA and the relative low number of differentially regulated genes in the group-based analysis we questioned how consistent the pharmacological response to rituximab was between RA patients. Therefore, we analyzed the pharmacological effects of each individual patient by comparison of the ratio of the post- (3 months) vs pre-therapy expression level for each gene (log-2 ratios). To search for differences in the pharmacological response between patients 154 genes were identified that revealed at least a two-fold difference in the rituximab-induced response in at least 3 patients. Cluster analysis categorized these 154 genes in 6 clusters (A, B, C, D, E and F, figure 1) that were differentially regulated by rituximab among RA patients.

MetaCore network analysis revealed that cluster A is characterized by the expression of genes involved in Interferon (IFN)-signaling (e.g. MxA, IFIT3, IFI44, RSAD2) and relates to antiviral actions of interferon. Genes mainly involved in translation (e.g. EEF1A1, RPL15, RPL39, RPS3a), MIF- and T-cell receptor signaling (e.g. CD8, ITK) and NK-cell cytotoxicity (e.g. ITPR3, CD2) were characteristic of cluster B. Cluster C represents genes involved in B-cell immunology (e.g. CD79a, CD79b, CD19, Igk chain V). Genes involved in ECM-remodeling (e.g. CTSG) and iron transport (e.g. LTF, LCN2) are characteristic for cluster D. Cluster E involves genes related to several processes including chemotaxis (e.g. CXCR1, CXCR2, FPR1), cell adhesion and angiogenesis. Finally, cluster F consisted of genes cytoskeleton and coagulation. Essentially similar genes are regulated at the 3 to 6 months time period albeit that the extend of the changes was not related to the 0 to 3 months time period. Altogether, these analyses show that pharmacological responses in RA patients under the influence of rituximab treatment are highly heterogeneous between patients.

Pharmacodynamics in relation to clinical response

Next we investigated the pharmacological differences between patients in relation to clinical response. Therefore, patients were stratified based on changes in Disease Activity Score (ΔDAS) at 6 months after the start of therapy in good responders (ΔDAS >1.2; n=7) and non-responders (ΔDAS <1.2; n=6) (figure 1B and 1C). Subsequently, we performed a cluster analysis using the set of 154 genes to search for genes that were differentially regulated by rituximab between responders and non-responders. Remarkably, the analysis revealed a selective increase in the expression of only cluster A genes (type I IFN-response genes) at 3 months following the start of rituximab therapy in those patients who had a good clinical response, whereas the ones with a similar or decreased expression of these genes exhibited a poor response. This association was most prominent for genes that constitute a subcluster of 6 genes consisting of RSAD2, IFI44, IFI44L, HERC5, LY6E and Mx1,
which were used for further analyses. Subsequently, we investigated the relationship between the rituximab-induced changes in the expression of this gene set and the clinical response status. Therefore, the expression levels of IFI44, IFI44L, HERC5, RSAD2, LY6E and Mx1 were averaged for each individual patient at each time point to reach an IFN type I response score. Rituximab-induced changes in the IFN type I response score over the 3 month time period, expressed as the ratio at 3 months vs. the baseline score (ratio $t_3/t_0$), were compared between the responders and non-responders and revealed a significant increase in the IFN-type I response score in the responders compared to the non-responders ($p=0.0492$, figure 2A).

Excluding seronegative patients from the analysis yielded essentially similar results ($p=0.05$, data not shown). Division of patients into two groups based on a cut-off of 1.1 fold induction (0.15 log2-based) of IFN-type I response activity resulted in a clear separation of good responders (high ΔDAS) and non-responders (low ΔDAS) ($p=0.0040$, figure 2B).

In addition, a trend was observed for the correlation between improvement in ΔDAS and the increase in IFN-type I response score (ratio $t_3/t_0$) ($p=0.09$, data not shown). Accordingly, similar results were observed when response status was assessed by the EULAR response criteria in good responders (n=4), intermediate responders (n=4) and non-responders (n=5) ($p=0.048$, figure 2C). The change in IFN-response score during rituximab therapy negatively correlated with the corresponding baseline level, although no significance was reached ($p=0.0576$ and $R=-0.53$). The IFN-response score returned to baseline values at 6 months after the start of therapy (figure 3). We also studied the relationship between the type I IFN-signature and clinical and laboratory parameters that are listed in table 1. This analysis revealed no associations between the baseline type I IFN-response activity and laboratory parameters. This is in line with results from other studies that reported on the absence of a relationship between the type I IFN-signature and clinical and laboratory parameters. [20-22] Also no significant associations were observed when we studied the relationship between IFN type I response activity and ESR/CRP as continuous variables at baseline and at 3 and 6 months (data not shown). Moreover, no association of the time between the last anti-TNF injection and start of rituximab treatment (at least 4 weeks), and baseline IFN-score or induction (ratio $t_3/t_0$) was observed, indicative that our results are not influenced by differences in the delay between treatments.

To provide further evidence that differential regulation of type I IFN-response activity during rituximab is associated with the clinical response status, we tested an independent cohort of 9 patients (5 non-responders and 4 responders based on ΔDAS). Therefore, we measured the expression of RSAD2, which is a representative IFN type I response gene that has an excellent correlation with the mean expression IFN type I score at baseline and 3 months after the start of
therapy (Pearson r=0.97; p=< 0.0001). Analysis of only RSAD2 expression data (ratio t3/t0) for the analysis of pharmacological differences between ∆DAS28-based responders and non-responders in the test cohort revealed essentially similar results compared to usage of the 6 genes set (p = 0.0406). Analyzing the t3/t0 ratio of RSAD2 in the validation cohort revealed a significant increase in the responders compared to the non-responders after 3 months of therapy (p=0.0317, figure 2D). Moreover, a correlation was observed between improvement of ∆DAS and increase in RSAD2 expression (ratio t3/t0) (p=0.045). These results confirm that differential regulation of type I IFN-response activity during rituximab is associated with the clinical response status. Thus, whereas rituximab depletes B-cells in all patients treated irrespective of their clinical response, our data show that a drug-induced increase of type I IFN-response activity is associated with clinical response (figure 3).

Figure 1. Cluster diagrams of genes that were differentially regulated by rituximab between RA patients. (A) Unsupervised (two-way) hierarchical cluster analysis of induced gene expression levels (ratio t3/t0) of a set of 154 genes revealed a marked interindividual variation in the pharmacological response to rituximab between RA patients. A total of 6 clusters of genes were differently regulated at 3 months following the start of rituximab therapy between patients. Pathway level analysis revealed that the clusters contained genes related to type I IFN biology (cluster A), Protein translation, MIF- and TCR-signaling and NK-cell cytotoxicity (cluster B), B-cell immunology (cluster C), ECM modeling and connective tissue degradation (cluster D), chemotaxis, adhesion and S100 family proteins (cluster E). Cluster F consisted of many genes with unknown function which together could not be classified into a pathway. (B) Supervised (one-way) cluster analysis revealed a set of type I IFN-response genes associated with clinical outcome. Patients were stratified based on changes in Disease Activity Score (∆DAS) at 6 months after the start of therapy. (C) Cluster of type I IFN-response genes, which is related to clinical responder status.
Figure 2. Differential regulation of type I IFN-response gene activity upon rituximab therapy in relation to clinical responder status. The expression levels of type I IFN-response gene activity were determined by cDNA-microarray and qPCR analysis in peripheral blood cells of RA patients before (t=0) and 3 months after (t=3) rituximab treatment. For each patient the expression of the IFN type I response gene activity at baseline (t0) and after 3 months after the start of rituximab treatment (t3) was determined and the ratio (t3/t0, log2 space) was determined. Data are shown as box-plots; each box showed the 25th to 75th percentiles. (A) The expression levels of 6 type I IFN-response genes (IFI44, IFI44L, HERC5, RSAD2, LY6E and Mx1) were determined by cDNA-microarray analysis in peripheral blood cells of 13 RA patients at t0 and t3. Students T-test analysis revealed a significant increase in the expression of the type I IFN-response score (ratio t3/t0) in responders compared to non-responders based on $\Delta$DAS > or < 1.2 ($p=0.0492$). (B and C) Patients were divided into two groups based on rituximab induced changes in type I IFN-response gene (IFI44, IFI44L, HERC5, RSAD2, LY6E and Mx1) activity (ratio t3/t0) at a cut-off of 1.1-fold (= 0.15 log2 based). The groups were compared to each other with respect to $\Delta$DAS improvement (B) or EULAR responder status (C). The 1.1-fold (= 0.15 log2 based) cut-off point marked a significant difference between responders and non-responders ($p=0.0040$ for $\Delta$DAS and $p = 0.0336$ for EULAR responder status). (D) The expression levels of RSAD2 were determined by qPCR in peripheral blood cells from RA patients of an independent validation cohort (n=9) at t0 and t3. For each patient the level induction (ratio t3 / t0, log2 space) was calculated. MannWhitney U test analysis revealed a significant increase in the expression of RSAD2 in responders compared to non-responders based on $\Delta$DAS > or < 1.2 ($p=0.0317$).
Figure 3: Pharmacodynamics of the IFN type I-response activity during rituximab treatment reveals marked differences between responders and non-responders. Shown are pharmacodynamic measurements in 13 RA patients of a set of 6 type I IFN response genes (IFI44, IFI44L, HERC5, RSAD2, LY6E, and Mx1) (left y-axis) and B-cell counts based on CD19 cytometry (right y-axis) at baseline, 3 (t3) and 6 months (t6). Patients were stratified in responders and non-responders based on ΔDAS (A) and EULAR (B) criteria. Pharmacodynamic analyses of the IFN type I-response activity during rituximab treatment revealed marked differences between responders and non-responders for baseline IFN-response activity (ΔDAS p = 0.0052; EULAR p = 0.016, respectively) and the rituximab induced increase in IFN type I activity at 3 months (ratio t3/t0) (ΔDAS p = 0.049; EULAR p = 0.048, respectively). The change in IFN-response gene activity during rituximab therapy negatively correlated with the corresponding baseline level, although no significance was reached (p = 0.0576= and R = -0.53).
DISCUSSION

Pharmacogenomic analyses demonstrated that despite the overall decrease in the expression of B-cell markers RA patients exhibited interindividual differences in their pharmacological responses upon rituximab therapy. Among these we observed a difference in the pharmacodynamics of only one gene signature during rituximab treatment between responders and non-responders. This signature represents type I IFN-response genes. Responders exhibited an increase in IFN type I-response activity after three months treatment with rituximab, whereas the IFN type I-response activity remained stable during treatment in the non-responders. The differential response correlated with baseline levels of IFN type I-response genes, which were low in responders and high in non-responders. These findings lead us to conclude that an increase in IFN type I-response activity during rituximab treatment is associated with a favorable response and may provide insight in the biological mechanism underlying the therapeutic response.

RA is a heterogeneous condition that is reflected by a heterogeneous response to therapy. As an exponent of that we observed that periodic analyses of the patients’ blood revealed rituximab-induced interindividual differences in the gene expression profiles. Our results revealed 6 gene signatures that are differentially regulated after rituximab treatment between the patients. These signatures contained gene sets that represent several distinct biological processes, including IFN-response gene activity, humoral immunity, cytotoxic T and NK-cell immunity and chemotaxis. The rituximab induced expression of genes in cluster B (T and NK cell mediated immunity) was inversely correlated with the expression of genes involved in ECM remodeling and connective tissue degradation (cluster D) (r = -0.62, P <0.0233) and cluster E (chemotaxis, cell adhesion) ( r = -0.96, P <0.0001). For SLE patients treated with rituximab an increase in NK-cell activity was found for patients who exhibited a sustained response.[23] Such an association between an increased NK-cell activity and clinical outcome could not be found for RA (data not shown). Differences in the reduction of B-cell numbers have been reported by Dass and colleagues [24], who claimed a significant association between less reduction and a poor clinical outcome. However, our data, based on cytometric and gene expression analysis, did not reveal significant differences in the expression of B-cell markers and genes between responders and non-responders at 3 and 6 months after the start of treatment.

Our data reveal that rituximab-induced differential regulation of only the IFN-response genes correlated with the ΔDAS28 and EULAR outcome. Regarding to pharmacodynamics of rituximab in relation to the type I IFN activity, two interesting observations were made in this study. Firstly, non-responders displayed an activated type I IFN-systems already before the start of treatment, which remains active during treatment. Secondly, good responders have low or absent IFN-response
activity at baseline and develop IFN-response activity during 3 months of therapy that is comparable to that of non-responders. The correlation between baseline type I IFN levels and clinical response is in line with previous findings [20]. Concerning the correlation between baseline level and clinical outcome, a simple explanation could be that the pathogenesis in IFNhigh patients is less dependent on B-cells compared to IFNlow patients. Alternatively, one might speculate that a high IFN-activity is associated with protection from depletion of pathogenic B-cells, especially located in tissues, due to concomitant increased expression of B-cell survival factors such as BAFF/BLyS.[25] Additional effects of IFNs on B-cell differentiation, such as in-situ differentiation in CD20 negative plasma blasts [26], could contribute to diminish the effects of anti CD20-depletion, which processes remain unseen in the peripheral blood compartment.

With respect to the increase of IFN-type I activity we know that regulation of type I IFN production and the consecutive IFN-response activity is initially mediated via the Toll-like receptors (TLRs), which are triggered by exogenous (infectious) agents and endogenous agents, such as nucleic acids and apoptotic/necrotic material.[27] Hence, subsequent release from apoptotic/necrotic material from depleted CD20+ B-cells may promote IFN-production and release, which might selectively take place in the IFNlow patients. Thus, interindividual differences in the regulatory processes involved in the IFN-biology, such as mentioned above, may be held responsible for the rituximab induced in the divergent regulation of IFN-type I activity. Such differences could have its origin in genetic variation in the type I IFN biology. Single nucleotide polymorphisms in several key components of the type I IFN system (such as IRF5, Tyk2, STAT4) have recently been identified to play a role in the differential of the IFN activity in e.g. SLE. [28,29]

The pharmacological induction of type I IFN-activity could be an important factor in the ameliorative effect of B-cell depletion therapy in RA. Such a role for type I IFN-activity in RA is highlighted by Treschow et al. [30], who showed that IFNβ-deficiency prolonged experimental arthritis. Additional evidence for a beneficial effect of type I IFNs in RA has been provided by de Hooge et al. [31] who demonstrated that STAT-1 deficiency resulted in exacerbation of experimental arthritis. Moreover, transfer of IFN-competent FLS was able to ameliorate arthritis in IFNβ-deficient recipients.[30] However, although treatment with recombinant-IFNβ revealed promising results in experimental arthritis, treatment of RA patients with IFNβ has been unsuccessful so far, which may be due to issues with dosing and pharmacokinetics.[32] Conversely, a pharmacological increase in the type I IFN activity by rituximab may lead to disease progression and/or an increase in disease activity in IFN-type I driven diseases such as systemic lupus erythematosus (SLE). Recent randomized, placebo-controlled trials of rituximab failed to meet their primary or secondary clinical endpoints for renal
and nonrenal SLE. [33] Our data suggest that rituximab might be less effective in those SLE patients who experience an increase in their type I IFN response activity levels during rituximab treatment.

A relation between the dynamics of the IFN system and clinical responsiveness has also been reported for infliximab, one of the TNF-blockers.[34] Data from an explorative study in RA revealed that an increase in IFN-response activity during therapy with infliximab is associated with a poor clinical outcome. The dynamics of this response don’t seem to correlate with the baseline IFN-response activity as was seen for rituximab. Moreover, the response pattern in relation to clinical outcome that was observed for infliximab is not in line with that for rituximab. Recent observations indicate that TNF-blockade involves complex crosstalk between TNF and IFN that may exert both stimulating and inhibiting effects on IFN-response activity.[35,36] Different than for rituximab, the differential effects on type I IFN-response activity associated with infliximab are believed to be mainly linked to intrinsic differences in this crosstalk. As a consequence, in the context of infliximab therapy the increased IFN-system seems to accompany a maintenance of inflammatory activity. This is in line with earlier observations that reveal an association between synovial inflammation and IFN-response gene activity.[37] Overall, these findings indicate that the dynamics of the type I IFN-response activity might have different clinical consequences in RA depending on the specific biological, thus consequently type of modulated immune-pathway, used.

In conclusion, our data provide insight in the pharmacological features associated with the clinical outcome of rituximab. An increase in type I IFN during therapy correlated with a favourable clinical outcome in RA. Further studies are required to define the underlying mechanism for this response association.

**Competing interests**

MTN has received research and speaking fees from Roche. WFL is member of advisory board van Roche, Abbott and Shering Plough (MSD). BACD received speaking fees from Pfizer and Abbott. CLV is an inventor on a patent application on the predictive value of IFN type I response activity for the clinical outcome of B cell depletion treatment. SV, HGR, TCTMvdPK, MWJS, BMEvB and AEV have no competing interests to declare.
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