B cells and B cell directed therapies in rheumatoid arthritis: towards personalized medicine
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Citation for published version (APA):
THE RELATIONSHIP BETWEEN THE TYPE I INTERFERON SIGNATURE AND THE RESPONSE TO RITUXIMAB IN RHEUMATOID ARTHRITIS
OBJECTIVE: To analyze the relationship between the type I interferon (IFN) signature and clinical response to rituximab in rheumatoid arthritis (RA) patients.

METHODS: Twenty RA patients were treated with rituximab (cohort 1). Clinical response was defined as decrease in disease activity score evaluated in 28 joints (DAS28) and according to EULAR response criteria at week 12 and 24. In peripheral blood mononuclear cells the presence of an IFN signature was analyzed measuring the expression levels of three interferon response genes by quantitative PCR. After comparison with healthy controls, patients were qualified as IFN high or IFN low. The data were confirmed in a second independent cohort (n = 31). Serum IFNα bioactivity was analyzed using a reporter assay.

RESULTS: In cohort 1, there was a better clinical response to rituximab in the IFN low group. Consistent with this finding, IFN low patients had a significantly stronger reduction in DAS28 and more often achieved an EULAR response at week 12 and 24 compared to IFN high patients in cohort 2. The pooled data showed a significantly greater reduction in DAS28 in IFN low patients at week 12 and 24 compared to the IFN high group and more often a EULAR response at week 12. Accordingly, serum IFNα bioactivity at baseline was inversely associated with the clinical response, although this result did not reach statistical significance.

CONCLUSION: The type I IFN signature negatively predicts the clinical response to rituximab treatment in RA, supporting the notion that IFN signalling plays a role in RA immunopathology.

Introduction
Type I interferons (IFNs) are cytokines that regulate antiviral immune responses. Surprisingly, among a range of auto-antibody associated autoimmune conditions a proportion of patients display a dominant type I interferon (IFN) signature in their peripheral blood mononuclear cells (PBMCs). These diseases include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), dermatomyositis, Sjögren’s syndrome, systemic sclerosis and multiple sclerosis. In several small cohorts of patients with these conditions the presence of a type I IFN signature was associated with the presence and the titer of auto-antibodies. It has therefore been hypothesized that patients with a type I IFN signature suffer from a pathogenetic subset in which type I IFN stimulating auto-antigens enhance the humoral immune response.

Rituximab has shown clinical efficacy in many of these conditions. Most of the evidence has been gathered in RA. However, rituximab does not induce a clinical response in all patients and does not decrease the level of all autoantibodies. Type I interferons have been associated with enhancement of B cell survival, since they stimulate B cell survival directly and through the production of BlyS and APRIL. Therefore, we hypothesized that patients with a type I interferon signature respond less well to rituximab treatment. We
tested this hypothesis in 2 independent cohorts of RA patients commencing rituximab treatment.

PATIENTS AND METHODS

PATIENTS AND TREATMENT PROTOCOL. Patients were included from 2 cohorts of RA patients that were reported previously. Patients had active RA (Disease Activity Score evaluated in 28 joints (DAS28) ≥ 3.2) despite methotrexate treatment. The study protocol was approved by the Ethics Committee of the participating centers (cohort 1: UMCU, cohort 2: AMC); all patients gave written informed consent. Patients were treated with 2 infusions of 1000 mg rituximab (day 1 and 15). Pre-medication with methylprednisolone was omitted in the AMC cohort as previously described. In both cohorts the DAS28-ESR was used to evaluate disease activity. The clinical response was defined by the decrease in DAS28 at week 24 compared to baseline. Paired serum and PBMC samples were collected at baseline.

BLOOD SAMPLING FOR RNA ISOLATION. Blood was drawn in heparin tubes. PBMCs were isolated using a Ficoll gradient and subsequently stored at liquid nitrogen until RNA isolation. RNA isolation was performed using the RNeasy mini kit (#74106 and QIAcube #9001293 (Qiagen, Venlo, the Netherlands). A DNAse digestion step was included in the protocol to remove genomic DNA. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, MA).

REALTIME PCR. RNA (0.5 μg) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers’ instructions. Quantitative realtime PCR (qPCR) was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA) using SybrGreen (Applied Biosystems). Relative expression of OAS1, ISG15 and Mx1 was normalized to 18S RNA expression. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. An IFN score was calculated by adding the relative expression values for the three IFN-inducible genes. We defined which patients showed an increased IFN response gene expression, by calculating the 95% limits of the healthy controls; normal values were defined as the mean expression of the 3 IFN-response genes, plus 1.96 times the standard deviation. An IFN score was calculated by adding the relative expression values for the three IFN-inducible genes. We defined which patients showed an increased IFN response gene expression, by calculating the 95% limits of the healthy controls; normal values were defined as the mean expression of the 3 IFN-response genes, plus 1.96 times the standard deviation.

REPORTER CELL ASSAY FOR TYPE I IFN. The reporter cell assay for type I IFN has been described in detail elsewhere. In this assay, reporter cells were used to measure the ability of patient sera to induce IFN-induced gene expression. The reporter cells (WISH cells, no. CCL-25; American Type Culture Collection) were cultured with 50% patient sera for 6 h and then lysed. cDNA was made from total cellular mRNA and gene expression levels were then quantified using qPCR. Forward and reverse primers for the genes MX-1 (myxovirus resistance 1), PKR (dsRNA-activated protein kinase), and IFIT-1 (IFN-induced protein with tetratricopeptide 1), which are known to be highly and specifically induced by IFNs, were used in the reaction and relative expression determined in comparison to a housekeeping gene control (HPRT1). The type I IFN activity was calculated by determining the mean and standard deviation of the relative expression of each of the three genes in a population of healthy individuals, determining the number of standard deviations above the mean of healthy donors for each of the three genes for each patient sample, and determining the sum of those three standard deviation values.

SERUM ELISAS. Serum levels of IgM-rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA; anti-CCP2 ELISA, Immunoscan RA, Mark 2, Euro Diagnostica, Arnhem, the Netherlands) were determined at baseline and weeks 4, 16, 24 and 36 after treatment. Baseline serum RAFF and APRIL levels were measured by ELISA (R&D and Bender).

STATISTICAL ANALYSIS. The Student’s t test and Mann–Whitney U test were used where appropriate for comparison of baseline data between the two cohorts and IFN high and IFN low patients. Linear and logistic regression analysis was used where appropriate for prediction analyses. The Wilcoxon signed rank test for paired data was used to analyze the decrease in auto-antibodies.
THE TYPE I IFN SIGNATURE IN PBMCs PREDICTS THE CLINICAL RESPONSE TO RITUXIMAB TREATMENT.

As a primary endpoint for analysis we used the clinical response at week 24 as measured by the decrease in DAS28 and EULAR response criteria. To gain more understanding about the dynamics of the clinical response we also analysed the clinical response at week 12.

First, we performed an exploratory analysis in the test cohort, cohort 1. Six of the 20 (30%) patients had an IFN high signature. The decrease in DAS28 after 12 and 24 weeks was markedly lower in the IFN high group compared to the IFN low group, although the difference did not reach statistical significance, possibly due to the relatively small number of patients (mean ± SD: -0.52 ± 0.75 compared to -1.49 ± 1.04; P = 0.056, R² = 0.19 at week 12 and -0.67 ± 0.99 compared to -1.70 ± 1.60; P = 0.15, R² = 0.11 at week 24, using linear regression analysis [Figure, panel A]). When analyzing the EULAR response, less IFN high patients achieved a EULAR response compared to IFN low patients (7 out of 18 versus 11 out of 13; P = 0.059, R² = 0.17 at week 24).

Subsequently, because we observed a comparable pattern in both cohorts, we pooled the data for univariate regression analysis. Using this approach in the test patients, the IFN signature was a significant predictor of the decrease in DAS28 after rituximab treatment (0.71 ± 0.8 for IFN high versus 1.6 ± 0.97 for IFN low; R² = 0.20; P = 0.001 at week 12 and 0.90 ± 1.48 for IFN high versus 1.96 ± 1.43 for IFN low R² = 0.12; P = 0.012 at week 24 [Figure, panel C]). Fewer IFN high patients achieved a EULAR response at week 12 compared to IFN low patients (9 out of 24 versus 20 out of 27; R² = 0.17; P = 0.01), but at week 24 this relationship did not reach statistical significance (EULAR response in 12 out of 24 versus 19 out of 27, respectively; R² = 0.06, P = 0.14).

Analysis of the relationship between the IFN signature and the relative decrease in DAS28 after rituximab yielded similar results (Figure, panel D-F). Using multivariate regression analysis in a forward model, we found that the IFN signature remained a predictor of the decrease in DAS28 to rituximab treatment, even when correcting for SJC at baseline (R² = 0.16, P = 0.003 and R² = 0.09, P = 0.029 at week 12 and 24, respectively). Consistent with these findings, serum IFNα bioactivity negatively predicted the decrease in DAS28, which did not reach statistical significance (P = 0.06, R² = 0.12 at week 12; P = 0.11, R² = 0.09 at week 24) and the achievement of a EULAR response at week 12 (P = 0.055, R² = 0.20 at week 12 and P = 0.24, R² = 0.07 at week 24).

Since type I interferons have been associated with enhancement of B cell survival in the tissues, we explored whether the IFN high signature might be associated with persistence of circulating autoantibodies. The levels of IgM-RF and ACPA decreased significantly in both IFN low and IFN high patients, but the reduction of both IgM-RF and ACPA appeared indeed more pronounced in the IFN low group. In IFN high patients the IgM-RF titer (n = 19) decreased from 490 (15 – 1700) to 434 (87 – 1393) kU/liter (P = 0.002) and the IgG-anti-CCP titer (n = 20) decreased from 490 (15 – 1700) to 434 (87 – 1393) kU/liter (P = 0.004). In IFN low patients the IgM-RF titer (n = 16) decreased from (median [IQR]) 95 (31–248) to 46 (13 – 103) kU/liter (P = 0.002) and the IgG-anti-CCP titer (n = 20) decreased from 490 (15 – 1700) to 434 (87 – 1393) kU/liter (P = 0.004). In IFN low patients the IgM-RF titer (n = 17) decreased from 618 (143–788) to 270 (63 – 396) kU/liter (P = 0.026).
Comparison of patient characteristics between the two cohorts.

<table>
<thead>
<tr>
<th>DEMOGRAPHICS (N=51)</th>
<th>AMC (N=31)</th>
<th>UMC (N=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, no. (%)</td>
<td>24 (77)</td>
<td>12 (60)</td>
<td>0.187</td>
</tr>
<tr>
<td>Age, median (range) years</td>
<td>54 (28-72)</td>
<td>59 (32-70)</td>
<td>0.354</td>
</tr>
</tbody>
</table>

| BASELINE DISEASE STATUS | | | |
|-------------------------| | | |
| IgM-RF positive, no. (%) | 26 (84) | 17 (85) | 0.915 |
| ACPA positive, no. (%)  | 28 (90)  | 15 (75)  | 0.177 |
| DAS28, mean (± SD)      | 6.4 ± 1.1 | 6.4 ± 1.1 | 0.985 |
| ESR, median (range) mm/hour | 37 (4-86) | 46 (7-124) | 0.189 |
| CRP, median (range) mg/dl | 20 (2-112) | 26 (7-110) | 0.839 |
| Disease duration, median (range) years | 12 (1-30) | 13 (3-50) | 0.429 |
| Symptomatic secondary Sjögren’s syndrome (%) | 3 (10) | 2 (10) | 0.99 |

Comparison of clinical and biological characteristics between IFN high and IFN low patients.

<table>
<thead>
<tr>
<th></th>
<th>IFN high (n=24)</th>
<th>IFN low (n=27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon serum bioactivity, median [IQR [n=28]]</td>
<td>0.49 (0.0 – 0.99)</td>
<td>0.00 (0.0 – 0.46)</td>
<td>0.02</td>
</tr>
<tr>
<td>DAS28, mean (± SD  [n = 51])</td>
<td>6.2 ± 1.0</td>
<td>6.6 ± 1.0</td>
<td>0.16</td>
</tr>
<tr>
<td>Swollen joint count, mean ± SD  [n = 51]</td>
<td>9 ± 5</td>
<td>14 ± 7</td>
<td>0.013</td>
</tr>
<tr>
<td>Tender joint count, mean ± SD  [n = 51]</td>
<td>14 ± 7</td>
<td>15 ± 6</td>
<td>0.43</td>
</tr>
<tr>
<td>VAS disease activity, mean (± SD [n = 51])</td>
<td>64 ± 23</td>
<td>69 ± 16</td>
<td>0.64</td>
</tr>
<tr>
<td>ESR, median (range [n=51]) mm/hour</td>
<td>38 (19-55)</td>
<td>45 (21-61)</td>
<td>0.50</td>
</tr>
<tr>
<td>CRP, median (range [n=51]) mg/dl</td>
<td>19 (5-112)</td>
<td>269 (2-117)</td>
<td>0.38</td>
</tr>
<tr>
<td>IgM-RF positive, no. (%) [n=51]</td>
<td>21 (88)</td>
<td>22 (81)</td>
<td>0.56</td>
</tr>
<tr>
<td>ACPA positive, no. (%) [n=51]</td>
<td>20 (86)</td>
<td>23 (83)</td>
<td>0.56</td>
</tr>
<tr>
<td>IgM-RF titer, median (range [n=51]) IU/l</td>
<td>81 (2-1128)</td>
<td>62 (2-1128)</td>
<td>0.57</td>
</tr>
<tr>
<td>ACPC titer, median (range [n=51]) IU/l</td>
<td>352 (0-6958)</td>
<td>240 (0-1366)</td>
<td>0.31</td>
</tr>
<tr>
<td>Serum BLyS level [n=28]</td>
<td>1265 (1093-1518)</td>
<td>1246 (964-1636)</td>
<td>0.76</td>
</tr>
<tr>
<td>Serum APRIL [n=28]</td>
<td>11 (6-24)</td>
<td>24 (14-50)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* IFN = interferon; IQR = interquartile range; IgM-RF = IgM rheumatoid factor; SD = standard deviation; ACPA = anti-citrullinated peptide antibodies; DAS28 = Disease Activity Score 28-joint assessment; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein, BLyS = B Lymphocyte Stimulator; APRIL = A Proliferation Induced Ligand.
In this study we found that patients with a type I IFN signature in PBMCs respond less well to rituximab treatment than patients without an IFN signature. A possible confounder in this study could have been the lower baseline disease activity, as measured by the swollen joint count, in IFN high patients. However, we found that the IFN signature also predicted the decrease in DAS28 when we controlled for the swollen joint count in a multivariate regression model. We found a more pronounced difference in the clinical response between IFN low and IFN high patients at week 12 compared to week 24 after rituximab treatment. This may be caused by the relatively small size of the patient cohorts. Alternatively, IFN high patients may respond more slowly to rituximab than IFN low patients.

The data presented here suggest that the type I IFN signature is not just an epiphenomenon in RA, but might be involved in its pathogenesis. RA is traditionally viewed as a TNFα driven disease. This concept is amongst others based on the effectiveness of TNFα blocking agents in RA and results from genetic studies in which an association was found between TNFα related genes and ACPA positive RA versus type I IFN related genes and ACPA negative RA. According to this model ACPA positive RA is predominantly driven by TNFα, based on in vitro observations that TNFα downmodulates the effects of type I IFNs and vice versa. However, the results from the current study, together with other observations, contradict this model.

First, in a recent genome wide association study both single nucleotide polymorphisms in TNF related genes and IFN related genes were associated with autoantibody positive RA. Furthermore, TNFα, IFNα and IFNβ are simultaneously expressed in RA synovial tissue. In line with these data we found that plasma TNFα levels were at least equal in IFN high compared to IFN low patients (data not shown). Taken together, these data suggest that the presence of TNFα and type I IFNs is not mutually exclusive. Instead of the single-cytokine based paradigm the data support a concept of multiple immune mechanisms that act simultaneously in RA.

In contrast to our hypothesis, we could not find a direct relationship between the response to rituximab, the IFN signature, increased levels of APRIL and BlyS and persistence of autoantibodies. This is supported by available data in the literature, which suggest a complex contribution of type I IFNs to RA pathogenesis and response to therapy. The production of type I IFN is not only stimulated by viruses, but also by apoptotic/necrotic material, RNA binding proteins, osteopontin and autoantigen-containing immune complexes. Furthermore, gene polymorphisms may contribute to excessive IFN signalling to subphysiologic activating stimuli. Subsequently, type I IFNs may not only stimulate the production of APRIL and BlyS and directly enhance B cell survival but also stimulate T cells and dendritic cells. At the same time, IFNβ is able to reduce the secretion of pro-inflammatory cytokines like IL-6, matrix metalloproteinases (MMPs), and prostaglandin E2 by fibroblast-like synoviocytes. In addition, IFNβ has anti-angiogenic properties and may inhibit osteoclastogenesis.
The relationship between the type I IFN signature and the humoral autoimmune response in RA was analyzed in a number of previous studies. In a first study the type I IFN signature was found in a subset of RA patients and correlated with autoantibody levels, similar to findings in other autoantibody associated diseases. Moreover, the type I IFN signature was identified in a subset of autoantibody positive arthralgia patients who later developed RA. A direct relationship between the IFN signature and autoantibody responses in RA could not be confirmed in a subsequent investigation in a larger cohort, in whom the IFN signature was found to occur equally often in seropositive and seronegative RA patients. However, the presence of an IFN signature was associated with persistence of ACPA after TNF blockade. Taken together with the association between polymorphisms in IFN-related genes and both seropositive and seronegative RA, the current and previous data suggest that IFN related autoimmunity does not directly determine humoral autoimmunity in RA, but may promote and sustain humoral autoimmunity responses in seropositive patients.

In RA the level of type I IFN bioactivity has been associated with the clinical response to TNFα blockade, although these results need to be replicated since they have been variable for different cohorts. Pilot data from an RA patient cohort treated with TNF antagonists suggested that relatively high plasma levels of type I IFN activity prior to initiation of therapy were associated with better clinical response than those agents that lower plasma type I IFN activity. Patients with high type I IFN activity may respond better to TNF blockade because of the anti-inflammatory effects of their disease-associated high IFNβ levels. Alternatively, patients with an IFN signature may have an overall higher inflammatory activity than IFN low patients and may respond better to TNF blockade because of higher TNFα activity. This is in line with the earlier finding that patients with higher levels of synovial inflammation and synovial TNFα expression respond better to TNF blockade. The concept of a lower inflammatory activity in IFN low patients is supported by the fact that IFN low patients do not show an alternative gene activation signature in their PBMCs, but have a gene signature comparable to healthy controls.

How can we explain the finding that patients with an IFN high signature respond less well to rituximab? First, IFN high patients could have a disease that is less B cell-dependent. This appears unlikely, since all patients in our study were RF and/or ACPA positive. Furthermore, current evidence suggests non-response to rituximab is associated with persistence of B cells and the deleterious effects of rituximab. Although we found similar systemic levels of BLyS and APRIL in IFN high and IFN low patients, type I IFNs may promote survival of pathogenic B cells in lymphoid tissue or bone marrow. This might involve contact-dependent interactions with T cells, dendritic cells and stromal cells and membrane-bound survival factors like CXCL12, VCAM, membrane-bound BLyS or heparan-sulphate bound APRIL. With regard to the clinical response to rituximab, although BLyS and APRIL are thought to rescue B cells from B cell depletion, systemic levels of BLyS and APRIL do not predict clinical response to rituximab. Nonetheless, the reason for the decreased clinical response to rituximab in IFN high patients remains speculative until more data become available.

The decreased clinical response to rituximab in IFN high RA patients seemingly contradicts the clinical responsiveness of IFN-associated diseases to rituximab. However, the presence of high type I IFN activity does not necessarily imply that these patients will respond better to rituximab treatment. In contrast, recent randomized controlled trials failed to show clinical efficacy of rituximab in patients with lupus nephritis.
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