How is autoimmunity against citrullinated proteins regulated?
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Type I interferons do not influence humoral autoimmunity in rheumatoid arthritis.

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Submitted for publication
Abstract

Objectives

As type I interferons (IFN) have recently been implicated in autoantibody-mediated diseases such as systemic lupus erythematosus and as half of the rheumatoid arthritis (RA) patients display a type I IFN\textsuperscript{high} signature, we investigated if type I IFN determine the autoantibody response in RA.

Patients and methods

Serum and peripheral blood mononuclear cells were obtained from 21 RA patients at week 0 and 24 of infliximab treatment, from 8 anti-citrullinated protein antibody (ACPA) positive individuals without arthritis, and from 10 ACPA negative healthy controls. The type I IFN signature was determined by peripheral blood cell gene expression analysis. ACPA IgG and IgM, rheumatoid factor (RF) IgM, anti-nucleosome IgM and anti-ds DNA were measured by ELISA.

Results

Nine RA patients had a type I IFN\textsuperscript{low} signature and 12 RA patients had a type I IFN\textsuperscript{high} signature. This signature was not related to the presence and titers of ACPA and RF during active disease. TNF blockade by infliximab induced a similar clinical response, a similar induction of anti-nuclear antibodies, and a similar decrease in RF titers in both groups. However, ACPA IgG and IgM levels were exclusively downmodulated in the type I IFN\textsuperscript{low} group. In contrast to the situation in RA after TNF blockade, the ACPA response in individuals without arthritis and inflammation was not related to an increase of type I IFN.

Conclusions

Type I IFN do not determine the humoral autoimmune response in RA, with exception of the persistence of ACPA levels after TNF blockade.
CHAPTER 3

Introduction

The family of the type I interferons (IFN) encompasses more than 20 related members which are mainly produced by plasmacytoid dendritic cells (1) and fibroblasts and mediate the host defence against viral pathogens (2). Over the last years, compelling evidence indicates that type I IFN also play a crucial role in autoimmune diseases such as systemic lupus erythematosus (SLE) (3-5). Firstly, peripheral blood mononuclear cells of SLE patients display a strong type I IFN expression profile (6;7). These findings are backed up by the association of genetic regulation of type I IFN with SLE (8;9). Secondly, activation of the type I IFN system correlates with disease activity and autoantibody titers in this condition (10-14). Thirdly, a direct causal relationship between type I IFN and systemic humoral autoimmunity has been formally proven in several experimental models of SLE (15-17) but also in humans as IFN alpha treatment can induce autoantibodies and lupus-like disease in non-autoimmune prone individuals (18).

Similar findings in other autoantibody-related diseases such as Sjögren’s syndrome (19) and dermatomyositis (20) suggest that the role of type I IFN is not restricted to SLE but may apply more generally to systemic humoral autoimmunity. In RA, a genetic association with interferon regulatory factor-5 is reported (21). We recently also described a type I IFN signature in a subset of patients with rheumatoid arthritis (RA) (22;23): approximately half of the RA patients showed a clear upregulation of type I IFN responsive genes in peripheral blood cells in comparison with healthy controls. The role of type I IFN in humoral autoimmunity in other systemic autoimmune diseases raises the question if this signature determines the serological profile in RA and, more specifically, the presence and/or levels of anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF).

Of particular interest in this context is the cross-regulation between type I IFN and TNF alpha, a key cytokine in RA. IFN beta downmodulates TNF alpha production in vitro (24;25) as well as experimental arthritis in vivo (26;27). Inversely, TNF alpha suppresses IFN alpha production by plasmacytoid dendritic cells in vitro (28) and blockade of TNF alpha in vivo leads to an increased activation of the type I IFN pathways in juvenile chronic arthritis and Sjögren syndrome (28;29). Therefore, we additionally investigated the role of type I IFN in the regulation of autoantibody levels during TNF blockade and in ACPA positive individuals without arthritis or inflammation.
Patients and methods

Patients

The study included 21 patients fulfilling the American College of Rheumatology criteria for RA (30) and having active disease defined as a Disease Activity Score using the 28 joint score (DAS28) of more than 3.2 despite adequate treatment with methotrexate (31). Peripheral blood was collected at baseline in Paxgene RNA isolation tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland). Serum was collected at baseline and after 24 weeks of infliximab treatment (3 mg/kg at week 0, 2, 6, 14, and 22). Clinical assessments included the DAS28 score as well as individual variables such as the swollen joint count, serum C-reactive protein (CRP) levels, and the erythrocyte sedimentation rate (ESR). The DAS28 was used to define clinical responders according to the EULAR response criteria (32).

We additionally included 10 ACPA negative healthy controls and 8 individuals with positive ACPA titers but without arthritis. The latter either presented with arthralgia without any clinical evidence for arthritis and a serum CRP level of less than 5 mg/L at our outpatient clinic (n = 5), or were first degree healthy relatives of RA patients (n = 3). Individuals with current or previous disease-modifying anti-rheumatic drug (DMARD) use or with a history of arthritis were excluded. Paired serum and PAXgene blood samples were collected.

All subjects provided written informed consent to participate in the study as approved by the Medical Ethical Committee of the Academic Medical Center/University of Amsterdam. Patient characteristics are summarized in Table 1.

Blood sampling for RNA isolation

2.5 ml blood was drawn in PAXgene blood RNA isolation tubes (PreAnalytix, GmbH, Germany) and stored at -20°C. Tubes were thawed for 2 hours at room temperature prior to RNA isolation. Next, total RNA was isolated using the PAXgene RNA isolation kit according to the manufacturers’ instructions including a DNAse (Qiagen, Venlo, Netherlands) step to remove genomic DNA.

Type I IFN signature measurement

As described previously (22), mRNA was extracted from peripheral blood cells of the 21 RA patients and analysed by microarray. The type I IFN pathway activation
was determined by calculating the average expression of 43 genes belonging to a type I IFN responsive gene cluster (further referred to as the continuous type I IFN measurements). The type I IFN\textsuperscript{high} signature was defined as a type I IFN pathway activation exceeding the 95% confidence interval for normal values as assessed in healthy individuals (22). This signature has been validated previously and correlated well with the expression of type I IFN induced genes as measured by realtime PCR (22).

### Realtime PCR

RNA (0.5 µg) was reverse transcribed into cDNA using the Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers’ instructions. Quantitative realtime PCR was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA) using SybrGreen (Applied Biosystems). Primers were designed using Primer Express software and guidelines (Applied Biosystems) and primer sequences are listed in Table 2. A standard curve was constructed in order to calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies. Expression levels
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<thead>
<tr>
<th>Symbol</th>
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Table 2: Primer sequences of primers used in quantitative realtime PCR.

of target genes were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Autoantibody measurements

ACPA IgG was measured by the anti-CCP2 ELISA kit (Eurodiagnostica, Arnhem, The Netherlands, cutoff 25 U/ml). In 3 patients (1 type I IFN$^\text{low}$ and 2 type I IFN$^\text{high}$) ACPA IgG titers could not be determined. ACPA IgM was measured with the anti-CCP2 ELISA kit after substitution of the secondary antibody with a goat anti-human
IgM antibody labelled with HRP (Novus Biologicals, Littleton, CO) diluted 1/1000 in PBS. For this modified ELISA, values are expressed as relative units using a dilution curve of a high positive sample as reference. RF IgM was determined with the human IgM RF kit (Sanquin, Amsterdam, The Netherlands, cutoff 12.5 U/ml). Anti-nucleosome antibodies were determined with the anti-nucleo ELISA (Generic Assays, Dahlewitz, Germany) substituting the secondary antibody with the goat-anti human IgM antibody labelled with HRP (Novus) diluted 1/1000 in PBS. Anti-dsDNA antibodies were measured with the anti-ds DNA IgM ELISA (Generic Assays). All ELISAs were performed in duplo according to the manufacturer’s instructions.

Statistics

Since the data were not normally distributed, the results were expressed as median and interquartile range. Accordingly, the Mann-Whitney U test and the paired Wilcoxon signed-rank test were used for comparison of unpaired and paired data, respectively. As the size of the groups was relatively small, these non-parametric tests exclude major influences of single outliers in the analysis. Correlations were calculated with the Spearman’s Rho test. Statistical significance was set at P < 0.05.

Results

Serum ACPA and RF levels are not associated with the type I IFN signature

As previously described, the type I IFN pathway activation was determined by calculating the average expression of 43 genes belonging to a type I IFN responsive gene cluster. The type I IFN\textsuperscript{high} signature was defined as a type I IFN pathway
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Of the 21 RA patients included in this study, 9 had a type I IFN<sub>low</sub> signature and 12 a type I IFN<sub>high</sub> signature. The levels of ACPA IgG (611 [166-791] U/ml versus 305 [36-868] U/ml), ACPA IgM (7.4 [5.2-31.6] relative U/ml versus 7.6 [3.4-26.4] U/ml), as well as RF IgM (29 [14-142] U/ml versus 37 [15-52] U/ml) were not different between the type I IFN<sub>low</sub> and the type I IFN<sub>high</sub> group (Figure 1). Accordingly, there was no difference between the type I IFN<sub>low</sub> group and the type I IFN<sub>high</sub> group in terms of seropositivity for ACPA IgG (9/9 versus 11/12) or RF IgM (7/9 versus 10/12) using the diagnostic cut-off levels (25 U/ml and 12.5 U/ml, respectively). Continuous rather than dichotomous analysis of the type I IFN expression data confirmed that there was no correlation with the serum levels of these autoantibodies (data not shown).

The type I IFN signature does not determine the clinical response to TNF blockade

As indicated in our previous report (22), there were no differences in disease activity (swollen joint count, CRP, ESR, and DAS) or disease duration between the type I IFN<sub>low</sub> and the type I IFN<sub>high</sub> group at baseline (Table 1). As all patients had active disease despite adequate treatment with methotrexate, treatment was started with infliximab. In agreement with previous reports, this treatment induced a significant decrease of the DAS28 (P < 0.001), the swollen joint count (P < 0.001), serum CRP levels (P < 0.001), and ESR (P = 0.002) between baseline and week 24 (Table 3). Analysis of the clinical response to treatment in function of the baseline type I IFN signature showed that the improvement of the DAS28 as well as of the individual disease activity parameters was similar in both groups (Table 3). Accordingly, 7 out of 9 patients

Table 3: Clinical response to treatment with infliximab. The data are represented as median (interquartile range) decrease between baseline and week 24. The total cohort was subdivided in a type I IFN<sub>low</sub> group and a type I IFN<sub>high</sub> group according to the microarray analysis of type I IFN responsive genes in peripheral blood cells. DAS = disease activity score. CRP = C-reactive protein. ESR = erythrocyte sedimentation rate.
were DAS28 responders (3 moderate responders and 4 good responders) in the type
I IFN\textsuperscript{low} group and 9 out of 12 were DAS28 responders (5 moderate responders and 4
good responders) in the type I IFN\textsuperscript{high} group. This was further confirmed by the lack
of correlation between the continuous type I IFN expression data at baseline and the
DAS28 response over 24 weeks (data not shown). Taken together, these data indicate
that the type I IFN signature at baseline does not determine the clinical response to
TNF blockade.

The type I IFN signature does not determine the induction of anti-nuclear
antibodies during TNF blockade

We and others previously reported the induction of anti-nuclear antibodies and
more specifically anti-dsDNA IgM antibodies during infliximab treatment (33;34). As
type I IFN play an important role in the induction of anti-nuclear antibodies
(ANA) in several animal models and human disease (15;16;18), we investigated
if the baseline type I IFN signature influenced the ANA induction by infliximab
expression in RA. We observed a significant increase in the anti-ds DNA IgM titers
from 3.9 (0-21.7) U/ml to 11.5 (2.2-106.4) U/ml (P < 0.001) between baseline and week
24. This increase was similar in the type I IFN\textsuperscript{low} group, where levels increased from
10.0 (4–34.2) U/ml to 42.1 (3.5–112.1) U/ml (P = 0.047) and the type I IFN\textsuperscript{high} group,
where there was an increase from 4.3 (0–18.8) U/ml to 9.4 (3.5–115.3) U/ml (P = 0.009)
(Figure 2A and B). Accordingly, there was no correlation between the continuous type
I IFN expression data at baseline and the increase of anti-ds DNA IgM titers during
treatment. Similar results were obtained studying the anti-nucleosome antibody
levels (data not shown).

The decrease of RF IgM during TNF blockade is not related to the type I IFN
signature

As previously described in other cohorts (35-37), the RF IgM levels were significantly
downmodulated by 24 weeks of treatment with infliximab (from 33 U/ml to 20 U/ml;
P = 0.020). This decrease was not related to the baseline type I IFN signature as it was
observed in both the type I IFN\textsuperscript{low} (from 29.2 (14.5 – 142.3) U/ml to 20.7 (8.3 – 36.5)
U/ml, P = 0.012) and the type I IFN\textsuperscript{high} (from 36.8 (15.0 – 52.0) U/ml to 27.4 (10.5 – 39.8)
U/ml, P = 0.042) group (Figure 2C and D). Accordingly, there was no correlation
between the continuous values of type I IFN at baseline and the changes in RF IgM
levels over 24 weeks (data not shown).
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The type I IFN signature influences the changes in ACPA levels during TNF blockade

The effect of TNF blockade on ACPA levels remains controversial as some but not all studies show a small but significant decrease of ACPA (35-38). In agreement with our previous studies (36), we found a slight but not significant decrease of serum ACPA levels during 24 weeks of infliximab treatment in the total RA cohort (305 [89-669] U/ml versus 245 [57-490] U/ml). However, analysis in function of the baseline type I IFN signature showed that the ACPA IgG levels were significantly downmodulated in the type I IFN low group (611 [166-792] U/ml versus 344 [100-517] U/ml, P = 0.023) but not in the type I IFN high group (272 [63-716] U/ml versus 245 [58-781] U/ml) (Figure 2).
2E and F). Taken into account the small sample size, a non-parametric statistical test was applied to exclude major influence of single values. To investigate if this differential effect of TNF blockade on RF IgM versus ACPA IgG in relation with the type I IFN signature was related to the difference in antibody system (RF versus ACPA) or the difference in isotype (IgM versus IgG), we additionally investigated ACPA IgM. As shown in Figure 2G and H, we observed a significant decrease of ACPA IgM in the type I IFN\textsuperscript{low} group (7.4 [5.2-31.6] relative units/ml versus 5.1 [4.2-15.8] relative units/ml, P = 0.008), but not in the type I IFN\textsuperscript{high} group (7.5 [3.5-26.2] relative units/ml versus 9.9 [4.8-19.9] relative units/ml). Although the size of the groups is relatively small, these data indicate that ACPA levels, irrespective of their isotype, are sustained only in the type I IFN\textsuperscript{high} group during TNF blockade.

**Type I IFN does not determine ACPA levels in individuals without inflammatory arthritis**

The observation that the type I IFN signature appears to influence the persistence of ACPA levels during TNF blockade but not the ACPA titers at baseline during active disease may relate to the proposed cross-regulation of TNF and type I IFN (26) as the putative relationship between type I IFN and ACPA may be masked and/or alleviated by high levels of proinflammatory factors such as TNF. Therefore, we investigated in ACPA positive individuals without any signs of inflammation or arthritis the association between the ACPA levels and the type I IFN signature. A set of type I IFN-response genes previously identified in the microarray experiments was assessed by quantitative PCR in peripheral blood cells of 8 ACPA positive (serum levels of ACPA IgG as determined by the anti-CCP2 ELISA: 1127 (567 – 2566 U/ml)) and 10 ACPA negative healthy controls. The type I IFN score, calculated as the mean relative expression level of type I IFN inducible genes such as RSAD2, IFI44L, OAS1, ISG15, IFITM3, Mx1, and IRF27, was similar in the ACPA positive and negative group (17.5 (14.4 – 33.6) versus 17.9 (16.0 – 20.8) arbitrary units) (Figure 3A). Accordingly, there was no difference in relative expression level of IFN alpha2 (1.9 (1.1 – 3.9) versus 1.7 (1.5 – 3.7) arbitrary units) and IFN beta (26.4 (8.5 – 46.4) versus 24.7 (20.4 – 33.7) arbitrary units) in the ACPA positive versus ACPA negative group (Figure 3B and C). Taken together, these data do not support the hypothesis that type I IFN are a major determinant of the ACPA response, even in the absence of proinflammatory cytokines.
ACPA and RF are the most specific autoantibodies associated with RA. Intriguingly, even after longer disease duration not all RA patients become seropositive for these autoantibodies. Taken together with the different genetic association in ACPA positive and ACPA negative disease (39-42), this suggests that seropositive and seronegative RA are distinct disease subsets with specific factors controlling the development of the humoral arm of this autoimmune disease. Four lines of circumstantial evidence lead us to the hypothesis that type I IFN may be one of these factors. Firstly, other autoantibody-associated diseases such as SLE and Sjögren’s syndrome are characterized by a strong type I IFN signature. Secondly, we recently demonstrated that also approximately half of the RA patients display this type I IFN\textsuperscript{high} gene expression signature (22). Thirdly, in a different type of gene expression analysis we identified a subgroup of RA patients with a gene expression profile reminiscent of that of poxvirus-infected macaques (43). Of major interest, this RA subgroup showed an enrichment of type I IFN-activated genes as well as increased titers of ACPA.

The present study was thus designed to test directly the association between the type I IFN signature and humoral autoimmunity in a homogenous and well characterized cohort of RA patients with active disease. A first important finding of the study is that in full-blown, active disease neither the presence nor the levels of ACPA or RF are related to the type I IFN signature. However, an intrinsic limitation of this cross-sectional analysis in active, established disease is the potential influence of confounding factors. The reciprocal regulation of TNF alpha, one of the pivotal proinflammatory cytokines in RA, and type I IFN is of particular interest in this context as high TNF levels may suppress or mask the effects of type I IFN (25;28).

**Figure 3:** (A) Mean relative expression level of type I IFN inducible genes in PBMC was not different between ACPA positive and ACPA negative individuals without arthritis. Relative expression levels of RSAD2, IFI44L, OAS1, ISG15, IFITM3, Mx1, and IRF27 were analysed. Relative expression level of the type I IFN score, IFN alpha2 (B) and IFN beta1 (C) in PBMC were not different between ACPA positive and ACPA negative individuals without arthritis.
To assess this issue, we additionally assessed the changes in autoantibody titers after blocking the key inflammatory TNF pathway by infliximab. Of interest in the context of the poor responsiveness of type I IFN-associated diseases such as SLE and Sjögren’s syndrome to TNF blockers (44;45), the clinical response to treatment in RA was similar in the type I IFN<sub>low</sub> and the type I IFN<sub>high</sub> patients. Although these data confirm and extend the lack of association with disease activity or severity in our original cross-sectional study (22), it needs to be emphasized that these pilot studies were neither designed nor powered to address this specific issue and thus larger, prospective studies are still needed to study associations with clinical features in more detail.

As expected from our previous studies (34;36), TNF blockade significantly modulated the autoantibody profiles with a clear induction of antinuclear reactivities, a significant decrease of RF IgM levels, but only a modest effect on ACPA titers. Furthermore, the induction of ANA by infliximab was not related to the type I IFN signature, confirming our recent data in spondyloarthritis (Cantaert et al, unpublished observations). Also the changes in RF were independent of type I IFN. In contrast, the baseline type I IFN signature did influence the ACPA response during infliximab therapy, with a decrease in titers in the type I IFN<sub>low</sub> patients but not in the type I IFN<sub>high</sub> patients. The different behaviour of ACPA compared to antinuclear antibodies and RF was not due to the isotype difference (IgG versus IgM, respectively) as we observed similar findings for ACPA IgM. Although these results should be confirmed in larger patient groups, this suggests that type I IFN could play a role in the persistence of the ACPA response and may provide an explanation for the discrepancies between different cohorts with respect to ACPA modulation by TNF blockade. Recently, polymorphisms in genes related to type I IFN signalling (such as interferon regulatory factor 5 (IRF5) or the dendritic cell immunoreceptor gene (DCIR)) are shown to be more associated with ACPA negative RA than ACPA positive RA (41;42;46), indicating a possible association (either direct or more probably indirect) between ACPA levels and type I IFN.

Our data would fit a model in which ACPA levels in RA depend on the combined positive effects of both type I IFN and TNF. Therefore, the type I IFN signature by itself cannot be related to ACPA levels in the presence of high TNF levels. Given the reciprocal inhibition of type I IFN and TNF, TNF blockade would lead to reduction of ACPA only in those patients with a relative low contribution of IFN to ACPA levels. In contrast, TNF blockade would have only minor effects on ACPA levels in patients with a relative high contribution of type I IFN.

The relation between ACPA and type I IFN after successful TNF blockade but not in active disease raises the question whether type I IFN also determines the ACPA response before the onset of clinical disease and/or inflammation. In an inception cohort of ACPA positive and negative individuals without clinical or biological
signs of inflammation, we found no relation between the ACPA status and the type I IFN signature. Moreover, in contrast to full-blown RA there were no signs of increased type I IFN in the ACPA positive individuals. Although the absence of good experimental models precludes the direct study of type I IFN during the induction of ACPA, these human data indicate that even in an early phase the ACPA response is not related to type I IFN and suggest that the type I IFN signature observed in a subset of RA patients may appear after the induction of the humoral autoimmune response. In the context of the therapeutic effect of type I IFN in experimental arthritis models, it is tempting to speculate that the upregulation of type I IFN is not a primary phenomenon but possibly an abortive attempt to control inflammation (25). An alternative but equally intriguing hypothesis which is now assessed in a prospective, longitudinal study is that type I IFN may determine the progression towards clinical arthritis in ACPA positive individuals (van Baarsen et al, unpublished data).

In conclusion, the present study indicates that the type I IFN profile does not determine humoral autoimmunity in RA, with perhaps the exception of the persistence of ACPA levels after TNF blockade. Importantly, even before the onset of clinical arthritis, the presence of ACPA is not related to elevated type I IFN. Further studies should clarify the pathophysiologic relevance and clinical correlates of the type I IFN signature in half of the RA patients, with special attention for the progression of ACPA positive healthy individual to active RA.

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References


