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Original article

Viral antigens elicit augmented immune responses in primary Sjögren's syndrome

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Abstract

Objectives. Infections have been suggested in the pathogenesis of primary SS (pSS). Systematic studies of immune responses to microbial antigens *in vivo* may be performed during vaccination. In the present study, we therefore longitudinally followed patients with pSS and controls during split-virion influenza vaccination to identify pSS-specific cellular, transcriptomic and serological responses.

Methods. Patients without treatment (pSS_{Untr}, $n = 17$), on hydroxychloroquine-treatment (pSS_{HCQ}, $n = 8$), and healthy controls ($n = 16$) were included. Antibody titres were determined by ELISA. Plasma proteins were measured by proximity extension assay. Monocyte gene expression was assessed by Nanostring. Routine laboratory tests were performed and clinical disease symptoms were registered by questionnaires.

Results. pSS_{Untr} developed higher vaccine-specific IgG titres compared with controls. Notably, anti-Ro52 autoantibody titres increased in pSS_{Untr} but remained unchanged in pSS_{HCQ}. No changes in disease symptoms including EULAR Sjögren's Syndrome Patient Reported Index score were registered. Twenty-four hours after vaccination, the leucocyte count in pSS_{Untr} decreased, with a concomitant increase of CCL7 in plasma. Transcriptomic analysis in monocytes revealed differential vaccination-related expression of the *NEMO/IKBKG* gene, and its higher induced expression in pSS_{Untr} associated with higher serological vaccine responses. Moreover, titres of vaccine-specific antibodies were associated with higher vaccination-induced NF-κB signalling and higher steady-state IFN signatures in monocytes, and with the levels of several plasma proteins with soluble PD-1 displaying the strongest association.

Conclusion. We observed augmented innate and adaptive immune responses in pSS following viral antigen exposure suggesting an underlying hyper-responsiveness to immune challenges, supporting a role for infections driving the immunopathology and acting as environmental risk factor for pSS.

Key words: Sjögren's syndrome, monocytes, interferon, NF-κB, PD-1

Rheumatology key messages

- The immune reaction pattern in autoimmune individuals may be systematically studied *in vivo* following vaccination.
- Patients with Sjögren's syndrome display augmented innate and adaptive immune responses after viral antigen exposure.
- Our data support the hypothesis of infections as an environmental risk factor for Sjögren's syndrome.

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CLINICAL SCIENCE

Introduction

Primary SS (pSS) is a systemic autoimmune disease characterized by immunological destruction of exocrine glands, primarily the salivary and lacrimal glands. The contribution of adaptive immunity to disease pathology is apparent from autoreactive B and T cells [1], but accumulating evidence points also to the importance of innate immunity in the initial stages of pSS development [2]. Monocytes are first-line effector cells and important mediators at the interface of innate and adaptive immunity, with roles as phagocytes, antigen presenting cells and cytokine producers. In pSS, monocytes present a type I IFN signature [3–5] with concomitant up-regulation of TLR7 in subsets of patients with high disease activity [6], produce higher amounts of the pro-inflammatory cytokine B-cell activating factor (BAFF) [7], and display impaired phagocytosis [8, 9].

A complex interplay between genetic predisposition and environmental factors has been suggested as the mechanism underlying autoimmune disease [1]. Specifically, viral infections have been proposed as risk factors [10, 11], and in pSS several viruses have been suggested to initiate the sequence of events leading to inflammation and disruption of the exocrine glands [12]. Moreover, viral infections have been suspected as acting as initial triggers of the persistent type I IFN signalling observed in the majority of patients with pSS [13]. In a recent study, we reported a link between infections and increased risk of pSS, thus further implying that microbial triggers may partake in the pathogenic process [14]. However, precise understanding of initial environmental immune triggers of autoimmune disease is challenging, also bearing in mind that pSS-associated autoantibodies are often present long before diagnosis [15, 16]. In the case of viral infections, the study of primary infections in human subjects poses great temporal and logistic challenges. To circumvent such difficulties, responses following vaccination can be monitored to enable systematic *in vivo* studies of immune reactions to, for example, viral antigens in a safe and time- and dose-controlled manner.

In a previous study, we noted that untreated anti-SSA seropositive patients with pSS had an accelerated plasmablast differentiation and developed higher vaccine-specific antibody titres compared with controls following squalene-adjuvanted H1N1 vaccination [17]. Whether a non-adjuvanted influenza vaccine may also induce higher vaccine-specific antibody titres is not known. Furthermore, the early transcriptional events following viral antigen exposure that may explain differential serological outcomes have not been defined. In the present study we therefore longitudinally followed patients with pSS and controls during non-adjuvanted split-virion influenza vaccination with the purpose of identifying differences in early, innate immune activation and the resulting cellular and serological responses.

Methods

Study population

The study included 25 anti-SSA seropositive female patients fulfilling the American-European Consensus Group criteria for pSS [18] and 16 age and sex-matched healthy controls (Table 1). Study participants were followed during seasonal influenza vaccination (Fluarix, GlaxoSmithKline, Solna, Sweden) containing inactivated A/California/7/2009 (H1N1)-, A/Switzerland/9715293/2013 (H3N2)-, and B/Phuket/3073/2013-like strains. One patient started HCQ treatment and was excluded from serological analyses.

Routine clinical laboratory tests were performed at the laboratory for clinical chemistry at Karolinska University Hospital, Solna, Sweden. Clinical symptoms were registered on visual analogue scales, and data on EULAR Sjögren's Syndrome Patient Reported Index score [19] were collected.

The study was approved by the Regional Ethics Committee in Stockholm and was carried out in compliance with the Declaration of Helsinki. All participants gave written, informed consent.

Antibody analyses

Hemagglutination inhibition assays were performed as previously described [20]. Vaccine-specific antibody levels were measured by ELISA. Briefly, 96-well MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with Fluarix vaccine (GlaxoSmithKline), 0.5 μg haemagglutinin for each strain. Sera were diluted 1 : 2000. For detection, ALP-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark), and goat anti-human IgA and IgM (Mabtech, Nacka Strand, Sweden) antibodies were used. Absorbance was measured at 405 nm. Arbitrary units were calculated from a standard serum curve.

Anti-EBV viral capsid antigen IgG were measured by ELISA according to manufacturers' protocol (IBL International GmbH, Hamburg, Germany) and anti-Ro52 autoantibodies were measured by ELISA as previously described [21].

Plasma protein quantification

Plasma protein levels were measured by proximity extension assay as previously described [22, 23] (Immuno-Oncology panel, Olink Bioscience, Uppsala, Sweden). Target proteins were detected by antibody pairs labelled with oligonucleotides that hybridize when binding epitopes in close proximity. Subsequently, DNA barcodes were quantified by real-time PCR. Protein levels are reported in arbitrary units (normalized protein expression, NPX).

Cell isolation

PBMC were isolated using Vacutainer Mononuclear Cell Preparation Tubes (BD Biosciences, Stockholm, Sweden). $CD14^+$ monocytes were isolated by CD14-

Table 1 Clinical characteristics of untreated and HCQ-treated patients with pSS, and healthy controls (HC)

	HC	pSS untr	pSS-HCQ
Individuals, *n*	16	17	8
Female, *n* (%)	16 (100)	17 (100)	8 (100)
Age at inclusion, years, mean (S.D.)	47.3 (8.4)	51.6 (13.1)[a]	52.5 (8.6)[a]
Age at diagnosis, years, mean (S.D.)	—	43.4 (15.7)	48.8 (9.7)
Autoantibody frequency, *n* (%)			
ANA	—	11/15 (73)	7 (88)
Anti-SSA antibodies	—	17 (100)	8 (100)
Anti-SSB antibodies	—	13 (76)	7 (88)
Medication, *n* (%)			
HCQ	0 (0)	0 (0)	8 (100)
≤5 mg prednisone/day	0 (0)	0 (0)	2 (25)
Extraglandular manifestations, frequency, *n* (%)			
RP	0 (0)	5/16 (31)	2 (25)
Arthritis	0 (0)	2 (12)	4 (50)
Dermal vasculitis	0 (0)	0 (0)	2 (25)
Major salivary gland swelling	0 (0)	4/16 (25)	0 (0)
Lymphadenopathy	0 (0)	4 (24)	0 (0)
Lymphoma	0 (0)	2 (12)	0 (0)
Hypothyroidism	0 (0)	1 (6)	1 (13)
Salivary gland biopsy, frequency, *n* (%)	—	7 (41)	3 (37)
Focus score, mean (S.D.)	—	3.2 (2.4)	4.3 (1.5)

[a]not significant compared with HC (student's *t test*, $P > 0.05$).

specific microbeads in an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany).

RNA purification and expression analysis

Total RNA was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany). Expression of 594 genes was measured (Human Immunology CodeSet v2, Nanostring Technologies, Seattle, WA, USA). Data was quality controlled and normalised using nSolver Analysis Software (v. 3.0.22, Nanostring Technologies). Genes selected for further analysis were expressed above a cut-off of mean + 2SD of eight negative controls and on average expressed above this cut-off in at least one of the groups in at least one of the time points, resulting in $n = 385$ genes. Interferon-regulated genes were identified in the Interferome database [24]. Principal component analysis was performed using R, and heatmaps were generated using Morpheus (https://software.broadinstitute.org/morpheus).

The IFN score was calculated as previously described [25] using genes *IFI35*, *IFITM1*, *IRF7*, *MX1* and *STAT1*. An IFN signature was considered positive when above the mean + 2SD of the control group. The NF-κB score was calculated in the same manner from expression levels of *BTK*, *NEMO*, *IRAK1*, *NLRP3*, *TLR4*, *TLR9* and *TRAF2*.

Statistical analysis

For differential gene expression analysis the Comparative MarkerSelection tool from GenePattern [26] was utilized, employing Student's *t test* at a specified false discovery rate of 10%. Linear mixed model analysis was applied using the lme4 package in R [27]. Variation of antibody levels was analysed by quantile regression for delta from baseline to day 28 using Stata (StataCorp LP, College Station, TX, USA). The Mann–Whitney *U* test was used when comparing two groups and the Wilcoxon test was used for paired data. For correlation analysis, Spearman rank correlation was used. Statistical calculations were performed in Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA) unless otherwise specified. *P*-values <0.05 were considered significant.

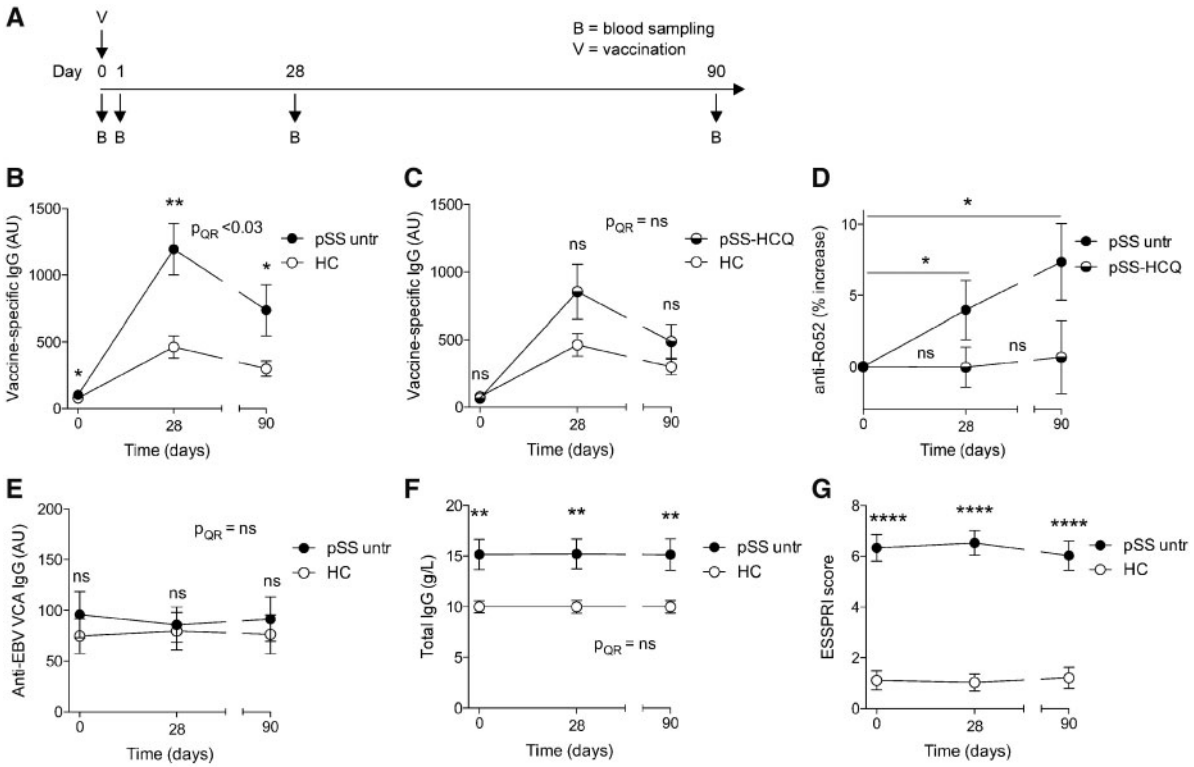
Results

Higher specific IgG response in patients with pSS following viral antigen exposure

To assess immunological perturbations associated with viral antigen exposure, we longitudinally followed untreated patients with pSS (pSS_{Untr}), HCQ-treated patients with pSS (pSS_{HCQ}) and age- and sex-matched healthy controls during seasonal influenza vaccination with a trivalent inactivated split-virion vaccine (Fig. 1A, Table 1).

First, we evaluated serological responses by measuring vaccine-specific antibody titres. We observed higher levels of vaccine-specific IgG titres in pSS_{Untr} compared with controls (Fig. 1B), but not in pSS_{HCQ} compared with controls (Fig. 1C). There was no statistically significant difference in antibody titres comparing pSS_{Untr} and pSS_{HCQ} (data not shown). Vaccine-specific IgA and IgM titres did not differ between pSS_{Untr} and controls

FIG. 1 Higher antibody response in untreated patients with pSS following viral antigen exposure



(A) Blood samples were collected from untreated patients with pSS (pSS untr, $n = 17$), HCQ-treated patient with pSS (pSS-HCQ, $n = 8$) and healthy controls ($n = 16$) before and after seasonal influenza vaccination. **(B, C)** Vaccine-specific IgG levels measured by ELISA. **(D)** Anti-Ro52 autoantibody levels measured by ELISA. **(E)** Anti-EBV viral capsid antigen antibody levels measured by ELISA. **(F)** Total IgG levels measured as part of routine laboratory blood testing. **(G)** EULAR SS Patient Reported Index score. Data are presented as mean (S.E.M.). QR, quantile regression; ns, not significant. *$P<0.05$, **$P<0.01$, ****$P<0.0001$ (Mann–Whitney U test, Wilcoxon signed-rank test).

(Supplementary Fig. S1A, available at *Rheumatology* online), and neutralizing anti-hemagglutinin antibody levels were comparable for two of the strains, but higher in pSS_{Untr} compared with controls for the A/Switzerland/9715293/2013-like strain (Supplementary Fig. S1B, available at *Rheumatology* online).

Bystander activation of memory B cells has been reported in untreated patients with pSS following squalene-adjuvanted H1N1 vaccination with increasing antibody titres to autoantigens as well as to other antigens unrelated to the vaccine [17]. To detect potential changes in autoantibody titres in the present study with a non-adjuvanted vaccine, autoantibody levels to one of the main pSS autoantigens, Ro52/TRIM21, were analysed. Following vaccination, levels of anti-Ro52 autoantibodies increased in pSS_{Untr}, but not in pSS_{HCQ} (Fig. 1D). To investigate changes in IgG titres to other microbial antigens unrelated to the vaccine, anti-EBV antibody titres were measured. Here, no increase was observed, neither in pSS_{Untr} nor pSS_{HCQ} (Fig. 1E). Total IgG levels did not change during the study period in either group (Fig. 1F).

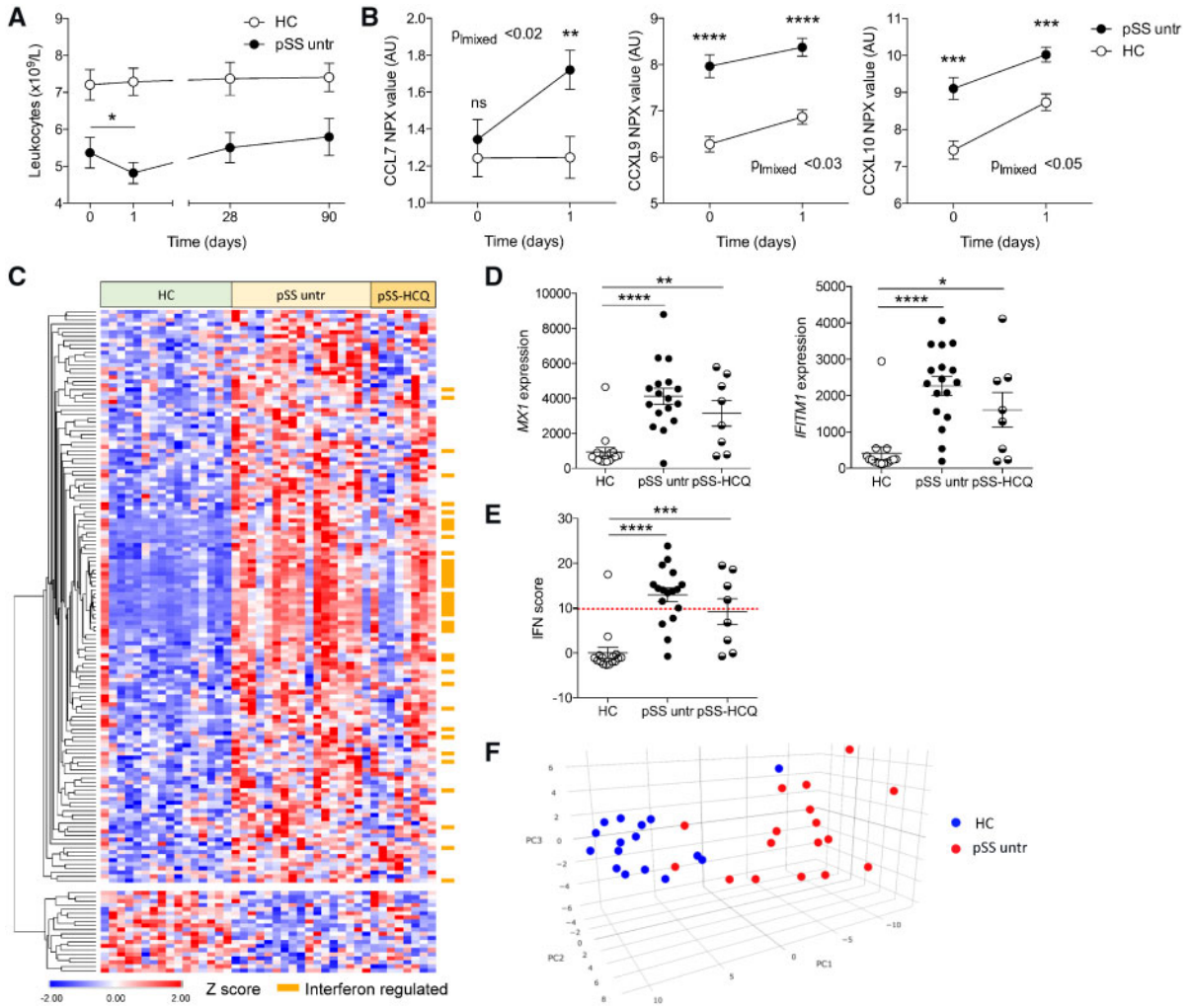
Potential changes in disease activity during the study period were followed through self-reported clinical parameters. No significant changes in EULAR Sjögren's Syndrome Patient Reported Index or other disease-related parameters were noted (Fig. 1G, Supplementary Fig. S2A–E, available at *Rheumatology* online).

Together, these data demonstrate a serological hyper-responsiveness following viral antigen exposure in untreated patients with pSS, possibly reduced by HCQ treatment.

Differential cellular and plasma proteome responses

To better understand differences in early immunological responses after viral antigen exposure in pSS_{Untr} compared with controls, routine laboratory tests as well as a comprehensive plasma proteome screen were performed on samples before and 24 h after vaccination. Despite the steady-state leukopoenia observed in pSS, a significant further decrease in total leucocyte blood count 24 h after vaccination was observed in pSS_{Untr}, but not in controls (Fig. 2A) or pSS_{HCQ} (data not shown). Significant changes in other routine laboratory tests were not observed (Supplementary Fig. S3A–E, available at *Rheumatology* online). To identify factors

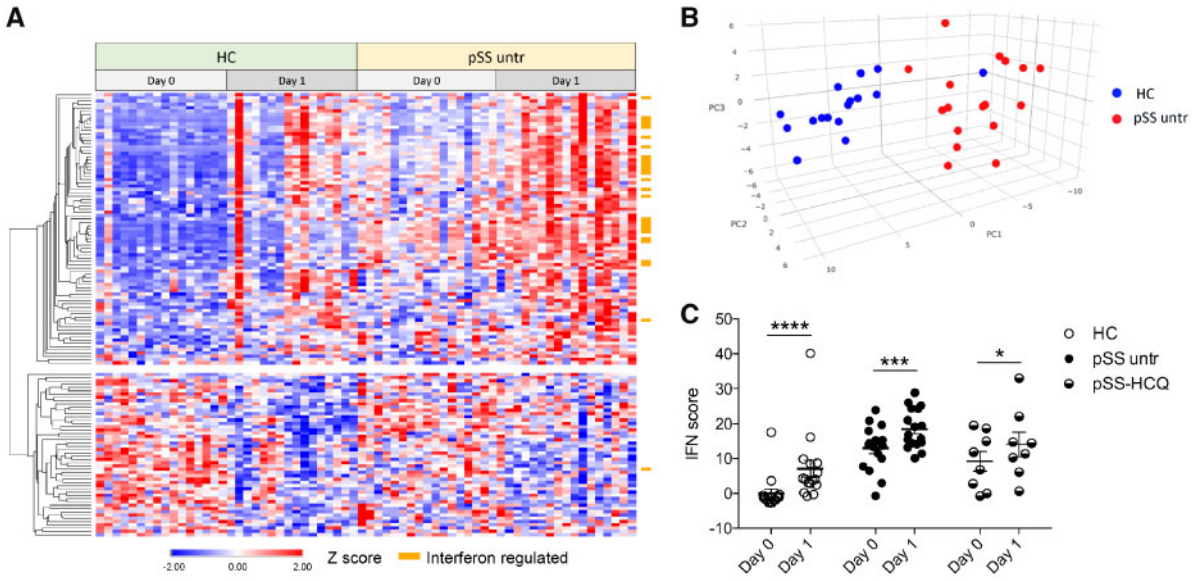
FIG. 2 Differential cellular and plasma proteome responses to viral antigens



(A) Total leucocyte count in blood. Counts were significantly higher in healthy controls compared with untreated patients with pSS (pSS untr) at all time points. **(B)** Linear mixed model analysis identified significant differences in vaccination-induced changes in protein levels of CCL7, CXCL9 and CXCL10 measured by proximity extension assay. **(C)** Gene expression in $CD14^{+}$ monocytes at day 0 analysed by Nanostring. Differentially expressed genes (*t* test, $P < 0.05$, false discovery rate 10%) comparing pSS untr and healthy controls are depicted in the heatmap for all three groups. **(D)** Gene expression levels of the type I IFN-regulated genes *MX1* and *IFITM1*. (**E**) *IFN* score calculated from the expression of five IFN-regulated genes. The red line represents the cut-off for type I IFN positivity defined as the mean plus two standard deviations of the healthy controls group. (**F**) Principal component analysis of differentially expressed genes in monocytes at day 0. Data are presented as mean (S.E.M.). Lmixed, linear mixed model. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Wilcoxon signed-rank test, Mann–Whitney *U* test).

related to differences in leucocyte trafficking, the plasma proteome was analysed by a catch-detection antibody pair-based proximity extension assay of 92 immune-related proteins [22]. Prior to vaccination, significantly higher levels of 34 proteins were observed in pSS_{Untr} compared with controls, and 24 h after vaccination the corresponding number was reduced to 24 proteins (Supplementary Fig. S4A, B, available at *Rheumatology* online). Employing linear mixed model analysis, differences in vaccination-induced changes of protein levels comparing pSS_{Untr} and controls were identified. The model identified significant differences in changes of C-C Motif Chemokine 7 (CCL7) also known as monocyte chemotactic protein 3 (MCP3), C-X-X Motif Chemokine 9 (CXCL9), and C-X-X Motif Chemokine 10 (CXCL10) (Fig. 2B). However, the only protein identified as significant by the model which also had a larger increase in pSS_{Untr} compared with controls was the monocyte-attracting chemokine CCL7, which increased in pSS_{Untr} while remaining at unchanged levels in the controls (Fig. 2B). Taken together, these data demonstrate differences in the early cellular

Fig. 3 Increased expression of type I IFN-regulated genes 24 h after viral antigen exposure



(A) Gene expression in CD14^{+} monocytes before (day 0) and 24 h after vaccination (day 1). Differentially expressed genes comparing day 0 and day 1 in untreated patients with pSS (pSS untr) or in healthy controls are depicted in the heatmap for both groups (*t* test, $P < 0.05$, false discovery rate 10%). Expression is normalized by gene across time points. **(B)** Principal component analysis of differentially expressed genes in monocytes at day 1. **(C)** IFN score calculated from the expression of five IFN-regulated genes. IFN scores at day 1 are normalized against day 0 to achieve relative quantification. Data are presented as mean (S.E.M.). $^{*}P<0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Wilcoxon signed-rank test).

response to viral antigen exposure, and suggest differences in monocyte recruitment.

Differential gene expression profile in monocytes from patients with pSS

Monocytes are among the cells first activated in immune responses, and to better characterize their state of activation in patients with pSS, we analysed gene expression by direct hybridization barcode technology defining the expression of 594 immune-related genes. Comparison of the transcriptomic profile between pSS_{Untr} and controls prior to vaccination revealed $n = 160$ significantly differentially expressed genes ($P < 0.05$, false discovery rate 10%) (Fig. 2C, Supplementary Table S1, available at *Rheumatology* online). As could be expected [4], several IFN-regulated genes were noted, such as *MX1* and *IFITM1* (Fig. 2D, Supplementary Table S1, available at *Rheumatology* online). Likewise, calculation of an IFN score based on the expression of five IFN-regulated genes revealed that a majority of the patients had high IFN scores (Fig. 2E). A principal component analysis of significantly differentially expressed genes revealed that pSS_{Untr} and controls formed two distinct, separate clusters (Fig. 2F). In all, these data demonstrate prominent differences in monocyte gene transcription comparing patients with pSS and controls, and establish the presence of an IFN signature in a majority of the patients.

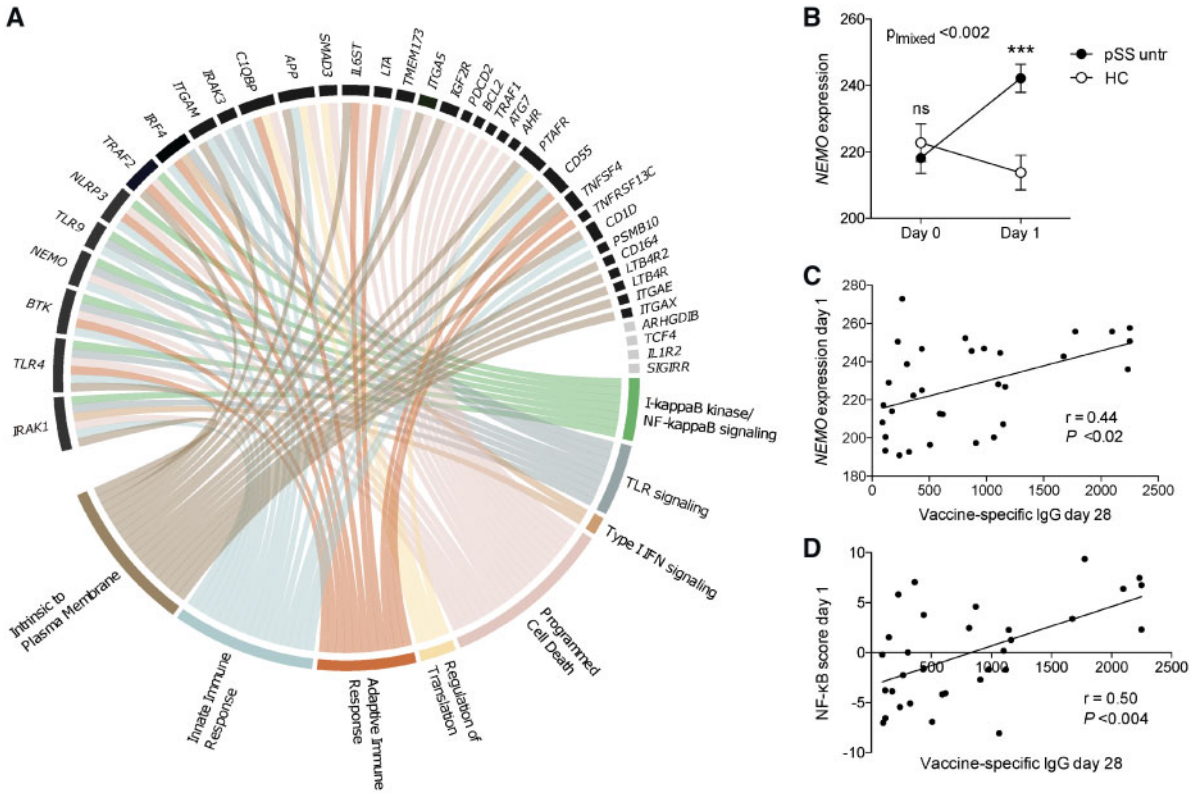
Induction of IFN-regulated genes in monocytes following viral antigen exposure

To examine the effect of viral antigen exposure on the gene expression profile in monocytes, we also analysed gene transcription in cells obtained 24 h after vaccine administration. We noted a marked up-regulation of IFN-regulated genes in both controls and pSS_{Untr} (Fig. 3A, C). Despite having been activated by the same immune trigger in terms of vaccination, pSS_{Untr} and controls still formed distinct clusters in a principal component analysis, albeit expression levels of IFN-regulated genes were more similar between the two groups than before vaccination (Fig. 3B).

Differential transcriptional responses in monocytes

To begin understanding the mechanisms by which higher antibody titres are generated in pSS_{Untr}, we investigated differences in the transcriptional responses in monocytes comparing pSS_{Untr} and controls. By linear mixed model analysis, we identified 38 genes with significantly different responses (Supplementary Table S2, available at *Rheumatology* online). Grouping by Gene Ontology (GO) terms revealed that the identified genes were involved in the immunological processes NF-κB signalling, TLR signalling, type I IFN signalling, programmed cell death, regulation of translation, adaptive immune response, innate immune response and intrinsic to plasma membrane (Fig. 4A). Notably, one of the most significant genes with a marked up-regulation in pSS_{Untr} was the *NF-κB*

FIG. 4 Differential transcriptional responses to viral antigen exposure in monocytes



(A) Significantly differentially regulated genes in CD14$^+$ monocytes in response to vaccination comparing untreated patients with pSS (pSS untr) and healthy controls were identified by linear mixed model analysis. The identified genes were grouped according to Gene Ontology terms and are depicted in the circular graph. **(B)** mRNA expression of *NEMO* in monocytes at day 0 and 1 in pSS untr and healthy controls. Data are presented as mean (S.E.M.). **(C)** Correlation between *NEMO* gene expression at day 1 and vaccine-specific IgG titres at day 28. **(D)** Correlation between the NF-κB score at day 1 and vaccine-specific IgG titres day 28. Lmixed, linear mixed model; ns, not significant. ***$P<0.001$ (Spearman rank correlation, Mann–Whitney *U* test).

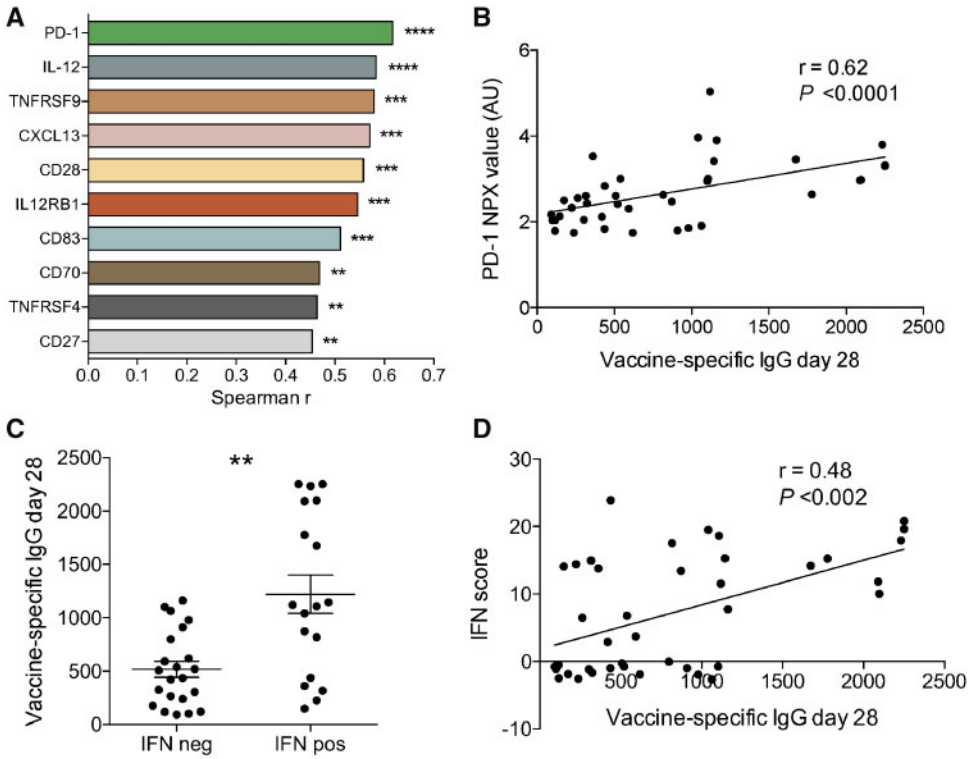
essential modulator (*NEMO*), a key regulator of NF-κB signalling also denoted *inhibitor of nuclear factor kappa B kinase subunit gamma* (*IKBKG*), which was strongly up-regulated in pSS_{Untr} while remaining at unchanged expression level in controls (Fig. 4B).

To further comprehend the relevance of the identified genes in relation to increased antibody responses, we correlated the top hits with antibody titres at day 28. Interestingly, *NEMO* gene expression at day 1 correlated with vaccine-specific IgG titres at day 28 ($r = 0.44$, $P < 0.02$) (Fig. 4C). To enable estimation of the activity of NF-κB signalling, expression levels of all identified genes annotated for NF-κB signalling were summarized into an NF-κB score for each individual in the same manner as when calculating the IFN score [25]. Indeed, the NF-κB score at day 1 also correlated with vaccine-specific IgG titres at day 28 ($r = 0.50$, $P < 0.004$) (Fig. 4D). These data imply that a stronger early activation of NF-κB signalling in monocytes may contribute to higher serological responses following viral antigen exposure in untreated patients with pSS.

Factors predicting the serological response to viral antigens

Having understood that altered transcriptional activity in monocytes is associated with higher vaccine-specific IgG titres, we proceeded to test which other factors may predict serological responses. First, we correlated plasma protein levels at day 0 with vaccine-specific antibody levels at day 28. The levels of several proteins were associated with the serological response (Fig. 5A), with PD-1, IL-12, TNFRSF9 and CXCL13 exhibiting the strongest correlations. Interestingly, the soluble form of PD-1, a protein extensively studied for its involvement in autoimmunity [28] showed the strongest correlation (Fig. 5B). A corresponding analysis of mRNA expression data in monocytes before vaccination revealed that expression of several genes also correlated with the serological response (Supplementary Fig. S5, available at *Rheumatology* online). Noting that several of these were IFN-regulated genes, we investigated whether the IFN score calculated in monocytes before vaccination was associated with a higher serological response. Indeed,

Fig. 5 Pre-vaccination factors predicting serological responses



(A) Plasma protein levels at day 0 were measured by proximity extension assay. The figure depicts correlations with vaccine-specific antibody titres at day 28, with cut-off set at Spearman $r > 0.45$. **(B)** Correlation between plasma levels of soluble PD-1 at day 0 and vaccine-specific antibody titres at day 28. **(C)** Vaccine-specific IgG titres in IFN score positive and negative individuals. Data are presented as mean (S.E.M.). **(D)** Correlation between the IFN score and vaccine-specific antibody titres at day 28. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Spearman rank correlation, Mann–Whitney *U* test).

the analysis revealed a higher response in individuals with positive IFN signature (Fig. 5C), and the IFN score correlated positively with vaccine-specific IgG levels at day 28 ($r = 0.48$, $P < 0.002$) (Fig. 5D). In all, these data show that increased levels of immune activation prior to vaccination is associated with a higher serological response following viral antigen exposure.

Discussion

It has been hypothesized that infections may act as environmental triggers in the early stages of autoimmune diseases [10]. The mechanisms through which pathogens would trigger autoimmunity, however, remain incompletely understood [13]. In the present study, we therefore detailed the cellular and serological responses in patients with pSS following exposure to viral antigens.

We observed higher titres of vaccine-specific antibodies in untreated patients with pSS than in controls, which extends our previous observations from squalene-adjuvanted vaccination to a non-adjuvanted vaccine [17]. By contrast, previous studies have frequently reported lower responses following vaccination in rheumatic patients [29–31]. This discrepancy may, however, be explained by the high frequencies of patients treated with immunomodulatory drugs in those studies.

Notably, we observed increasing titres of anti-Ro52 autoantibodies in untreated patients with pSS but not in HCQ-treated patients. In the current study, however, the relative increase in anti-Ro52 autoantibodies was less than following squalene-adjuvanted vaccination [17]. This difference may relate to the fact that the vaccine in the study by Brauner *et al.* contained the adjuvant squalene and was given with a booster dose after three weeks. Notably, a separate study also reported increased autoantibody levels in patients with pSS at 1-year follow up after H1N1 vaccination [32]. Taken together, our data imply that vaccination with non-adjuvanted vaccines may be preferable when vaccinating untreated patients with autoimmune disease, as to avoid off-target B-cell activation. Despite the increasing titres of anti-Ro52 autoantibodies in untreated patients with pSS, we did not observe any aggravation of disease-related symptoms. Indeed, the EULAR Sjögren's Syndrome Patient Reported Index score remained stable in the patients throughout the course of the study, supporting findings that vaccinations do not

exacerbate disease activity in rheumatic diseases [33]. Our results support that patients with pSS are able to respond to vaccination without unwanted sequelae, which is in line with previous reports [32, 34].

To better understand differences in cellular and plasma proteome responses, we collected routine laboratory tests, and performed a comprehensive screen of 92 plasma proteins. Intriguingly, at 24 h after vaccination we observed a decrease in total leucocyte count with a concomitant up-regulation of the monocyte-attracting chemokine CCL7 in untreated patients with pSS. Mouse studies have shown that CCL7 is critical for recruitment of monocytes [35]. Thus, these results could reflect an increased recruitment of immune cells to the site of injection in the patients with pSS, and also point to the importance of monocytes in differential innate responses.

Wanting to begin understanding the mechanisms underlying stronger serological responses, we assessed differential vaccination-induced transcriptional changes in monocytes. Here, we were able to identify transcriptional features that associated with the magnitude of the humoral response against the viral antigens. Specifically, higher induced expression of the gene *NEMO* in monocytes was associated with a higher vaccine-specific antibody response. As the regulatory subunit of the IKK complex, the NEMO protein activates NF-κB by phosphorylating the inhibitory IκBα, which then dissociates from the NF-κB complex, leaving the p50 and p60 subunits free to translocate into the nucleus and activate gene transcription [36]. In the present study, the time points for blood sampling did not support analysis of protein production resulting from the NF-κB activation, but notably we observed a prominent peak in serum levels of TNFα, which is a prototypic cytokine induced by NF-κB pathway signalling, in patients with pSS but not controls seven days after influenza vaccination in a previous study [17]. Additionally, monocytes from patients with pSS were previously shown to have decreased expression of the NF-κB inhibitor IκBα, further implicating dysregulated NF-κB signalling [37]. Interestingly, mutations in *NEMO* are associated with the disease incontinentia pigmenti [38], and several types of immunodeficiencies [39]. In fact, mutations in *NEMO* were shown to result in defect NF-κB activation and impaired response to TLR agonists [40], as well as deficits in B-cell responses [41]. The stronger up-regulation of *NEMO* expression following viral antigen exposure in untreated patients compared with controls and the association of higher induced expression of *NEMO* with higher vaccine-specific antibody titres suggests its importance in the pathology of pSS, thus warranting further investigation.

Further, the plasma proteome was investigated to identify factors of importance for generation of higher vaccine-specific responses. Here, levels of soluble PD-1 showed the strongest association. The membrane-bound form of PD-1 is a receptor involved in suppressing T-cell inflammatory activity [42]; however, in its soluble form PD-1 is believed to functionally antagonize its membrane-bound form [28]. Interestingly, levels of soluble PD-1 are higher in the blood of patients with systemic sclerosis and rheumatoid arthritis compared with controls, also correlating with disease parameters [43–45]. Consequently, our data suggest the involvement of the PD-1/PD-L:s pathway in the hyperactive immune status of patients with pSS and indicate a role in promoting enhanced humoral responses.

We noted that a higher steady-state activation of the type I IFN system before vaccination correlated with a higher vaccine-specific IgG response. Aberrant activation of the IFN system has been suggested to drive autoantibody production [46], and there is a clear association between presence of a type I IFN score and higher monocyte gene expression of the B-cell activating cytokine BAFF [4], supporting a mechanism where increased IFN activity drives B-cell hyper-responsiveness. Also, our results are in line with data showing that pre-activation of naïve B cells from controls with type I IFN renders the cells hyper-responsive to endosomal TLR activation [17].

Considering previous data that naïve B cells from HCQ-treated patients are less prone to differentiate into antibody producing plasmablasts [17], we analysed the serological response of HCQ-treated patients. We did not observe significant differences in vaccine-specific IgG titres in HCQ-treated patients compared with either untreated patients or controls. The fact that these observations did not reach statistical significance may relate to a lack of statistical power, as the HCQ-treated group only encompassed eight patients. However, we observed a tendency for a lower vaccine-specific antibody response in the HCQ-treated compared with the untreated group, which is likely related to previous observations that HCQ treatment reduces immunoglobulin levels in patients with pSS [47–51].

Limitations of this study include that anti-SSA seropositive patients were studied and the results are therefore valid for a specific subset of patients with pSS. Also, because only one vaccine was investigated, it remains unclear whether the results are generalizable to other types of vaccinations or microbial infections.

In conclusion, we observed higher antibody responses in untreated patients with pSS following non-adjuvated viral antigen exposure, and found that a higher serological response is associated with higher early activation of NF-κB signalling in monocytes as well as with indications of heightened immune activation prior to vaccination. The augmented innate and adaptive immune responses suggest an underlying hyper-responsiveness to immune challenges, and imply the importance of viral infections as an environmental risk factor for autoimmune disease.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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