




Dysregulated Fc γ receptor IIa-induced cytokine production in dendritic cells of lupus nephritis patients

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Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease of unknown etiology. SLE is a chronic disease characterized by relapses alternating with periods of low disease activity or remission. The clinical manifestations are heterogeneous, ranging from mild rash and arthralgia to severe or life-threatening forms of the disease affecting the kidneys or central nervous system [1–4].

One of the key factors associated with SLE pathogenesis is excessive production of type I interferons (IFNs).

Summary

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology. One of the key factors associated with SLE pathogenesis is excessive production of type I interferons (IFNs). This could result from increased activation of type I IFN-stimulating pathways, but also from decreased activation of type I IFN-inhibitory pathways. Recently, we have identified that immunoglobulin (Ig)G immune complexes strongly inhibit type I IFN production in healthy individuals by inhibitory signaling through Fc γ receptor IIa (Fc γ RIIa) on dendritic cells (DCs). Because, in SLE patients, immune complexes are characteristically present, we assessed whether Fc γ R-induced suppression of type I IFN is functional in DCs of SLE patients. We divided the SLE patients into one group without, and one group with, previous major organ involvement, for which we chose nephritis as a prototypical example. We show that DCs of lupus nephritis patients displayed impaired Fc γ R-mediated type I IFN inhibition compared to SLE patients without major organ involvement or healthy controls. We verified that this impaired type I IFN inhibition was not related to differences in disease activity, medication, Fc γ RIIa expression or expression of IFN regulatory transcription factors (IRF)1 and IRF5. In addition, we identified that DCs of lupus nephritis patients show increased Fc γ R-induced interleukin (IL)-1 β production, which is another important cytokine that promotes kidney inflammation. Taken together, these data indicate that DCs of lupus nephritis patients display altered Fc γ R-mediated regulation of cytokine production, resulting in elevated levels of type I IFN and IL-1 β . This dysregulation may contribute to the development of nephritis in SLE patients.

Keywords: dendritic cells, Fc γ receptor, lupus nephritis, systemic lupus erythematosus

Consequently, type I IFN is considered to be an important therapeutic target, which is supported by recent trials that target the type I IFN receptor (IFNAR) (anifrolumab) [5]. Type I IFNs can be produced by many different cell types, usually in response to stimulation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), that recognize nucleic acids. Predominantly specialized antigen-presenting cells (APCs) such as dendritic cells (DCs) are potent inducers of type I IFNs. The cause of elevated type I IFN expression in SLE patients is not completely clear, but appears to result from unknown defects in the

Table 1. Demographic and clinical characteristics of the systemic lupus erythematosus (SLE) patients in this study

	SLE patients – nephritis (<i>n</i> = 8)	SLE patients + nephritis (<i>n</i> = 8)
Age, mean ± s.d. (years)	38 ± 15	40 ± 13
Men/women	0/8	3/5
Disease duration, mean ± s.d. (years)	10 ± 9	11 ± 8
ESR, mean ± s.d. (mm/h)	27 ± 26	22 ± 22
CRP, mean ± s.d. (mg/liter)	5 ± 7	6 ± 5
Low complement		
C3	3	1
C4	1	0
dsDNA elevated	3	3
SLEDAI, median ± IQR	1.5 ± 4.5	0 ± 4
Class of nephritis*		
IIIa (focal)	–	2
IV (diffuse segmental or global)	–	3
V (membranous)	–	3
Duration nephritis, mean ± s.d. (years)	–	7 ± 6
Medication		
Prednisolone ≤ 10 mg	3	5
Prednisolone > 10 mg	1	1
HCQ	7	8
Azathioprine	1	0
Mycophenolate mofetil	0	5
Belimumab	2	0

*Some patients had class IIIa/IV nephritis combined with class V. The class that was leading for therapy is depicted in the table. Values are the number of patients. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; dsDNA = double-stranded DNA; SLEDAI = systemic lupus erythematosus disease activity index; IQR = interquartile range; HCQ = hydroxychloroquine; s.d. = standard deviation.

control of type I IFN responses [6]. Hence, identification of these defects is of great importance, as it would improve our understanding of SLE pathogenesis.

Increased levels of type I IFN in SLE patients could be caused by increased activation of type I IFN-inducing pathways, but could also result from impaired functionality of pathways that suppress type I IFN. Recently, we have identified an inhibitory pathway that specifically suppresses type I IFN production by human DCs [7]. This suppression is induced by inhibitory signaling through Fcγ receptor IIa (FcγRIIa, or CD32a), a receptor that recognizes immunoglobulin (Ig)G immune complexes. The physiological function of inhibitory signaling by FcγRIIa in healthy individuals is to down-regulate type I IFN responses during the late phase of viral infections, which is triggered by anti-viral IgG antibodies that opsonize viruses and thereby form immune complexes [7].

SLE is also characterized by the presence of immune complexes, which results from the production of autoantibodies to nuclear antigens that form immune complexes, which are known to deposit in various tissues and organs [1,2,8,9]. Normally, these immune complexes would inhibit type I IFN responses. However, as the type I IFN response is elevated in SLE patients, in this study we speculate that this type I IFN inhibition may be impaired in SLE patients. Therefore, we investigated whether FcγR-induced suppression of type I IFN is still functional in DCs of SLE patients.

Here, we show that DCs of lupus nephritis patients displayed impaired FcγR-induced suppression of type I IFN when compared to healthy controls (HCs) or SLE patients without kidney involvement. Furthermore, we verified that this impaired type I IFN inhibition was not related to differences in disease activity, medication, FcγRIIa expression or expression of IFN regulatory transcription factors (IRF)1 and IRF5. Surprisingly, while FcγR-induced type I IFN suppression was impaired, FcγR-induced amplification of proinflammatory cytokine IL-1β was increased in lupus nephritis patients. Taken together, these data indicate that DCs of lupus nephritis patients display dysregulated FcγR-induced cytokine production, resulting in elevated levels of type I IFN and IL-1β.

Materials and methods

Patients

The study included 22 HCs and 16 patients with a clinical diagnosis of SLE who all fulfilled the 2012 Systemic Lupus International Collaborating Clinics Classification (SLICC) criteria. Considering the heterogeneity of the disease, we divided the SLE patients into two groups; one without major organ involvement (*n* = 8) and one with previous major organ involvement, for which we chose nephritis as a prototypical example (*n* = 8). Demographic and clinical data are shown in Table 1.

Cells and stimulation

Monocytes were isolated from heparinized peripheral blood from healthy donors or SLE patients by density gradient centrifugation on Lymphoprep (Nycomed, Zurich, Switzerland) and Percoll (Pharmacia, Uppsala, Sweden). DCs were generated by culturing monocytes for 7 days in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Basel, Switzerland) containing 5% fetal bovine serum (FBS) (Biowest, Nuaille, France) and 86 µg/ml gentamicin (GIBCO, Carlsbad, CA, USA) supplemented with 20 ng/ml recombinant human granulocyte-macrophage colony-stimulatory factor (GM-CSF) (Invitrogen, Carlsbad, CA, USA) and

2 ng/ml recombinant human IL-4 (Miltenyi Biotec, Bergisch Gladbach, Germany). At day 3, half the medium was replaced by fresh medium containing the same cytokines.

At day 7, cells were harvested, washed and stimulated in 96-well culture plates (40 000–50 000 cells/well) with 20 µg/ml Poly I:C (Sigma-Aldrich, St Louis, MO, USA) and with or without 3.5 µg/ml mycophenolic acid (MPA; Sigma-Aldrich). To stimulate the cells with IgG immune complexes, we incubated the cells in IgG-coated plates, which were generated by incubating 96-well Maxisorp plates overnight with 2 µg/ml human IgG (Nanogam) that were subsequently blocked with phosphate-buffered saline (PBS) containing 10% FBS. Please note that these plate-coated IgG immune complexes have been previously extensively compared to IgG immune complexes that were generated in other ways, i.e. (1) IgG-coated beads, (2) heat-aggregated IgG immune complexes and (3) IgG opsonized viruses (or bacteria), which all induce very similar cytokine responses (including IFN- β and IL-1 β) [7,10–12].

Ethics statement

The study was approved by the medical ethics committee of the Academic Medical Center in Amsterdam (project number NL44031.018.13) and written informed consent was obtained from each SLE patient before enrollment. The study was conducted in compliance with the International Conference on Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki.

Buffy coats obtained after blood donation by healthy donors were anonymously provided by Sanquin blood supply (Sanquin, Amsterdam, the Netherlands). Ethical review and approval was not required for these samples, which is in accordance with the local legislation (the Medical Research Involving Human Subjects Act and the AMC Medical Ethics Review Committee). All samples were handled anonymously.

Enzyme-linked immunosorbent assay (ELISA)

For analysis of cytokine production, supernatants were harvested 6 h after stimulation and stored at -20°C . Cytokine levels in supernatants were measured by ELISA, using an IFN- β ELISA kit (PBL Assay Science, Piscataway, NJ, USA) or antibody pairs for IL-1 β (CT213-c, CT213-d; U-CyTech, Utrecht, the Netherlands).

Quantitative reverse transcription–polymerase chain reaction (RT–PCR)

For mRNA-level analysis DCs were lysed at several time-points: $t = 0$ h; $t = 1.5$ h; $t = 3$ h; or $t = 6$ h, after which mRNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Fremont, CA, USA). Quantitative

RT–PCR was performed on the StepOnePlus™ real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using TaqMan gene expression assays for *IFNB1* (Hs01077958_s1), *FCGR2A* (Hs01013401_g1), *IRF1* (Hs00971965_m1), *IRF5* (Hs00158114_m1), *IL1B* (Hs00174097_m1), *SYK* (Hs00895377_m1) and *GAPDH* (4310884E), according to the manufacturer's protocol (Thermo Fisher Scientific). The following Sybr Green primers were used for the PCR reactions: *FCGR1* (CD64): 5'-CTT CTC CTT CTA TGT GGG CAG T-3' and 5'-GCT ACC TCG CAC CAG TAT GAT-3'; *FCGR3* (CD16): 5'-CGC AAG CTT TGG TGA CTG GTC CAC TC-3' and 5'-CGC TCT AGA TCA TGG GCT TTT CC CT-3' and *GAPDH*: 5'-GAA GGT GAA GGT CGG AGT C-3' and 5'-GAA GAT GGT GAT GGG ATT TC-3'. mRNA levels were normalized to Ct-values of the housekeeping gene *GAPDH* and folds were calculated compared with an unstimulated control sample ($t = 0$ h).

Flow cytometry

Fc γ RIIa expression was determined by staining the DCs with 5 µg/ml anti-Fc γ RIIa (CD32a; IV.3; StemCell Technologies, Vancouver, Canada) followed by an Alexa Fluor 647-labeled rabbit anti-mouse antibody (Thermo Fisher Scientific). Cells were analyzed by flow cytometry (Canto II; BD Biosciences).

Data analysis

Data were analyzed for statistical significance using the unpaired two-tailed Student's *t*-test or unpaired one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test with GraphPad Prism version 7 software (GraphPad Software, San Diego, CA, USA). All relevant data are contained within this paper and are available from the authors upon reasonable request.

Results

Impaired Fc γ R-induced type I IFN suppression in DCs of lupus nephritis patients

Previously, we identified that in healthy individuals IgG immune complexes strongly inhibit type I IFN production by DCs via inhibitory signaling through Fc γ RIIa [7]. Because immune complexes consisting of IgG autoantibodies bound to autoantigens are continuously present in SLE patients, and SLE is strongly associated with increased levels of type I IFNs, in this study we investigated whether DCs of SLE patients display any abnormalities in Fc γ R-induced suppression of type I IFN. We studied both a group of patients with nephritis and a group without major organ involvement. To assess potential differences in Fc γ R-induced suppression in these groups, we stimulated

DCs from both HCs and SLE patients with Poly I:C or Poly I:C combined with complexed IgG (c-IgG). We observed that the suppression of type I IFN by immune complexes was similar between HCs and SLE patients without major organ involvement, at both protein and mRNA level (representative examples in Fig. 1a,b). Notably, in lupus nephritis patients this type I IFN suppression was impaired (Fig. 1a,b). We assessed this difference in greater detail by determining *IFNB* mRNA expression for multiple donors by determining the relative suppression of type I IFN through FcγR stimulation by dividing IFN-β expression upon co-stimulation (i.e. Poly I:C + c-IgG) by IFN-β expression upon single stimulation (i.e. Poly I:C alone). Indeed, we observed that DCs of lupus nephritis patients showed significantly impaired type I IFN suppression compared to HCs (Fig. 1c).

In addition to aberrant responses to FcγR co-stimulation, DCs of lupus nephritis patients may also already respond differently to individual TLR stimulation. To test this, we assessed *IFNB* mRNA expression levels after Poly I:C stimulation. However, *IFNB* mRNA levels did not significantly differ between HCs and SLE patients with or without nephritis (Fig. 1d), indicating that DCs of lupus nephritis patients do not respond differently to individual TLR ligation.

Taken together, these data indicate that FcγR-induced type I IFN suppression is impaired in DCs of lupus nephritis patients, but not in SLE patients without major organ involvement.

Impaired FcγRIIa-induced type I IFN suppression in lupus nephritis patients is not caused by medication or different expression of FcγRs, IRF1 and IRF5

As almost all SLE patients use specific medication, impaired type I IFN suppression could be therapy-related. We therefore assessed the differences in medication between the two groups of SLE patients. A clear difference in medication between the two groups of SLE patients was the use of mycophenolate mofetil (MMF) in the majority of lupus nephritis patients (Table 1). Therefore, in order to determine whether MMF affects FcγR-induced type I IFN suppression we stimulated DCs from HCs in the absence or presence of mycophenolic acid (MPA), the active metabolite of MMF [13]. We used 3.5 µg/ml MPA, as serum levels in the range of 1–3.5 µg/ml indicate adequate therapy in patients [14]. As shown in Fig. 2a, MPA did not affect FcγR-induced type I IFN suppression. These findings indicate that the impaired type I IFN suppression in lupus nephritis patients is not related to the use of MMF.

In addition, the expression of FcγRs may be different in lupus nephritis patients. To assess whether FcγR expression is different between HCs and SLE patients with or without nephritis, we determined FcγR mRNA expression

on DCs of HCs and SLE patients. The main receptor that is responsible for the suppression of IFN-β production by DCs is FcγRIIa [7], the expression levels of which were similar between the different groups (Fig. 2b). To validate this finding on protein level, we assessed FcγRIIa expression using flow cytometry, where we also did not observe significant differences in FcγRIIa expression between SLE patients with or without nephritis (Fig. 2c). In addition to the 'activating' receptor FcγRIIa, DCs also expressed the 'inhibitory' receptor FcγRIIb (Fig. 2b). However, FcγRIIb expression levels also did not significantly differ between the different groups (Fig. 2b), neither when comparing individual expression of the receptor (Fig. 2b) nor when calculating the ratio of FcγRIIa/FcγRIIb (i.e. 'activating'/'inhibitory') (Fig. 2d). Hence, the impaired type I IFN suppression in DCs of lupus nephritis patients does not seem to result from altered expression of FcγRs.

FcγRIIa is considered to suppress type I IFN by reducing the expression of transcription factor IRF1 [7]. Therefore, lack of a reduction of IRF1 expression upon co-stimulation could explain the impaired suppression of type I IFN in DCs of lupus nephritis patients. To test this, we calculated the relative inhibition by dividing IRF1 expression upon co-stimulation (i.e. Poly I:C + c-IgG) by IRF1 expression upon single stimulation (i.e. Poly I:C alone). However, the reduction of *IRF1* mRNA levels did not differ between HCs and SLE patients with or without nephritis (Fig. 2e).

Another transcription factor that plays a central role in the activation of innate inflammatory immune responses is IRF5. In addition, IRF5 has been implicated in the pathogenesis of lupus nephritis [15,16]. As disease-associated single nucleotide polymorphisms (SNPs) of IRF5 are associated with increased IRF5 expression [16], we assessed IRF5 mRNA levels in the different groups. However, *IRF5* mRNA levels did not significantly differ between HCs and SLE patients (Fig. 2f), indicating that IRF5 expression levels are unlikely to be involved in the impaired type I IFN suppression.

Taken together, these data show that the impaired type I IFN suppression in lupus nephritis patients is not due to differences in medication or FcγR, IRF1 and IRF5 expression.

FcγRIIa-induced amplification of IL-1β is increased in lupus nephritis patients

In our previous study we showed that FcγRIIa stimulation activates two parallel pathways. Type I IFN responses are suppressed by the 'inhibitory' pathway, which is mediated by Syk- and PI3K-independent signaling. Conversely, the 'activating' pathway signals through Syk and PI3K, which amplifies the production of proinflammatory cytokines such as IL-1β [7,11,12]. As our data demonstrate that in

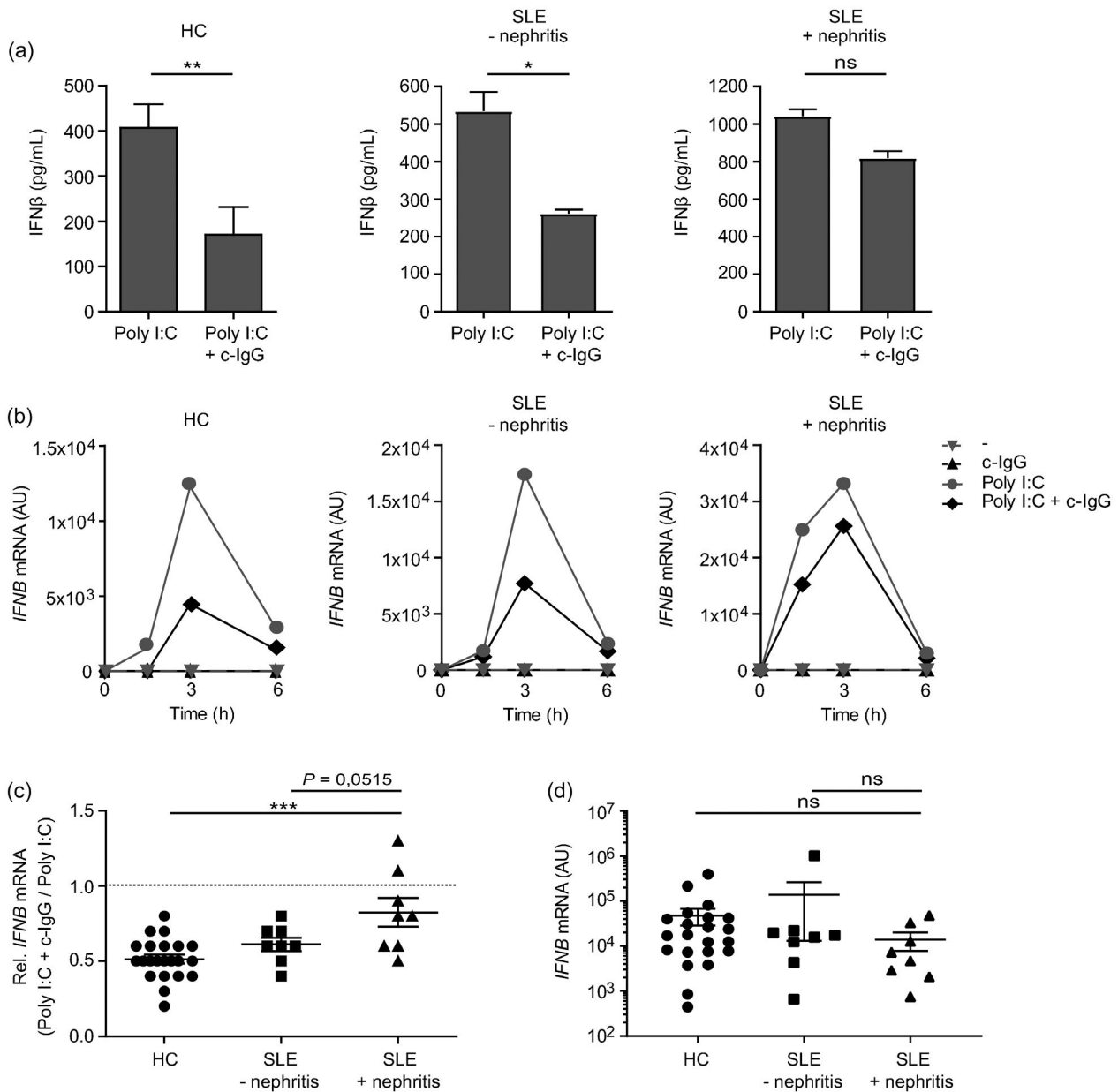


Fig. 1. Impaired Fc gamma receptor IIa (Fc γ RIIa)-induced type I interferon (IFN) suppression in dendritic cells (DCs) of lupus nephritis patients. (a–c) DCs of healthy controls (HCs) and systemic lupus erythematosus (SLE) patients with or without nephritis were stimulated with Toll-like receptor (TLR)-3 ligand Poly I:C and/or complexed immunoglobulin (IgG) (c-IgG). (a) IFN- β protein expression was determined after 6 h. Representative donors [mean \pm standard error of the mean (s.e.m.)] in triplicate of seven (HC; SLE – nephritis) or three (SLE + nephritis) independent experiments with different donors. * $P < 0.05$, ** $P < 0.01$, paired two-tailed Student's t -test. (b) mRNA expression was determined at indicated time-points. Representative donors of 22 (HC) or eight (SLE – nephritis; SLE + nephritis) independent experiments with different donors. (c) Relative expression of *IFNB* by DCs was calculated by dividing mRNA expression at the peak of the response (Poly I:C + c-IgG/Poly I:C; $t = 3$ h). (d) DCs of HCs and SLE patients with or without nephritis were stimulated with Poly I:C. *IFNB* mRNA expression was measured at the peak of the response ($t = 3$ h). (c,d) Every dot represents one donor. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, unpaired one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test.

lupus nephritis patients the inhibitory pathway (that suppresses type I IFN) is impaired, we subsequently examined whether the activating pathway (that increases IL-1 β) is

also impaired in lupus nephritis patients. To test this, we stimulated DCs from both SLE patients with or without nephritis with Poly I:C and/or c-IgG and measured IL-1 β

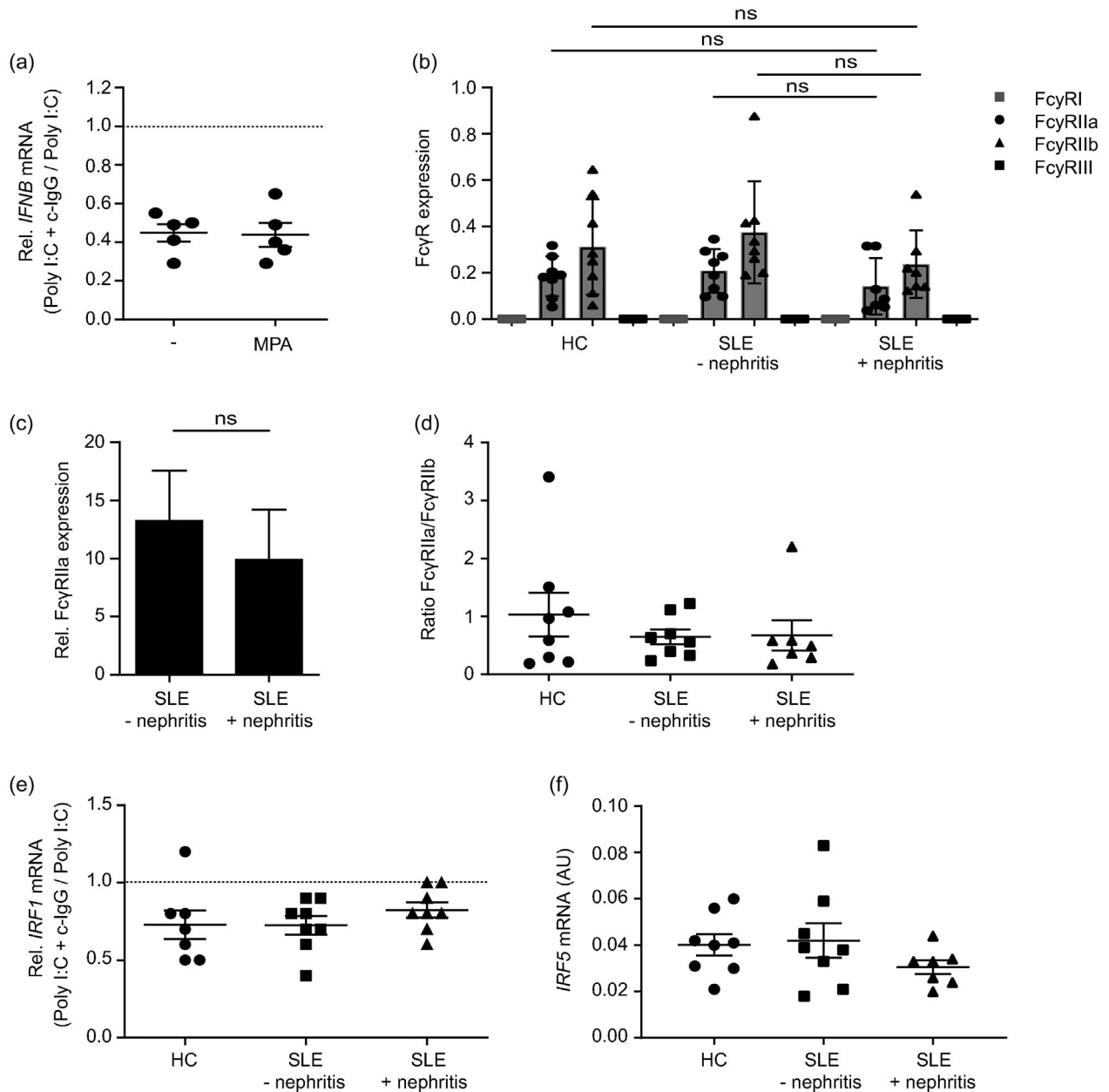


Fig. 2. Impaired Fc gamma receptor IIa (FcyRIIa)-induced type I IFN suppression in lupus nephritis patients is not due to medication or differences in FcyR, interferon (IFN) regulatory transcription factors (IRF)1 or IRF5 expression. (a) Dendritic cells (DCs) of healthy controls (HCs) were stimulated with Toll-like receptor (TLR)-3 ligand Poly I:C with or without complexed immunoglobulin (Ig)G (c-IgG) and/or mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF). Relative expression of *IFNB* by DCs was calculated by dividing mRNA expression at the peak of the response (Poly I:C + c-IgG)/Poly I:C; $t = 3$ h). Each pair of dots represents one donor; five donors in total. Paired two-tailed Student's *t*-test. (b) FcyR expression by DCs of HCs and systemic lupus erythematosus (SLE) patients with or without nephritis was determined at $t = 0$ h. (c) FcyRIIa expression on DCs of SLE patients with or without nephritis (both $n = 3$) was measured by fluorescence activated cell sorter (FACS). Data shown are relative FcyRIIa expression as determined by mean fluorescence intensity (MFI; FcyRIIa divided by isotype control); unpaired two-tailed Student's *t*-test. (d) Ratio of FcyRIIa/FcyRIIb was calculated by dividing FcyRIIa expression by FcyRIIb expression ($t = 0$ h). (e) DCs of HCs and SLE patients were stimulated with TLR-3 ligand Poly I:C with or without c-IgG. Relative expression of *IRF1* by DCs was calculated by dividing mRNA expression at the peak of the response (Poly I:C + c-IgG)/Poly I:C; $t = 3$ h). (f) *IRF5* expression was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) at $t = 0$ h. (b,d,e,f) Each dot represents one donor; unpaired one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test.

protein and mRNA levels. Surprisingly, we observed that IL-1 β amplification by Fc γ R co-stimulation was enhanced in lupus nephritis patients compared to SLE patients without major organ involvement (Fig. 3a,b). Next, we assessed the difference between HCs and the two subsets of SLE patients by calculating the relative amplification of IL-1 β . The amplification of *IL1B* mRNA was significantly elevated in lupus nephritis patients when compared to HCs or SLE patients without major organ involvement (Fig. 3c). Similar to IFN- β induction, DCs of lupus nephritis patients did not display different *IL1B* mRNA levels upon individual TLR-3 stimulation (Fig. 3d), indicating that the response to Fc γ R stimulation, in particular, is different in these patients.

Under particular circumstances, IFN- β has been described to promote IL-1 β production [17]. To determine whether the enhanced IL-1 β production in lupus nephritis patients is related to enhanced IFN- β production, we assessed the correlation between the inhibition of IFN- β and amplification of IL-1 β for the HC and patient groups (Fig. 3e). Remarkably, for all three different groups (i.e. HC, SLE without nephritis or SLE with nephritis), a stronger suppression of IFN- β (i.e. less IFN- β production) correlated with a stronger amplification of IL-1 β (Fig. 3e). Hence, these data argue against the hypothesis that in these cells elevated IFN- β levels are responsible for the enhanced IL-1 β production. However, these data confirm that the response of lupus nephritis patients is different from the HCs and SLE patients without nephritis, particularly by displaying less IFN- β suppression and more IL-1 β amplification (Fig. 3e).

The amplification of IL-1 β upon Fc γ RIIa-TLR co-stimulation is dependent upon signaling via the kinase Syk [18]. Although both Fc γ RIIa and TLRs have been described to signal via Syk, IL-1 β amplification induced by Fc γ RIIa-TLR cross-talk is specifically dependent upon Fc γ RIIa triggering [18,19]. Therefore, we next determined whether Syk expression is increased in DCs of lupus nephritis patients. However, *SYK* mRNA expression levels were similar between HCs and SLE patients (Fig. 3f). Combined, these data indicate that while Fc γ RIIa-induced type I IFN suppression is impaired, Fc γ RIIa-induced amplification of IL-1 β is increased in lupus nephritis patients.

Discussion

SLE is a complex autoimmune disease that is strongly associated, in the majority of patients, with high production of type I IFNs and other inflammatory mediators [5,20]. These cytokines are known to be induced by the presence of complexes formed by nuclear antigens and anti-nuclear autoantibodies. Increased responsivity to these SLE-associated immune complexes by immune cells of

patients would enhance the pathology even further, thereby increasing the risk of complications and severe organ damage. Here, we provide evidence of such increased responsivity of DCs of lupus nephritis patients to the combination of PRR ligands and IgG immune complexes, resulting in increased levels of type I IFN and IL-1 β .

Lupus nephritis is characterized by the accumulation of circulating and *in-situ*-formed immune complexes in the glomerulus. These immune complexes promote inflammation in the glomeruli and tubule-interstitium, which can lead to chronic scarring and end-stage kidney disease if not treated early and successfully [21]. The main receptors that recognize these immune complexes are members of the Fc γ R family, which are expressed by various local myeloid immune cells, including DCs, macrophages and monocytes [12]. Our data indicate that DCs of lupus nephritis patients respond aberrantly to the combination of PRR stimuli and IgG immune complexes, which may thereby contribute to increased local inflammation in the kidneys.

Importantly, we showed that the aberrant cytokine production by DCs of lupus nephritis patients was not due to differences in disease activity, medication or expression levels of Fc γ RIIa, Syk, IRF1 or IRF5. However, regarding the drug MMF, it should be acknowledged that immune cells of patients treated with MMF will be exposed to MPA for a longer period of time than we can possibly mimic in our *in-vitro* cultures, and therefore we cannot exclude the potential long-term effects of MPA exposure. The aberrant cytokine production by DCs of lupus nephritis patients was specifically apparent in response to Fc γ R co-stimulation. In contrast, no differences in cytokine production were observed upon individual stimulation of TLR-3. This is in line with a previous study that showed that TLR-4 stimulation induces similar levels of cytokines by cells of lupus nephritis patients compared to HCs and non-inflammatory renal disease patients [22]. Combined, these data indicate that particularly cytokine production by Fc γ RIIa is aberrantly regulated in lupus nephritis patients.

It has previously been shown that copy number variants in Fc γ Rs that are associated with lupus nephritis are characterized by reduced Fc γ R expression on myeloid cells [3,23]. This reduced Fc γ R expression has been hypothesized to worsen pathology by reducing the clearance of SLE-associated immune complexes [3]. In addition, this reduced Fc γ RIIa expression could explain the reduced suppression of type I IFN by Fc γ RIIa. However, we observed no differences in Fc γ RIIa protein and mRNA expression between lupus nephritis patients and other SLE patients. Potentially, Fc γ R protein expression differs to some extent between the groups, but may not have been observed because of the relatively small samples size used in this study. However, even that seems unlikely to explain the aberrant cytokine

production by DCs of lupus nephritis patients as the Fc γ RIIa-induced amplification of IL-1 β production was increased, which suggests increased (instead of suppressed) activation of Fc γ RIIa. In this regard, it is also important

to realize that Fc γ Rs in fact seem to be critical for the induction of renal inflammation, as Fc γ R knock-out mice are completely protected from lupus nephritis, even in the presence of autoantibodies [3,24].

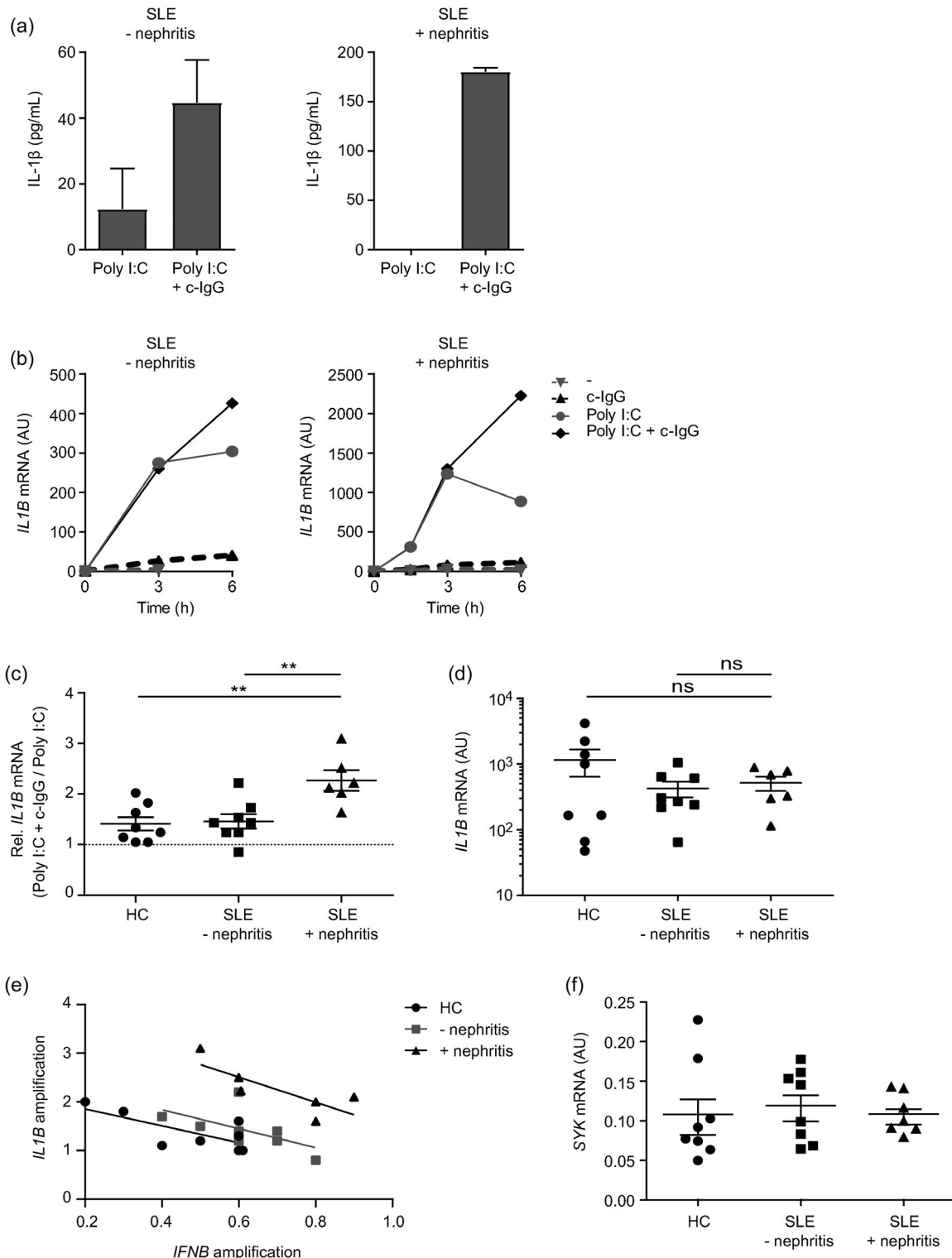


Fig. 3. Fc gamma receptor IIa (FcγRIIa)-induced production of interleukin (IL)-1β is increased in lupus nephritis patients. (a) Dendritic cells (DCs) of systemic lupus erythematosus (SLE) patients with or without nephritis were stimulated with Toll-like receptor (TLR)-3 ligand Poly I:C with or without complexed immunoglobulin (Ig)G (c-IgG). IL-1β protein expression was determined after 6 h. Representative donors [mean ± standard error of the mean (s.e.m.) in triplicate] of seven [healthy control (HC) HC; SLE – nephritis] or four (SLE + nephritis) independent experiments with different donors. (b, c, e) DCs of HCs and SLE patients were stimulated with Poly I:C with or without c-IgG. (b) mRNA expression was determined at the indicated time-points. Representative donors of eight (HC; SLE – nephritis) or six (SLE + nephritis) independent experiments with different donors. (c) Relative expression of *IL1B* by DCs was calculated by dividing mRNA expression at the peak of the response ((Poly I:C + c-IgG)/Poly I:C; *t* = 6 h). (d) DCs of HCs and SLE patients were stimulated with Poly I:C. *IL1B* mRNA expression was determined at the peak of the response (*t* = 6 h). (e) Correlation of *IFNB* and *IL1B* amplification. Each dot represents one donor. (f) *SYK* mRNA expression levels were determined in DCs of HCs and SLE patients at *t* = 0 h. (c,d,f) Each dot represents one donor. **P* < 0.05, ***P* < 0.01, unpaired one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test.

In addition to copy number variants in FcγRs, SNPs have also been associated with SLE and lupus nephritis, particularly a SNP in *FCGR2A* that results in an arginine instead of a histidine at position 131 (H131R). FcγRIIa-R131 has a reduced binding affinity for IgG subclasses [25] and is therefore hypothesized to worsen the pathology in lupus nephritis patients as a result of impaired clearance of IgG immune complexes in the kidneys [3]. However, while the *FCGR2A* R131 SNP reduces the phagocytic function of FcγRIIa, we have previously shown that this SNP does not affect FcγRIIa-induced cytokine production [12,26]. Hence, the *FCGR2A* R131 SNP seems to contribute mainly to pathology in lupus nephritis patients by decreasing FcγRIIa-dependent phagocytosis, but most probably not by increasing FcγRIIa-induced proinflammatory cytokine production.

Although the etiology of lupus nephritis is still unknown, it is known that both systemic and intrarenal events are important in the pathogenesis [3]. Within the kidney, the combination of nuclear antigens and autoantibodies mainly promote inflammation by activation of myeloid immune cells such as DCs, monocytes and macrophages [27]. However, systemically, SLE-associated nuclear antigens and immune complexes mainly drive inflammation by activation of plasmacytoid DCs (pDCs), which predominantly reside in blood and peripheral lymphoid organs and largely control IFN-α production [28,29]. In this regard, it is important to realize that, specifically for type I IFN production (but not for other cytokines), FcγR stimulation has opposite effects on myeloid immune cells (such as conventional DCs, Langerhans cells, monocytes and macrophages) versus pDCs. While FcγRIIa stimulation suppresses PRR-induced type I IFN (mainly IFN-β) production by conventional DCs [7], FcγRIIa increases type I IFN (mainly IFN-α) production by pDCs [30–33]. We identified that specifically the inhibitory function of FcγRIIa on type I IFN production by myeloid DCs is impaired, which will lead to increased type I IFN production mainly in local tissues. As such, particularly aberrant FcγRIIa responses by myeloid immune cells, instead of pDCs, may provide an explanation for the development of organ complications such as nephritis in this subset of SLE patients.

Notably, while suppression of type I IFNs by FcγRIIa was impaired, FcγRIIa-induced amplification of proinflammatory cytokine IL-1β was actually increased in lupus nephritis patients. In addition to type I IFN, IL-1β is also an important factor in the pathogenesis of lupus nephritis, which is illustrated by increased IL-1β expression in biopsies of lupus nephritis patients and by knock-out mouse models for SLE that are protected against kidney inflammation [9,34]. It is still unclear why in lupus nephritis patients the inhibitory function of FcγRIIa (that suppresses type I IFN) is impaired, while the activating function of FcγRIIa (that increases proinflammatory cytokine production) is overactive. It is tempting to speculate on a shift in the balance between the two different pathways that are induced by FcγRIIa. In healthy individuals, FcγRIIa signals through both a Syk-dependent (activating) pathway and a Syk-independent (inhibitory) pathway, leading to increased production of proinflammatory cytokines (IL-1β, TNF, etc.) and simultaneous inhibition of type I IFNs, respectively [7,10,12,18]. Within the different experimental groups (HCs, SLE without nephritis and SLE with nephritis) IFN-β suppression correlated with IL-1β amplification, indicating that, in general, stronger Syk-dependent signaling correlates with stronger Syk independent signaling within an individual. In DCs of lupus nephritis patients, however, FcγRIIa stimulation appears to mainly lead to (over)activation of the Syk-dependent pathway, while the Syk-independent pathway is dysfunctional (see also Fig. 4 for a schematic representation).

While we verified that Syk expression is not different in lupus nephritis patients, a limitation of this study is that we were not able to directly compare Syk activation by phosphorylation because of the limited number of available cells from these patients. Therefore, direct evidence for increased Syk activation in DCs of lupus nephritis patients is still unavailable, and will be an important topic for future research. In addition, in DCs of lupus nephritis patients we observed a trend towards reduced expression of FcγRIIa and IRF5 and an increase in IRF1. Although these differences were not statistically significant, future studies using larger numbers of patients may demonstrate

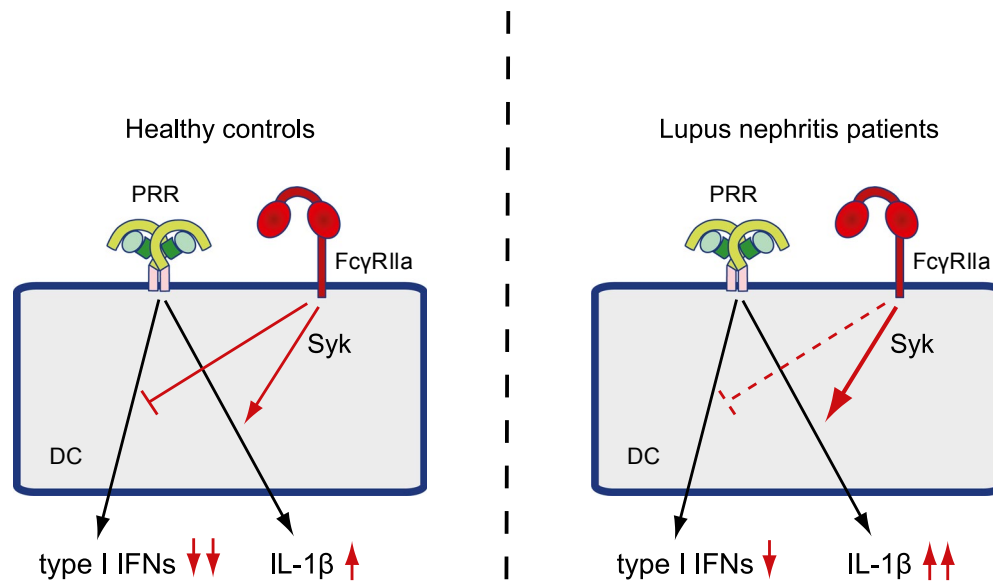


Fig. 4. Graphic summary of dysregulated Fc gamma receptor IIa (Fc γ RIIa)-induced cytokine production in dendritic cells (DCs) of lupus nephritis patients. In healthy individuals, immune complexes stimulate Fc γ RIIa, which signals through two parallel pathways: an inhibitory pathway that suppresses type I interferon (IFN) and is Syk-independent, and an activating pathway which amplifies the production of proinflammatory cytokines such as IL-1 β and is Syk-dependent. In lupus nephritis patients these pathways are dysregulated, leading to (over)activation of the Syk-dependent pathway (resulting in more IL-1 β), while the Syk-independent pathway is dysfunctional (resulting in higher type I IFN levels).

significant changes in expression of these proteins. In this regard, increased IRF1 expression (important for type I IFN transcription) may provide an explanation for the reduced inhibition of IFN- β . However, how reduced expression of Fc γ RIIa and IRF5 could cause increased IFN- β and IL-1 β expression is still unclear.

Taken together, our data demonstrate that Fc γ R-mediated cytokine production is dysregulated in DCs of lupus nephritis patients, resulting in increased production of disease-associated cytokines such as type I IFN and IL-1 β .

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Author contributions

Conceptualization: J. d. D.; methodology: M. N., R. F., L. S., W. H. and J. B.; resources: R. F., A. H. and M. S.; writing: original draft, M. N., J. d. D.; writing, review and editing: M. N., R. F., L. S., A. H., R. V., D. B., J. d. D.; supervision: D. B. and J. d. D.

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