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Intracellular interferon-β and distinct type I IFN expression patterns in circulating SLE B cells

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Abstract

In systemic lupus erythematosus (SLE), type I interferons (IFN) promote induction of type I IFN stimulated genes (ISG) and can drive B cells to produce autoantibodies. Little is known about the expression of distinct type I IFNs in lupus, particularly high-affinity IFNβ. Single cell analyses of transitional B cells isolated from SLE patients revealed distinct B cell sub-populations, including type I IFN-producers, IFN-responders and mixed IFN-producer/responder clusters. Anti-Ig plus TLR3 stimulation of SLE B cells induced release of bioactive type I IFNs that could stimulate HEK-blue cells. Increased levels of IFNβ were detected in circulating B cells from SLE patients compared to controls and were significantly higher in African American (AA) patients with renal disease and in patients with autoAbs. Together, the results identify type I IFN producing and responding sub-populations within the SLE B cell compartment and suggest that some patients may benefit from specific targeting of IFNβ.

INTRODUCTION

Activation of the type I IFNs, consisting of 13 IFNα and one high-affinity IFNβ sub-types, is highly associated with the development of SLE as well as clinical disease manifestations (1). Type I IFNs can be produced by most cell types though their activity in SLE is most often measured indirectly using the presence of specific type I IFN inducible transcripts, termed the type I IFN signature (1, 2). Previous studies have identified unique type I IFN signatures among different immune cell populations (3–7) but cell-specific expression patterns and roles of distinct type I IFNs in SLE, especially high affinity IFNβ, remain elusive (8), largely due to their low levels of transcription and circulation (9).

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In autoimmune mice, IFNAR deficiency ameliorates germinal center (GC) and autoantibody development (10, 11). Although a previous study in NZB mice reported no difference in anti-chromatin Abs or renal disease in *Ifnb*$^{-/-}$ mice (12), these findings have been challenged by reports that IFNβ is elevated and dysregulated in SLE (13, 14) as well as the identification of distinct IFN signatures not restricted to IFNα (15). Serum detection of IFNβ was recently associated with disease flares, particularly in in African American (AA) patients, a population with increased disease prevalence, severity and robust type I IFN dysregulation (14, 16). Autocrine IFNβ signaling has been identified as a mechanism of type I IFN dysregulation in SLE mesenchymal stem cells (13), and B cells have also been shown to produce type I IFNs in SLE and other diseases (9, 17, 18). In this study, we examined expression patterns of type I IFNs and their target ISGs in circulating B cells and identified a potential role for B cell-associated IFNβ in SLE.

METHODS

Clinical Samples.

All SLE subjects met the American College of Rheumatology 1997 revised criteria for SLE (19) and were recruited from the UAB Lupus Clinic. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep/SepMate, StemCell Technologies). Clinical data were determined by the UAB clinical laboratory and attending physician. All data were collected in a double blinded manner.

Study approval.

These studies were conducted in compliance with the Helsinki Declaration and approved by the institutional review board at UAB. All participants provided informed consent.

Flow Cytometry analysis.

Human antibodies included BioLegend BV510-anti-CD24 (ML5), PE-anti-CD303 (clone 201A), BV510-anti-IgM (clone MHM-88), Pacific-Blue-anti-CD4 (clone RPA-T4), PE-Cy7-anti-CD10 (clone HI10a), BV650-anti-CD27 (O323), Pacific Blue-anti-CD19 (HIB19), PE-Cy7-anti-CD38 (HB-7); Southern Biotech PE-IgD (IADB6), and PBL Assay Science FITC-anti-IFNβ (MMHB-3). Dead cells were excluded from analysis with APC-eFluor® 780 Organic Viability Dye (eBioscience). For intracellular staining, cells were stained with ef780 viability dye, followed by fixation in 2% PFA and 70% ice-cold methanol permeabilization prior to staining. Purity was validated by post-sort analysis of FACS sorted cells to verify that >99% of cells fell into the sort gate after resorting. FACS data were acquired with an LSRII FACS analyzer (BD Biosciences) and analyzed with FlowJo software (Tree Star Ashland, OR).

Super-resolution structured illumination microscopy (SIM).

Negative selection purified B cells (StemCell Technologies) were fixed in 2% paraformaldehyde and permeabilized with Triton X-100, followed by blocking with 2% BSA. Cells were stained with AF647 goat-anti-human IgM (Southern Biotech), FITC mouse-anti-human IFNβ (PBL Assay Science clone MMHB-3) or polyclonal rabbit-anti-

human IFNβ (Abcam), followed by anti-rabbit IgG AF488 secondary Ab. DAPI nuclear stain was included for nucleus determination (200 ng/mL).

Super-resolution imaging was carried out using the Nikon N-SIM-E super-resolution microscope (resolution capable of 120 nm *x-y* and 300 nm *z*). Images were acquired with a 100× 1.49 NA objective, Orca-Flash 4.0 sCMOS camera (Hamamatsu), 488, 561, and 640 nm laser excitation, a multi-pass dichroic cube and specific emission filters. DAPI images were acquired with a widefield excitation and are at conventional resolution. Images were acquired and reconstructed with Nikon Elements software. Staining intensity and localization of IFNβ were carried out using Fiji/ImageJ.

Real-time quantitative RT-PCR.

RNA isolation, cDNA synthesis, and real-time PCR reactions were carried out as described previously (20, 21). Primers for GAPDH are: forward, GCACTCACTGGAATGACCTC, backward, TTCTTCGCACTGACACACTG. All primers used for single cell analysis are described in Supplemental Table 1.

Single cell gene expression analyses:

Gene expression analysis of single transitional B cells ($CD24^+CD38^+IgD^+CD27^-$) obtained from SLE PBMCs was performed using the Fluidigm single cell capture and BioMark RT-PCR analysis system (Fluidigm Co., South San Francisco, CA) as described in detail previously (22). Single cell gene expression clustering analysis was carried out using the ClustVis online web tool (23). Single cell gene expression data can be retrieved at https://biit.cs.ut.ee/clustvis/?s=zlbtSCZKfAcPrUg

In vitro B-cell secretion of type I IFN assay.

B cell production of functional type I IFNs was analyzed using coculture of purified primary B cells (5.0×10^5 cells per well) with HEK-Blue IFNα/β cells which expressed an inducible secreted embryonic alkaline phosphatase (SEAP) (InvivoGen, San Diego, USA). B cells purified using the Human Pan B Cell Isolation Kit (Miltenyi Biotech) were were then unstimulated or stimulated for 10 min with an F(ab')2 anti-hIg (IgM+IgG) antibody (ThermoFisher) (10 μg/mL) plus 5 μg/mL poly(I:C) or 5 μg/mL CL264 (InvivoGen) before HEK-Blue IFNα/β cells (2.5×10^4 cells per well) were added into the medium. Supernatants were collected at the 24 hr time point and were incubated 1 hr with the Quanti-Blue™ colorimetric enzyme assay reagent for determination of absorbance at OD_{650}.

Statistics.

Results are mean ± standard deviation (s.d.) or mean ± standard error of the mean (s.e.m.) as described in figure legends. P values of less than 0.05 were considered significant. Unless otherwise indicated, all analyses were performed using GraphPad Prism software (La Jolla, CA).

RESULTS and DISCUSSION

Type I IFN producing and response genes in SLE transitional B cells.

We previously showed that type I IFN expression is a prominent feature of T1 B cell development in BXD2 autoimmune mice and that T1 B cell IFNβ acts in an autocrine priming mechanism to promote *Ifna* and ISG expression (22). Analysis of type I IFN gene expression from SLE patients revealed a significant increase in the expression of *IFNB*, *IFNA1*, *IFNA14*, *IFNA17* and *MX1* in transitional (Tr) B cells from African American (AA) patients with SLE (Supplemental Figure 1A). To determine whether distinct type I IFN and ISG gene expression patterns were present in SLE B cells, Tr B cells from three female AA SLE subjects as described in Supplemental Fig. 1B were FACS sorted and analyzed for expression of *IFNB*, *IFNA*, and *ISGs* (Fig. 1A). Hierarchical clustering analysis revealed three prominent clusters with distinct gene signatures, including a mixed IFN and ISG producer/responder signature ($IFN_{P/R}$), an IFN-responder signature (IFN_R) and an IFN-producing signature (IFN_P) (Fig. 1A, B, Supplemental Table 1). Cells from all 3 patients were equally represented in each cluster, revealing the presence of these major clusters in different SLE patients (Fig. 1C).

Cells within the $IFN_{P/R}$ cluster expressed the highest levels of Tr B cell marker *CD24* (Fig. 1D, upper left). Cells within the $IFN_{P/R}$ cluster also expressed higher levels of *IFNB*, *IFIT1*, *IRF7, IRF9, ZBP1, IFNA1*, *IFNA7*, and *CCND1*, compared to the IFN_R or IFN_P cluster or both (Fig. 1D, Supplemental Table 1). This is consistent with our previous observations in BXD2 mice where *IFNB* expression was upregulated in early T1 B cells (22). Cells within the IFN_R cluster expressed higher levels of *IFNAR1*, *MX1*, *IFIT2*, *PKR, RIG1 and CCND2* (Fig. 1E). The IFN_P cluster was characterized by higher levels of *IFNA4*, *IFNA5*, *IFNA10*, *IFNA14, IFNA16* and *IFNA17* (Fig. 1F). Expression of *TLR3* and *TLR7*, but not *TLR9* were different among the groups (Supplemental Fig. 1C). Single cell gene expression and cell identities were validated by analysis of CD20, CD3, and CD303 expression (Supplemental Fig. 1D). Together, the results reveal heterogeneous type I IFN producing and responding signatures in circulating transitional B cells, suggesting that B cells are not only type I IFN targets, but also producers of type I IFNs in SLE.

Increased intracellular IFNβ in SLE B cells.

FACS staining (Fig. 2A) and confocal imaging (Fig. 2B) of IFNβ revealed increased levels of IFNβ in B cells from SLE patients compared to HCs. Quantification of IFNβ MFI revealed that while transitional and naïve B cells from SLE patients exhibited significantly increased IFNβ compared to HCs, in CD4 T cells and $CD303^+CD4^{low}$ pDCs (24), IFNβ levels were not significantly increased in SLE compared to HCs (Fig. 2A). Staining of IFNβ was specifically inhibited by pre-incubation with human IFNβ, but not mouse IFNα (Supplemental Fig. 2A). We next determined the sub-cellular localization of IFNβ in SLE B cells. Two anti-IFNβ antibodies detected a mainly cytoplasmic distribution of IFNβ in IgM^+ B cells (Fig. 2C, Supplemental Fig. 2B). Although significantly less prominent compared to the cytoplasmic localization, IFNβ was also detected in the nucleus (Fig. 2C) as previously reported for other cell types (25, 26). Western blot analysis of cytoplasmic extracts from isolated SLE B cells *ex vivo* further confirmed the presence of a 25 and 50 kDa band

(Supplemental Fig. 2C) consistent with the predicted molecular weight of human IFNβ monomer and dimer (27, 28). Together, these results confirm the presence of cytoplasmic IFNβ in SLE B cells.

To determine if B cells produced biologically active type I IFNs, an HEK IFNα/β reporter cell line assay was carried out. Stimulation of the human B cell lymphoma cell line Ramos with TLR3 ligand poly(I:C) induced the highest IFNβ response compared to a TLR7 ligand (CL264) and a TLR9 ligand (ODN-2006) as measured by both intracellular FACS for IFNβ (Supplemental Fig. 2D, left) and by the HEK reporter assay (Supplemental Fig. 2D, right). Stimulation of purified SLE B cells with anti-Ig + TLR3 induced an increased HEK reporter response compared to B cells derived from healthy controls (Fig. 2D). As an additional control, levels of IFNβ measured in the FACS assay were correlated with IFNβ protein secretion as measured by the HEK IFNα/β reporter assay (Fig. 2E). These results are consistent with previous findings by Gram et al (29) which reported type I IFN secretion by human B cells upon poly(I:C) stimulation and further suggest the importance of further evaluation of in vivo TLR3 ligands including U1 RNA that may be associated with B cell type I IFN secretion in SLE patients (30).

Intracellular IFNβ is associated with autoantibody production, renal disease and AA race.

The higher expression and production of IFNβ from SLE B cells suggest that B cell intracellular IFNβ may be an important factor associated with SLE pathogenesis. We identified that patients who were seropositive for anti-dsDNA at the time of specimen collection exhibited significantly increased levels of IFNβ (MFI) in transitional and $CD27^+$ memory B cells, compared to SLE patients who were seronegative for anti-dsDNA at the time of collection (Fig. 3A). Subjects who were positive for anti-Sm at any time during their disease course exhibited a significant increase in IFNβ levels in transitional and $CD27^+$ memory B cells, whereas subjects who were seropositive for anti-SSA exhibited increased IFNβ expression in transitional, naïve and $CD27^+$ B cells (Fig. 3B–C). These data suggest an association between IFNβ expression and enhanced survival of B cells exhibiting reactivity with nucleic acid/protein complexes able to co-activate BCR and TLR signaling (31).

SLE subjects with a history of renal disease also exhibited a significant increase in IFNβ in transitional and naïve B cells compared to SLE patients without a history of renal disease (Fig. 3D). Interestingly, intracellular IFNβ in transitional and naïve B cells was significantly higher in AA compared to non-AA patients (Fig. 3E). Levels of IFNβ in SLE B cells were generally significantly higher compared to healthy controls (HC), even in comparisons between autoAb-negative or otherwise low severity patients and HCs (Fig. 3A–E). B cell endogenous IFNβ levels were not significantly different in SLE subjects with low complement or other clinical parameters including SLEDAI or hydroxychloroquine (HCL) treatment, but were significantly higher compared to healthy controls (Fig. 3F–H).

These results suggest that B-cell IFNβ is most strongly associated with increased autoantibodies and renal disease and that polymorphisms in the IFNβ enhanceosome genes or other upstream genes may predispose some patients to the development of type I IFN dysregulation and autoimmune disease (32). This notion supported by recent population

level studies which identified the *IFNB* locus as a *trans*-regulatory hotspot that controlled antiviral networks enriched in genes differentially expressed in AA vs. European American healthy volunteers (33). Together, the present findings support the importance of cell-specific analyses of both IFNs and IFN response genes in patients of defined ancestral backgrounds, as type I IFN expression may not be highlighted in analyses of patient groups with diverse genetic ancestry. It is important to note that the present PCR-based targeted single-cell gene expression analysis approach was selected in order to detect IFN pathway genes which exhibit a broad expression range spanning from lower expressed type I IFN genes to higher expressed ISGs. The detection of type I IFN genes may be more challenging in conventional RNA-seq analyses as detection of these genes can be limited by read-depth. The present single-cell analyses reveal a new level of understanding in type I IFN dysregulation, as the proper regulation of these type I IFN-producing and -responding populations in early B cells may influence functional cell trajectories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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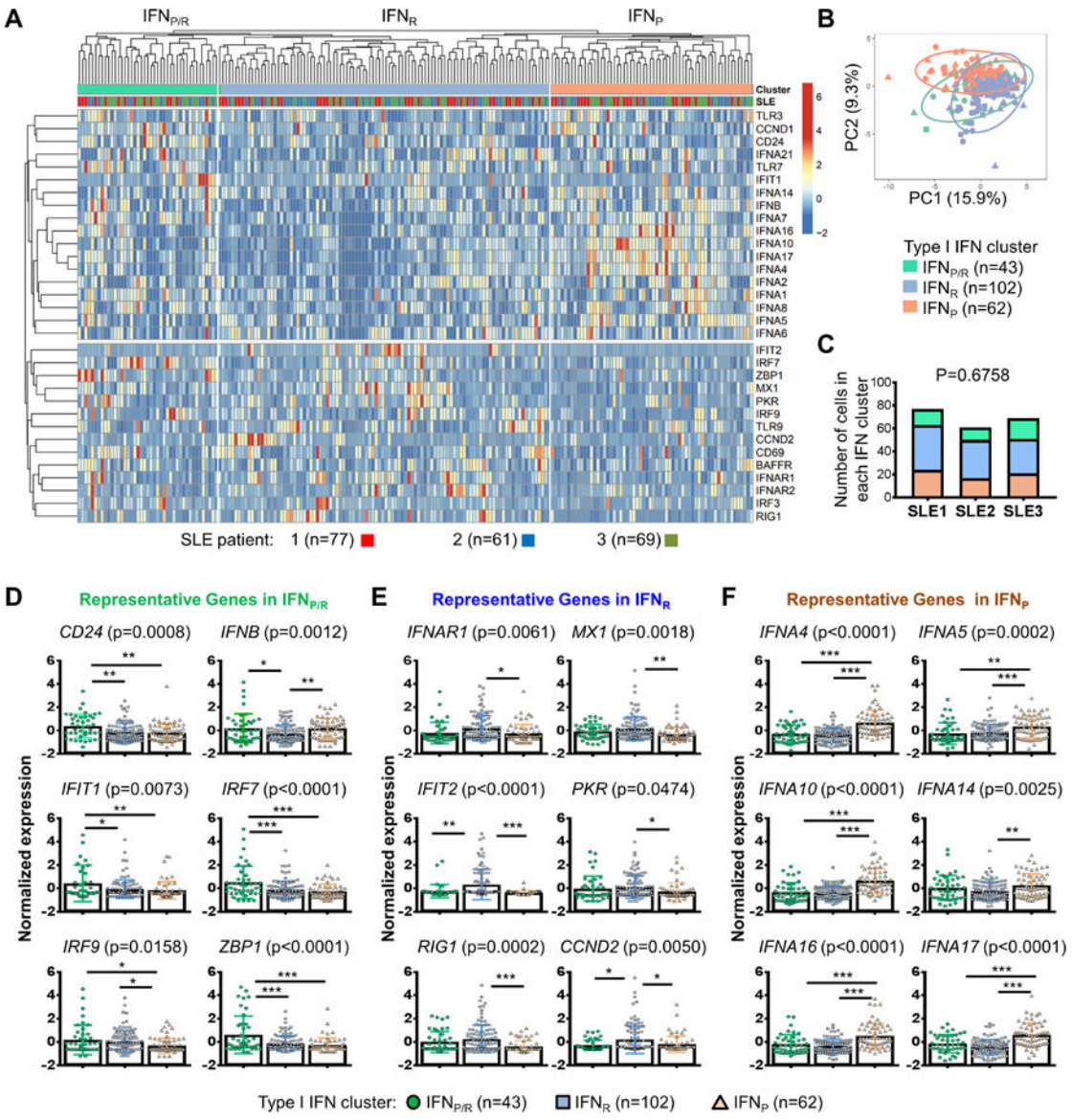

Figure 1. Type I IFN and ISG gene expression in single transitional B cells from SLE patients.

Transitional B cells ($CD24^+CD38^+IgD^+CD27^-$) isolated from PBMCs of 3 SLE patients (see Supplemental Fig. 1B) were prepared for single cell gene expression analysis. (**A**) Heat map of hierarchically clustered type I IFN gene expression clustering in individual B cells (n = 207). The top row above the heat map is color-coded to denote cell clustering and SLE patient origin. (**B**) Principal component analysis of SLE transitional B cell clusters. The X and Y axis show principal component (PC)1 and PC2 that explain 15.9% and 9.3% of the total variance, respectively. PCA was carried out based on PC1 and PC2 segregation of 32 genes in the $IFN_{P/R}$, IFN_P, or IFN_R B cell clusters. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. **(C)** Bar graph showing the number of single cells designated to each type I IFN cluster based on the gene expression profile from each individual SLE patient (Chi-square analysis). (**D-F**) Dot plots showing the normalized expression of representative genes in the mixed type I IFN

producing and responding cluster ($IFN_{P/R}$) (**D**), the type I IFN responding cluster (IFN_R) (**E**), and the type I IFN-producing (IFN_P) (**F**) cluster of B cells as defined by hierarchical clustering. All results are mean ± standard deviation. Significant differences among means were analyzed using a one-way ANOVA test with p value shown on the top of each graph. Differences between groups were analyzed using Tukey's multiple comparisons test. Results are shown as mean ± SD (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ between the indicated groups).

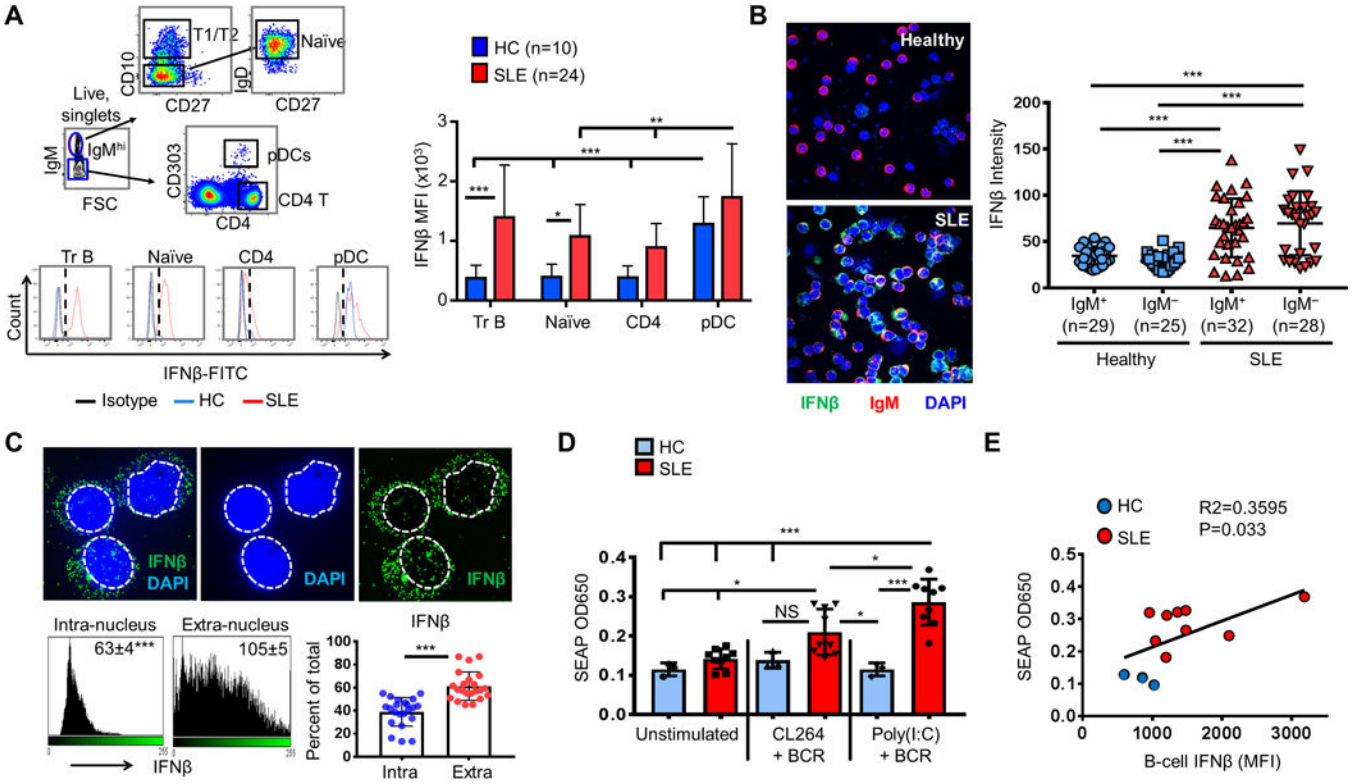

Figure 2. Increased expression of intracellular IFNβ in B cells from a subset of SLE patients.

(**A**) Gating strategy (upper left), representative histograms (lower left), and summary of IFNβ MFI (right) in circulating transitional (Tr) B cells, naïve B cells, CD4 T cells, and pDCs in SLE compared to HC. (**B**) Confocal microscopy imaging (left) and bar graph quantitation of IFNβ intensity (right) in purified B cells from a representative HC or an SLE patient (objective lens = 20×). (**C**) SIM super-resolution imaging and analysis of IFNβ intracellular localization in representative B cells from SLE patients. Top: representative images showing intra- versus extra-nuclear staining of IFNβ. The nucleus-cytoplasmic border was defined by DAPI staining (dotted white line). Bottom: ImageJ quantitation of intra and extra-nuclear intensity (left) and distribution (right) of IFNβ (n = 22 cells from 3 SLE patients). (**D**) HEK-blue reporter cell analysis of type I IFN secretion by B cells from SLE (n=9) or HC (n-3) under the indicated conditions of stimulation. (**E**) Correlation of FACS detection of baseline (*ex vivo*) B-cell IFNβ (MFI) with HEK-blue analysis of IFNβ secretion from anti-Ig plus poly(I:C) stimulated B cells.

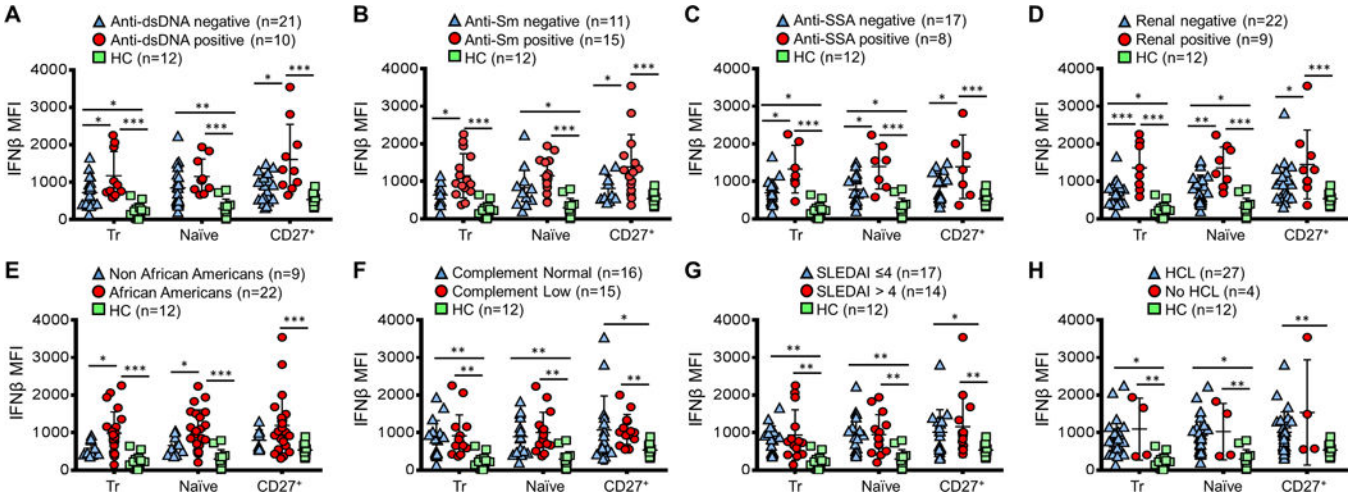

Figure 3. Elevated B cell IFNβ in autoantibody positive AA SLE patients.

The MFI of IFNβ in the indicated populations of B cells in healthy controls (HC) or in SLE patients segregated by (**A**) positivity of anti-dsDNA, (**B**) historic positivity of anti-Sm or (**C**) anti-SSA (**D**), the presence of renal disease, (**E**) race: non-African Americans versus African Americans, and (**F**) complement (C3/C4) normal versus low, (**G**) SLEDAI ≤4 versus >4 (and (**H**) with or without hydroxychloroquine (HCL) treatment. All clinical characteristics, except anti-Sm and anti-SSA, were collected at the time of PBMC sample collection (Results are mean ± SD. Statistical differences were determined by Mann–Whitney U test; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ between the indicated comparisons).