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Neutrophil-Mediated IFN Activation in the Bone Marrow Alters B Cell Development in Human and Murine Systemic Lupus Erythematosus

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Inappropiate activation of type I IFN plays a key role in the pathogenesis of autoimmune disease, including systemic lupus erythematosus (SLE). In this study, we report the presence of IFN activation in SLE bone marrow (BM), as measured by an IFN gene signature, increased IFN regulated chemokines, and direct production of IFN by BM-resident cells, associated with profound changes in B cell development. The majority of SLE patients had an IFN signature in the BM that was more pronounced than the paired peripheral blood and correlated with both higher autoantibodies and disease activity. Pronounced alterations in B cell development were noted in SLE in the presence of an IFN signature with a reduction in the fraction of pro/pre-B cells, suggesting an inhibition in early B cell development and an expansion of B cells at the transitional stage. These B cell changes strongly correlated with an increase in BAFF and APRIL expression in the IFN-high BM. Furthermore, we found that BM neutrophils in SLE were prime producers of IFN-α and B cell factors. In NZM lupus-prone mice, similar changes in B cell development were observed and mediated by IFN, given abrogation in NZM mice lacking type-I IFNRI. BM neutrophils were abundant, responsive to, and producers of IFN, in close proximity to B cells. These results indicate that the BM is an important but previously unrecognized target organ in SLE with neutrophil-mediated IFN activation and alterations in B cell ontogeny and selection. The Journal of Immunology, 2014, 192: 906–918.

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that affects multiple target organs. Both the innate and adaptive arms of the immune system contribute to the pathogenesis of this autoimmune disorder (1, 2). With respect to innate immune system dysregulation, inappropriate activation of type-I IFN plays a critical role in the pathophysiology of SLE (3, 4). IFN, a key mediator molecule capable of mounting a first line of antiviral response also possesses multiple immune-modulatory properties that include differentiation of monocytes into APCs, activation of T lymphocytes, and differentiation of B lymphocytes into Ab-producing plasma cells (5, 6).

Plasmacytoid dendritic cells (pDCs) are the major producers of type-I IFN in response to infection by a wide array of viruses. pDCs express TLR7 and -9, which recognize ssRNA and demethylated CpG, respectively, leading to the initiation of JAK/STAT signaling cascade, resulting in abundant secretion of type-I IFN (7). Several lines of evidence indicate the connection between type-I IFN and development of SLE in murine and human studies. Administration of type-I IFN to mice accelerates the development of autoimmune arthritis associated with glomerulonephritis (8). In humans, elevated levels of IFN in the serum of lupus patients were reported almost three decades ago (9). An important link between IFN and SLE was revealed by studies of patients receiving IFN-α as a therapeutic agent against malignant carcinoid tumors or viral hepatitis, with a subset developing autoimmune phenomena, including Abs against dsDNA and clinical lupus (10). The role of IFN activation in the initiation and propagation of the disease has been further highlighted by the seminal finding of upregulation of IFN-inducible...
genes in the peripheral blood (PB) of SLE patients (11, 12). Both pDCs and, more recently, neutrophils (13) have been implicated as drivers of IFN activation in SLE.

Within the adaptive compartment of the immune system, dysregulation of B cells has been shown to play a critical role in SLE (14). Because the disease is characterized by the generation of large amounts of autoantibodies directed against chromatin and other self-Ags, the loss of B cell tolerance clearly plays a key role (15). B cells contribute to the immune pathogenesis and end-organ damage in SLE via both Ab-dependent and -independent pathways. In an autoimmune setting, B cells can present self-Ag, activate T cells, and produce proinflammatory cytokines including TNF-α and IL-6, in addition to secreting autoantibodies (16–18). Autoantibodies produced by B cells and RNA- and DNA-containing immune complexes in SLE stimulate pDCs to produce large quantities of IFN-α (19–22) and also contribute to the more recently identified neutrophil activation characteristic of the disease, thereby establishing a critical link between the adaptive and innate compartments of the immune system (13).

Interestingly, it has been demonstrated previously that IFN-α impairs B cell lymphopoiesis in the bone marrow (BM) of young normal mice (23). Moreover, lupus-prone mice exhibit an age- and autoantibody-related decline in B cell lymphopoiesis at the same stage as the inhibition mediated by IFN and an expansion of IFN-producing, TLR9-expressing pDCs in the BM (24). Overall, these results raise the intriguing possibility that B cell lymphopoiesis may be altered in human lupus due to the presence of TLR-stimulating, interferonogenic autoantibodies that have direct BM-mediated effects. Although limited studies have found impaired BM stromal cell function in human SLE (25), the idea that the BM may be an important target organ in SLE is largely unexplored. Given aberrant type-I IFN activation in the PB compartment in human SLE, we examined IFN-regulated gene expression in the BM and changes in B cell development. In the current study, we report for the first time, to our knowledge, the presence of type-I IFN activation in the BM microenvironment in human SLE in association with a reduction in the commitment of precursor cells into the B cell lineage and increased differentiation of B cells into the transitional compartment. We also find that neutrophils are a prime producer of type-I IFN in the SLE BM and produce key mediators that affect B cell development, including APRIL and BAFF. Data in murine lupus further implicate neutrophils as a key BM population both stimulated by and producing type-I IFN and mediating altered lupus further implicate neutrophils as a key BM population both stimulated by and producing type-I IFN and mediating altered

Materials and Methods

Study population and sample procurement

Detailed written informed consent was obtained from all patients and healthy donors, in accordance with protocols approved by the Human Subjects Institutional Review Board of the University of Rochester Medical Center. Clinical data included a comprehensive medical history, medications profile, clinical laboratory tests, and assessment of SLE disease activity by the SLE Disease Activity Index (SLEDAI) (Table 1) (26). Autoantibodies were measured by clinical multiplex assay (Bio-Rad). Research BM aspirates and paired PB samples were drawn from 28 SLE patients and ≥20 normal controls (NC). BM mononuclear cells (BMNCs) and PBMCs were isolated from heparinized BM aspirate and heparinized PB by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). To avoid PB contamination of marrow aspirates, aspirate volumes per draw were limited with careful top position adjustments during the procedure. When nucleated cell differentials were performed between the first and last marrow draws, the percentage of nucleated RBCs was 33 ± 4.7% in the first draw and 27 ± 4.5% (n = 4; mean ± SEM) in the last draw, suggesting minimal change in PB admixture through the procedure. Preparation of BM smears and flow cytometry assessment also demonstrated the expected high frequency of precursor populations in the BM aspirates.

RNA preparation and quantitative PCR

For RNA isolation, PB or BM aspirate was placed in PAXgene tubes (PreAnalytix, Franklin Lakes, NJ) and total RNA isolated according to the manufacturer’s protocol with on-column DNase treatment. RNA yield and integrity were assessed using an Agilent Lab-on-a-Chip Bioanalyzer (Agilent Technologies, Palo Alto, CA). In some experiments, CD10+ cells were first purified from the BM aspirate. Total cellular mRNA was reverse-transcribed to cDNA immediately following purification using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was used to quantify specific cDNA using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA).

The expression of five IFN-regulated genes was measured (Clorf29, IFIT1, IRF7, GIP2, and CEBl) by quantitative PCR (qPCR) (12). In a subset of patients, examination of three IFN-inducible genes—IFIT1, IRF7, and GIP2—was performed, as this was sufficient to assess IFN activation. These three to five genes were selected because they exhibited the highest correlation with a full 82-gene IFN signature generated by microarray (R² = 0.988) (J.W. Bauer and E. Baeucher, unpublished observations). Data were normalized to a housekeeping gene (GAPDH) that was not differentially regulated in the PB compartment in human SLE. Data in murine studies, one IFN-regulated gene (Ms-1), IFN-α, IFN-β, BAFF, and APRIL, and one housekeeping gene (β-actin) were quantified by real-time PCR as described.

Measurement of serum and BM chemokine and cytokine levels

BM supernatant and PB serum were collected from heparinized BM aspirates and PB drawn in serum tubes, respectively, following a centrifugation. Serum (light,uffy coat) (G1P2) chemiluminescence-based immunoassays were used to measure serum levels of CCL19 (MIP-3β), CXCL10 (IP-10) and CCL2 (MCP-1) were measured by multiplex (Milliplex MAG 29; Millipore). These chemokines are IFN regulated, and they exhibited the strongest correlations with disease activity in our prior publications (27). Additional cytokines and chemokines were measured on the MAG 29. Concentrations (picograms per milliliter) were determined using seven-point standard curves that were generated from reconstituted samples. Each assay was run in duplicate. BAFF levels were measured by ELISA per the manufacturer’s instructions (Quantikine; R&D Systems, Minneapolis, MN).

Analysis of IFN activation in purified cell populations

PB from five SLE patients and three NC was collected, and gene expression patterns were compared between four different cell populations. Blood was collected in Vacutainer CPT Cell Preparation Tubes with sodium citrate, and cell fractions were prepared as follows. 1) Whole blood (WB): RNA was purified from 1.5 ml WB after lysis in Tri Reagent RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. 2) RBCs: WB was centrifuged for 15 min at 2000 × g. The RBC pellet was suspended in an equal volume of distilled PBS plus 2% FBS after removal of the plasma and buffy coat. The RBC suspension was passed through a Streamlock filter (StreamLock Technologies) to remove BM neutrophil fractions using similar methods. For murine studies, one IFN-regulated gene (Ms-1), IFN-α, IFN-β, BAFF, and APRIL, and one housekeeping gene (β-actin) were quantified by real-time PCR as described.

PBMCs: the CPT tube was centrifuged for 3 min. The RBC pellet was passed through a LeukoLock filter (Ambion Life Technologies) to remove residual

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**Isolation and analysis of BM neutrophils**

BM neutrophils were isolated using previous published protocols (28, 29). In brief, Percoll gradient solutions with various densities were prepared from Percoll (Sigma-Aldrich) using 10× PBS (Invitrogen). Cells isolated in the top gradient (called M1) contained mostly immature neutrophils, whereas cells isolated in the lower gradient (M2) consisted primarily of mature neutrophils. Phenotype was confirmed by flow cytometry as previously described (30). In some instances, CD66b magnetic isolation was used for neutrophil purification. Morphology was confirmed by Giemsa staining and BAFF, APRIL, and IFN protein expression defined by immunofluorescence or flow cytometry. Murine BM neutrophils were isolated as described previously (31). Briefly, BM was flushed from femurs and tibias with HBSS with 15 mM EDTA. Cells were then spun on a discontinuous Percoll gradient (52, 69, and 78%) at 1500 × g for 30 min. Cells from the 69–78% interface were isolated, and RBCs were lysed.

**Analysis of BM pDCs**

BMMCs and PBMCs were stimulated with CpG 2216 (type A: potent pDC stimulator) or CpG 2006 (type B: potent B cell stimulator) or TLK 7/8 ligand R848 (5 μg) for 2 h at 37°C, followed by protein transport inhibition (brefeldin A) for 2 h and staining for surface Line1 (CD3, CD14, CD16, CD19, CD20, and CD64), CD304, CD123 markers, and intracellular IFN-α.

**Flow cytometry analysis of human B cell populations**

Flow cytometry was performed on a subset of patient samples. Single-cell suspensions of Ficoll-isolated BMMCs or PBMCs (10^6/sample) were labeled at 4°C with predetermined optimal concentrations of fluorophore-conjugated mAbs against CD19, IgD, CD27, IgM, CD24, and CD38 surface markers. Pair-matched isotype controls were also used. Previously, the mAbs were formalin-fixed and labeled at 4°C with predetermined optimal concentrations of fluorophore-conjugated mAbs against CD19, IgD, CD27, IgM, CD24, and CD38 surface markers. Pair-matched isotype controls were also used. The mAbs were formalin-fixed and labeled at 4°C with predetermined optimal concentrations of fluorophore-conjugated mAbs against CD19, IgD, CD27, IgM, CD24, and CD38 surface markers. Pair-matched isotype controls were also used.

**Bioinformatics analysis of phenotyping data**

Samples were hierarchically clustered based on B cell subset proportions using simple distance [a metric useful for compositional data described by Atchison (40)] and complete linkage. Matlab (Mathworks, Natick, MA) was used for bioinformatics analyses and visualization.

**Statistical analysis**

The nonparametric Mann–Whitney U test was used to compare the distribution of continuous variables between pairs of groups. Three group comparisons were conducted using nonparametric ANOVA (Kruskal-Wallis) followed by Dunn post hoc tests. For comparison of frequency within groups, Fisher exact test was used. Spearman nonparametric correlation coefficient and associated p values were computed to assess the association between pairs of continuous variables. All tests were two-sided, and p values ≥0.05 were considered statistically significant. Statistical analyses were performed using Prism software (GraphPad).

**Results**

**SLE BM displays an IFN signature and elevated IFN-regulated chemokines**

An IFN signature has previously been reported in the PB of SLE patients, but the activation of this cytokine in the BM has not been well studied. Therefore, the IFN signature was assessed in BM and paired PB from SLE patients (n = 28) and NC (n = 20) by ex-
An IFN score was calculated for each sample as the summation of the relative expression of these three genes normalized to a housekeeping gene (GAPDH). Next, we divided the SLE patients into two groups based on the BM IFN signature: 1) IFN high (IFN score $\geq$ mean + 2 SD NC); or 2) IFN low (IFN score $<$ mean + 2 SD NC). Notably, an IFN-high signature was demonstrated in the BM of more than half of the SLE patients (57%) (Table I) and correlated with that in the PB ($R^2 = 0.78, p < 0.0001, \text{Spearman correlation}$) (Fig. 1A). Examination of the fold change of individual genes (GIP2, IFIT1, and IRF7) in IFN-high and IFN-low SLE (expressed relative to a normal BM) revealed interesting differences, with the highest expression in IFIT1 followed by GIP2 and IRF7 (Fig. 1B, 1C). IFN-high SLE patients expressed significantly higher levels of all three IFN-inducible genes in the BM compared with NC and IFN-low SLE patients ($p < 0.0001$, Kruskal–Wallis followed by Dunn post hoc tests). The relationship between the BM and PB IFN signature was further reflected at the individual gene level (BM versus PB expression: $R^2 = 0.80, 0.82$, and 0.82 for GIP2, IFIT1, and IRF7, respectively; $p < 0.0001$, Spearman correlation). Remarkably, the BM demonstrated higher expression of IFN-inducible genes compared with PB (Fig. 1B, 1C).

The magnitude of this difference varied by gene, suggesting interesting tissue-specific differences in IFN activation and gene expression (BM versus PB for IFN high group: 45.7- versus 18.5-fold, $p = 0.005$ for GIP2; 108.5- versus 54-fold, $p = 0.013$ for IFIT1; 6.3- versus 5.4-fold, $p = 0.04$ for IRF7, Wilcoxon signed-rank test).

Table I reveals that the clinical, demographic, and immunologic features of the BM IFN-high and -low SLE groups as a whole were similar. However, a notable difference was the enrichment for more autoantibody specificities in the IFN-high group. Thus, the mean number of autoantibodies present was significantly higher in the IFN-high group ($p = 0.003$) (Table I). There was also a higher frequency of autoantibodies against RNA binding proteins (RNP, Sm, Ro, and La) in the IFN-high group (12 of 16 positive for at least one) versus the IFN low group (3 of 12) ($p = 0.02$), but no significant differences for anti-DNA (9 of 16 versus 3 of 12; $p = 0.13$, Fisher exact test). Additionally, on specific analysis of the IFN-high group, significant correlations were observed between the degree of IFN activation in the BM and peripheral lymphopenia (correlation coefficient $R^2 = -0.58$ for IFN-high group total BM score and absolute lymphocyte counts; $p = 0.019$) and disease activity (correlation coefficient $R^2 = 0.48$ for IFN-high group IFIT1 in the BM and SLEDAI, $p = 0.05$, Spearman correlation).

Given an increased expression of IFN-inducible genes in the BM and paired PB of SLE patients, we next analyzed the levels of IFN-regulated chemokines in the BM supernatants and PB serum in SLE patients with or without an IFN signature. In particular, the levels of the three IFN-regulated chemokines were examined—CLC2 (MCP-1), CCL19 (MIP-3b), and CXCL10 (IP-10)—that have previously been shown to be expressed in elevated levels in the sera of SLE patients (27). The expression levels of MIP-3b and IP-10 were significantly different on three-group comparison (NC, IFN-low SLE, and IFN-high SLE; Kruskal–Wallis) ($p = 0.004$ and $p = 0.012$, respectively), with higher levels of MIP-3b in the

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An IFN high compared with NC and IFN-low (BM) (Dunn post hoc test) and $p = 0.0003$ for three-group comparison with significance on IFN-high compared with NC and IFN-low (PB), $^*p = 0.0008$, $**p = 0.0004$ (Kruskal–Wallis three-group comparison with significance maintained in high versus NC and high versus low on Dunn multiple comparison test).

**FIGURE 1.** IFN signature in the BM of SLE patients. IFN signature was measured in terms of relative expression of the three IFN-inducible genes GIP2, IFIT1, and IRF7 (which was shown to reflect the microarray gene expression technology) normalized to a housekeeping gene (GAPDH), using a healthy control blood sample that was included in each run, and (for BM) recalibrated against a healthy BM. IFN score (summation of scores of all three genes) in the SLE PB correlated significantly with the BM with an $R^2 = 0.78; p < 0.0001$ ($n = 26$) (Spearman correlation). SLE patients were divided into IFN-high ($n = 16$) and IFN-low ($n = 12$) groups based on the BM IFN scores (IFN-high group: IFN score $\geq 2$ SD above the normal mean) ($n = 20$ NC). Dotted lines show the cutoff between IFN-high and IFN-low groups in BM and PBL as determined from the IFN score (A). Fold change of individual IFN-inducible genes in IFN-high and IFN-low SLE groups are shown in (B) (BM) and (C) (PB), respectively ($\pm$ SEM). IFN-high SLE patients expressed significantly higher levels of all three IFN-inducible genes compared with NC and IFN-low SLE patients. $^*p < 0.0001$ for three-group comparison (Kruskal–Wallis) with significance on
IFN-high SLE BM supernatant compared with NC (p < 0.005, Dunn post hoc) and higher levels of IP-10 in the IFN-high and -low SLE compared with NC (p < 0.05, Dunn post hoc) (Fig. 2A). In the PB, MIP-3b (p = 0.008) and IP-10 (p = 0.042) were also significantly different on three-group comparison (p values by Kruskall–Wallis in Fig. 2B), with significantly higher levels in the IFN-high SLE, as has been previously reported (27). Thus, elevated IFN-inducible chemokines provide further evidence for IFN activation in lupus BM.

**IFN signature in BM-derived cell populations**

To provide further evidence that IFN is actually produced in SLE BM, we purified specific cell fractions from the PB including RBCs. This cell population lacks nuclei, and thus the presence of an IFN signature would suggest erythropoiesis in a high IFN BM environment. Purified populations of RBCs, PBMCs, and Grans from SLE patients with a high IFN signature (n = 5) and NC (n = 3) were compared. The expression levels of cell-type specific genes (HBA in RBCs, TRBC1 in lymphocytes, and PGLYRP1 in Grans; Supplemental Fig. 1) verified the purity of the cell populations. Relative expression levels of two IFN-regulated genes, IFI27 and IFI44, were elevated in all cell fractions in SLE compared with NC donors (Mann–Whitney) (Fig. 3). In the WB RNA, the average IFI27 expression was 270-fold higher in SLE compared with NC, with the average IFI44 expression 18-fold higher. Interestingly, these genes showed different levels of expression in different cell fractions. For example, the average expression level increases in SLE of IFI27 were higher in RBC (340-fold) relative to WB (250-fold) and somewhat lower in PBMC (76-fold) and Grans (55-fold). Because erythrocytes extrude their nuclei before entering the circulation from the BM, this suggests that erythropoiesis occurred in a high IFN environment. As an additional verification of the BM IFN signature, we also sorted CD10+ precursor B cells from an SLE BM sample and demonstrated the presence of the IFN signature (data not shown).

**Association of IFN activation in SLE BM with changes in B cell development**

It has previously been reported that type-I IFN in murine BM has an impact on B cell lymphopoiesis (23). Thus, we next explored whether IFN activation in human SLE affects the early B cell developmental pathway in the BM. Although the BM is a microenvironment for the generation of early B cells, it also serves as a niche for mature B cells as memory and long-lived plasma cells home to the BM compartment. Thus, it is critical to separate B cell precursors from mature B cells when studying the BM cell composition. B cell ontogeny initiates in the BM and follows a sequential pathway of hematopoiesis: stem cell → pro/pre-B → pre-B → immature B → early transitional B cell followed by migration to the periphery for further differentiation into a mature B cell phenotype. As expected, a significant fraction of early B cells identified by CD24++CD38++ surface expression was found in the BM (Fig. 4A). In parallel, these cells were also positive for CD10 expression consistent with a B cell precursor phenotype (Fig. 4B). The fraction of early B cells overall did not vary significantly across NC, IFN-low, and IFN-high BM. However, further classification of early B cells into pro/pre-, immature, and transitional (type 1 and 2) B cells based on the expression of IgD and IgM revealed interesting differences with a reduction of CD19+, CD24+++, CD38++, IgD+, IgM- pro/pre-B cells in the early B cell compartment in IFN-high SLE BM (mean ± SD, high SLE BM versus low SLE BM versus NC BM: 60.3 ± 24.1 versus 83.6 ± 2.9 versus 79.3 ± 5.6, respectively; p = 0.019 by non-parametric ANOVA Kruskal–Wallis with significant differences

**FIGURE 2.** IFN-regulated chemokines are upregulated in SLE BM with an IFN signature. IFN-regulated chemokines IP-10 (CXCL10), MCP-1 (CCL2), and MIP-3b (CCL19) were assessed in BM supernatant (A) and PB serum or plasma (B) in NC (n = 23) and SLE patients with low (n = 12) or high (n = 16) IFN signatures as defined in Fig. 1 (mean ± SEM). *p = 0.012 for three-group comparison (Kruskal–Wallis) (BM) and 0.042 (PB) with significance on IFN-high and IFN-low compared with NC (Dunn post hoc test), **p = 0.004 (BM) and p = 0.008 (PB) for three-group comparison with significance on IFN-high compared with NC.

**FIGURE 3.** IFN signature in SLE RBCs reflects erythropoiesis in an IFN-rich BM environment. RBCs, PBMCs, and Grans were purified as described in the Materials and Methods, RNA prepared, and the expression of two IFN-regulated genes, IFI27 and IFI44, measured. Gene expression in IFN-high SLE patients (n = 5) and NC (n = 3) is represented relative to the average expression level in WB from NC after normalization to housekeeping genes. All SLE values are significantly different: p = 0.03 compared with NC (mean ± SEM) (except for IFI27 in Grans) (Mann–Whitney).
between IFN-high and IFN-low SLE BM on Dunn post hoc analysis) (Fig. 4C). Immature B cells (CD19+, CD24hi, CD38hi, IgD+, IgM+) represented a small fraction of the early B cell compartment across all BM samples, without any significant group differences (Fig. 4D).

Strikingly, transitional B cells (T1 and T2, identified by CD19+, CD24hi, CD38hi, IgD+, and IgM+ expression) were significantly overrepresented in the early B cell compartment in IFN-high SLE BM, whereas IFN-low SLE and NC BM displayed comparable levels of T1/T2 B cells (27, 4.6, and 7.5% in IFN-high SLE, IFN-low SLE, and NC, respectively; p = 0.011 by Kruskal–Wallis with significant differences between IFN-high and IFN-low SLE BM on Dunn post hoc analysis; Fig. 4E). With the reduction in the fraction of pro/pre-B cells and expansion of early transitional (T1 and T2) B cells in the IFN-high SLE BM, we wanted to further examine the transitional B cell compartment in detail. We have previously shown that, similar to mouse, transitional B cells exist in a phenotypic continuum in humans, namely T1 (CD19+, IgD+, CD27+, MTG+, CD24hi, and CD38++) and T2 (CD19+, IgD+, CD27+, MTG+, CD24hi, and CD38++) and T3 (CD19+, IgD+, CD27-,
FIGURE 5. Modulation of transitional B cell compartment in SLE BM with an IFN signature. CD19⁺, IgD⁺, CD27⁻ naive B cells were examined for MTG-retaining transitional B cells. These cells were further divided into transitional B cell subsets T1, T2, and T3 based on the relative expression of CD24 and CD38 markers. Representative examples of distribution of transitional B cells in the NC BM, IFN-low SLE BM, and IFN-high SLE BM are shown in (A). Representation of transitional subsets as fraction of total transitional B cells is shown in (B). SLE BM with an IFN signature consisted of significantly higher fractions of transitional T2 B cells in the transitional compartment. (C) Cluster analysis reveals a correlation between pro/precontraction and T1/T2 expansion in the BM (Spearman coefficient $-0.93; p < 0.0001$). The stacked-bar plot represents the relative proportions of the early B cell subsets (CD24CD38 high). Samples were clustered (by flow data) based on simplicial distance and complete linkage. Sample group is... (Figure legend continues)
As early B cells generated in the BM were significantly influenced by IFN activation, we wanted to examine the relationship between IFN activation and mature B cell subsets in the BM that are believed to be generated in the periphery and home to the BM niche. Interestingly, we found significant differences in the CD27+ expressing memory cells in SLE high IFN BM, with a significant reduction of CD27+/IgD+ USM and a trend toward expansion of CD27+/IgD− switched memory (SM) B cells (p = 0.05 for three-group uniform sampling method comparison Kruskal–Wallis) (Supplemental Fig. 2). Clustered mature B cell compositions show that these B cell changes are related to number of autoantibodies as well as gene and chemokine expression (Supplemental Fig. 2). Thus, IFN-high samples cluster together and correlate with relative expansion of SM (Spearman coefficient between BM IFN score and SM, 0.42; p = 0.031) and more autoantibodies (Spearman coefficient between number of autoantibodies and SM, 0.70; p < 0.0001; Spearman coefficient between number of autoantibodies and double negative memory, 0.50; p = 0.02). In the PB compartment, there were similar disturbances in the memory B cell subsets in the IFN-high SLE (p = 0.02 and 0.008 for USM and SM, respectively, compared with NC PB, Mann–Whitney; data not shown). Thus, IFN may have effects on PB B cell activation and differentiation in addition to direct BM effects.

BAFF and APRIL are increased in the SLE IFN-rich BM microenvironment

It has been reported that type I IFN counteracts the IL-7–dependent growth and survival of murine BM B lineage cells at the pro-B cell stage (43) and is also required for the earliest recognizable stages of human B cell lymphopoiesis (44). However, IL-7 was not significantly different in the SLE BM supernatants compared with healthy controls (Fig. 5, Supplemental Fig. 3), not necessarily surprising given that IFN effects may be downstream of IL-7. There were significantly higher levels of TNF-α in the SLE BM supernatants (21.3 ± 4.7 pg/ml for SLE versus 10.2 ± 1.5 pg/ml for NC; p = 0.003; Supplemental Fig. 3), which is notable because proinflammatory cytokines such as IL-1 and TNF-α have also been shown to inhibit human lymphoid progenitors (44) (IL-1 and IFN-γ were not different, data not shown). Additionally, levels of G-CSF, a stimulator of granulopoiesis, were significantly increased in SLE BM (68.4 ± 11.8 pg/ml for SLE versus 32.0 ± 5.6 pg/ml for NC; p = 0.005). Higher levels of G-CSF and TNF-α appear to be related to IFN activation (Fig. 5B) (Spearman correlation shown by symbols below the dendrogram. High SLEDAI (≥4) is indicated by a circled group label. IFN signature gene expression for BM is shown as heat maps below the CD19 plot. Responses for each gene (shown for BM) were scaled based on its maximum. X indicates missing data. Similarly, log10 chemokine expression in BM is displayed, also scaled to the maximum. Presence or absence of autoantibodies is indicated with closed or open circles, respectively, underneath the gene expression data. IFN-high SLE samples cluster together to the right with high transitional (Spearman coefficient 0.40; p = 0.043) and contracted pro/pre-B cells (Spearman coefficient −0.44; p = 0.025) and more autoantibodies (Spearman coefficient 0.61; p = 0.001). There is also a correlation between BAFF and contracted pro/pre-B (p = 0.016), expanded T1/T2 (p = 0.05), and IFN signature (p < 0.0001). *p ≤ 0.01 compared with NC and IFN-low SLE BM, *p = 0.007 for three-group comparison (Kruskal–Wallis) with significance on IFN-high compared with IFN-low and NC (Dunn post hoc test).
relation coefficient relative to the BM IFN score of 0.58 and 0.46, respectively; \( p = 0.001 \) and \( p = 0.008 \).

We next sought to understand why transitional B cells are increased in the SLE IFN-high BM in contrast to the inhibition seen in early B cell development. One mechanism could be increased BAFF, a key B cell survival cytokine that is known to be regulated by type-I IFN (8). We did not observe a difference in BAFF protein levels in BM supernatants from SLE patients and controls or between IFN-high and IFN-low SLE (522 ± 656 pg/ml for IFN-high SLE versus 488 ± 645 pg/ml for IFN-low SLE; Supplemental Fig. 3). However, the cellular expression of BAFF in the BM aspirates may be a more accurate reflection of BAFF activation. Indeed, qPCR of BAFF mRNA levels showed significantly higher expression in the IFN-high SLE group. On cluster analysis (Fig. 5B), there was a correlation between BAFF and the contracted pre-/pro- (\( p = 0.016 \)) and expanded T1/T2 (\( p = 0.05 \)) B cells in the BM. BAFF also strongly correlated with the IFN signature (Spearman coefficient 0.65, \( p = 3.8E-6 \)). Similarly, the gene expression for the related cytokine APRIL was significantly increased in the IFN-high SLE BM (Fig. 6).

**Neutrophils are a prime producer of IFN-α in SLE BM**

Given that pDCs are major producers of IFN-α, we postulated that apoptotic fragments and immune complexes in the BM complexes may serve as ligands for TLR9 and TLR7 on pDCs, leading to abundant IFN production. Notably, pDC fractions were not significantly different between SLE and NC BM, and pDC production of IFN in response to TLR9 or TLR7 stimulation was not significantly different (data not shown). Recent studies indicate that neutrophils play a critical role in the induction and propagation of autoimmune responses in SLE by providing autoantigen and immunostimulatory molecules that activate pDCs (30, 45, 46). In addition to potentiating type-I IFN production by pDCs, SLE neutrophils may produce IFN as well (30). Remarkably, we found that the Percoll-isolated M2 (mature) neutrophil fraction in SLE BM had a significantly higher expression of IFN-α when compared with healthy control M2 BM neutrophils (Fig. 7; \( p = 0.036 \), Mann–Whitney U test). APRIL expression was also higher in SLE BM neutrophils. In contrast, IFN-β was not significantly elevated. Notably, there was a significant correlation (Spearman) between neutrophil IFN-α expression and both APRIL (\( p = 0.0004 \)) and BAFF expression (\( p = 0.0001 \)) (Fig. 7B), suggesting the IFN-α may be driving APRIL and BAFF. The expression of APRIL (Fig. 7C) and BAFF at the protein level was confirmed by immunofluorescence.

**Lupus-prone mice display disturbances in BM B cell development and neutrophils**

To directly address the putative role of type-I IFNs in altered BM B cell development in lupus, we examined lupus-prone female NZM2328 mice (predisease onset, age 12 wk). Compared to age- and sex-matched BALB/c mice, NZM mice had profound alterations in the BM B cell compartment with significant reductions in precursors B cells (particularly at the pre-stage) and subsequent B cell progeny (immature and mature) (Fig. 8A). Interestingly, similar to human SLE, transitional B cells were in contrast increased. These B cell developmental differences were abrogated in NZM mice lacking type-I IFN receptor (Fig. 8A). Notably, total

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**FIGURE 7.** Neutrophils in SLE BM produce increased levels of IFN-α and APRIL. (A) Neutrophils were isolated from BM as described in the Materials and Methods for SLE and healthy controls (n = 8). IFNα was measured by qPCR and compared with IFNβ. APRIL and BAFF were also measured. Expression was normalized to the housekeeping gene GAPDH and then to the control PB neutrophils (mean ± SEM). (B) Linear regression analysis of the relationship of IFNα RNA levels and APRIL or BAFF RNA levels in the BM cell populations in (A) plotted on a log scale. (C) Giemsa staining (left panel; original magnification ×40, cropped for higher magnification) demonstrating purity and morphology of isolated neutrophils from the BM. By immunofluorescence (original magnification ×20, cropped for higher magnification), BM neutrophils (green elastase +) express APRIL (red) protein (overlay yellow) (representative of two experiments). *\( p = 0.036 \) compared with matched healthy control population (Mann–Whitney).
FIGURE 8. Murine SLE is associated with disturbances in B cell lymphopoiesis related to BM IFN activation and driven by neutrophils. (A) NZM mice age 12 wk were compared with age-matched NZM mice lacking type-I IFNR (I-NZM) and BALB/C mice (n = 5/group). B cell subsets in the BM were defined as follows: pro- (B220<sup>low</sup>IgM<sup>2</sup>CD43<sup>+</sup>), pre- (B220<sup>+</sup>IgM<sup>2</sup>CD43<sup>low</sup>), immature (B220<sup>+</sup>IgM<sup>+</sup>CD23<sup>2</sup>), T1 (B220<sup>+</sup>AA4.1<sup>+</sup>IgM<sup>hi</sup>CD23<sup>2</sup>), T2 (B220<sup>+</sup>AA4.1<sup>+</sup>IgM<sup>+</sup>CD23<sup>+</sup>CD24<sup>hi</sup>), and mature (B220<sup>+</sup>IgM<sup>+</sup>CD23<sup>+</sup>AA4.1<sup>+</sup>CD24<sup>lo</sup>)). Data are shown for B cell subsets expressed relative to the total live lymphocyte gate, except for T1 and T2, which are expressed relative to the early B cells (AA4.1<sup>+</sup>). Similar trends were observed when expressed relative to B220<sup>+</sup> B cells for all subsets including the T1 and T2. p values were calculated by nonparametric ANOVA (three-group comparison Kruskal–Wallis with Dunn post hoc test). (B) BM and spleen cells (data not shown) were isolated from the above groups of mice (n = 7/group). Neutrophils were isolated from BM as described in the Materials and Methods. IFN<sub>a</sub>, IFN<sub>b</sub>, APRIL, and BAFF were measured by qPCR and expression normalized to the housekeeping gene and then to BALB/C controls (mean ± SEM). (C) Neutrophils were isolated from a lupus-prone mouse BM (n = 2) and stimulated in vitro with IFN-α (1000 U/ml) for 4 h prior to qPCR. Expression is normalized to housekeeping gene and then to media control cultured. (Figure legend continues)
BM and spleen cells from these NZM mice displayed significantly higher in vivo expression of IFN-α expression by qPCR compared with BALB/c controls (p = 0.03 for BM and p = 0.019 for spleen, shown for BM, Mann–Whitney U test) (Fig. 8B). Changes in type-I IFN–regulated genes (Mx-1) were more variable. However, BM neutrophils displayed significant upregulation of Mx-1 (10-fold; p = 0.01), as well as increases in IFN-α, IFN-β, and BAFF (p = 0.03, 0.03, and 0.04, respectively; p = 0.08 for APRIL). Moreover, stimulation of BM neutrophils with IFN-α in vitro induced further upregulation of both IFNα and BAFF (Fig. 8C). In bone sections, we also detected numerous Gr-1+ myeloperoxidase+ neutrophils in NZM mice in close contact with B220+ B cells (Fig. 8D) and also expressing APRIL and BAFF. In combination, these data confirm that neutrophils are a major cell population mediating IFN production and the secretion of cytokines that alter B cell development in the BM and further highlight the IFN-driven nature of the process.

Discussion

Although SLE is known to be associated with a type I IFN signature in the PB (11, 12), the precise site and mechanism of IFN pathway activation remain unclear. In this study, we demonstrate for the first time, to our knowledge, that the BM in human SLE is a primary site of IFN activation based on the expression of IFN-inducible genes and chemokines. Notably, IFN activation in SLE BM was strongly associated with changes in B cell lymphopoiesis, initiated in the early stages of immune cell development in the BM microenvironment, and likely mediated at least in part by IFN-induced BAFF upregulation. Further, we demonstrate that neutrophils in SLE BM are a prime producer of IFN, stimulating APRIL, BAFF, and further IFN expression, thus providing multiple direct and indirect links between neutrophils and B cell development and survival. Overall, our results highlight the importance of the BM as a target organ in SLE and provide a novel connection between IFN activation and B cell selection.

Genomic approaches have demonstrated that the majority of SLE patients display a type I IFN signature based on WB or PBMC gene expression profiling. The signals generating IFN activation in SLE are relatively well characterized. Thus, RNA- and DNA-containing immune complexes that are abundant in SLE activate pDCs via TLRs (20–22) to produce large quantities of IFN-α. Although pDCs are paradoxically decreased in the PB of SLE patients (6), it is speculated that they may accumulate in sites of inflammation such as the skin (47, 48) or kidney (49). Other than these few publications, however, there is surprisingly little literature addressing the primary site of IFN activation in SLE and no consideration of the BM as an important location. We hypothesized in this paper that the BM might be an ideal organ for the production of IFN given the presence of long-lived plasma cell populations as a putative source of interferogenic autoantibodies, Ag in the form of apoptotic/dying cells, and BM pDCs as IFN producers. Indeed, BM DCs from lupus-prone mice have been demonstrated to produce IFN in a TLR9-dependent fashion (24). Other reports indicate that distinct cell types, including BM-resident macrophages (43), may also produce IFN. Similarly, a mouse model of viral lung infection demonstrated alveolar macrophages as prime producers of IFN-α (50).

Interesting recent data have highlighted the role of neutrophils in amplifying IFN activation (45) both by directly producing IFN (30) and indirectly by stimulating pDCs. This may be particularly relevant in the BM microenvironment where neutrophils develop. Neutrophil extracellular traps (NETs; weblike structures released by neutrophils as a means of immobilizing and killing invading microbes), which contain nuclear material (DNA and histones) and neutrophil proteins, are increased by IFN in SLE and in turn activate pDCs to produce high levels of IFN in a DNA- and TLR9-dependent manner (45, 46, 51). Additionally, in one of the few studies examining SLE BM, Nakou et al. (52) have demonstrated a cell death and granulopoiesis signature but not an IFN signature. The authors speculated that the absence of an IFN signature might be related to differences in the patient populations examined. Our data provide new evidence that BM neutrophils contribute to local IFN production in SLE with potentially critical effects on developing B cells.

IFN-α acts at the center of an amplification loop in SLE, contributing to B cell abnormalities, promoting the differentiation of activated B cells into plasmablasts (5), and, in conjunction with TLR stimulation, triggering B cell expansion (53) and a lowered activation threshold for autoreactive B cells (54). Our data suggest a novel role for IFN-α activation in the BM of SLE patients by decreasing B cell lymphopoiesis, contributing to B cell lymphopenia, and potentially decreasing the stringency of B cell–negative selection. This is in accord with data from Lin et al. (23) and Wang et al. (43) demonstrating that type-I IFN–producing macrophages in murine BM oppose IL-7–induced survival of B cell precursors at the pro-B stage, possibly by Bcl-2–driven apoptosis of pro-B cells mediated by type-I IFN (55). Similarly, in NZM lupus-prone mice with elevated levels of IFN in the BM, we find a loss of precursor B cells, most striking at the pre-B cell stage. These findings are consistent with published literature that NZB mice have a loss of pre-B cells in the BM that develops with age and autoantibody onset (24, 42). However, the specific association between an IFN signature and B cell development in murine lupus and human SLE has not been previously reported.

Our findings indicate that, similar to mouse models, perturbations in early B cell development are observed in human SLE in the BM compartment and are likely at least partly mediated by IFN. We also found disturbances in several cytokines and chemokines in the SLE BM. An increase in TNF-α and G-CSF in the SLE BMs, cytokines known to impair B lymphopoiesis, suggests additional IFN-independent mechanisms for inhibition of B cell development (56). Other studies have demonstrated increased apoptosis of CD34+ BM stem cells in SLE and impaired function of stromal cells (25). It has also been appreciated recently that hematopoietic progenitors express functional TLRs, with TLR ligands blocking B lineage differentiation (57), providing another potential IFN-independent mechanism for inhibition of B cell lymphopoiesis in SLE.

Although we noted an inhibition at the pro/pre-B cell stage, an increase of early transitional B cells into the T2 compartment was observed in the IFN-activated BM. The precise molecular mechanisms involved in this process are not fully understood, but this is likely to have critical implications for the breach in B cell tolerance in SLE. The selection of newly formed B cells into the mature follicular compartment occurs via these intermediate subsets or...
transitional B cells (58), a key target of negative selection of autoreactivity (59, 60). Among the numerous signals regulating the development from one transitional stage to another and ultimately selection into the mature repertoire, BAFF is key (61) and was notably increased in IFN-high SLE BM in the current study. BAFF knockout mice have a block in B cell development at the T1 stage (62), and transgenic expression of BAFF results in dramatic increases in the late transitional compartment (63), loss of B cell tolerance, and development of an SLE-like phenotype (64). Although numerous studies have demonstrated increased levels of BAFF in the human SLE PB, none have examined this phenomenon in the BM, where critically, B cells are developing. Intriguingly, IFN has been reported to increase the expression of BAFF by human neutrophils (65), providing a mechanistic link between our observations of BM IFN activation and enhanced B cell differentiation into the T2 compartment. The demonstration in this study that lupus-prone NZM mice have elevated IFN and BAFF in the BM and a notable increase in the transitional compartment supports this mechanism. In turn, it has previously been demonstrated that APRIL produced by neutrophils promotes the survival of human plasma cells in mucosa (66) and BM (67) and possibly the retention of memory B cells (68, 69). Further, IFN is known to stimulate APRIL production by DCs (67). We now provide evidence that BM neutrophils in human and murine lupus respond to IFN, upregulating IFN target genes APRIL and BAFF. The relevance of these findings for the human BM microenvironment in SLE merit further study but are in accord with the higher APRIL expression by neutrophils in the human SLE BM observed in this study and the increase in BM memory B cells. Additionally, G-CSF and TNF-α were both noted to be elevated in the SLE BM and have also been described to stimulate BAFF production by neutrophils (70). These cytokines also impair B lymphopoiesis (56), providing a potential explanation for both the inhibition of early B cell development and enhanced transitional B cell selection observed in this study. Intriguingly, it was recently published that BM neutrophils from NZM mice have enhanced NET formation (71). Thus, it is tempting to speculate that some of the effects of neutrophils on the BM B cell compartment are mediated through NET formation.

In conclusion, our results indicate that the majority of lupus patients have local production of IFN-α in the BM by resident neutrophils that also contribute to elevated levels of the B cell survival factors BAFF and APRIL in an IFN-dependent manner. We suggest that the BM may be an ideal organ for the production of IFN given the presence of long-lived plasma cell populations producing autoantibodies, apoptotic B cells providing nuclear Ags, and neutrophils as both direct IFN producers and potentiators of pDC and B cell activation. In the setting of IFN activation, an inhibition of early B lymphopoiesis may occur, and with low numbers of precursor B cells entering the transitional B cell compartment, the stringency of negative selection may be compromised leading to enhanced recruitment of autoreactive clones into the mature B cell repertoire.

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Disclosures
The authors have no financial conflicts of interest.

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