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B Cell-Intrinsic Role for IRF5 in TLR9/BCR-Induced Human B Cell Activation, Proliferation, and Plasmablast Differentiation

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Upon recognition of antigen, B cells undergo rapid proliferation followed by differentiation to specialized antibody secreting cells (ASCs). During this transition, B cells are reliant upon a multilayer transcription factor network to achieve a dramatic remodeling of the B cell transcriptional landscape. Increased levels of ASCs are often seen in autoimmune diseases and it is believed that altered expression of regulatory transcription factors play a role in this imbalance. The transcription factor interferon regulatory factor 5 (IRF5) is one such candidate as polymorphisms in IRF5 associate with risk of numerous autoimmune diseases and correlate with elevated IRF5 expression. IRF5 genetic risk has been widely replicated in systemic lupus erythematosus (SLE), and loss of Irf5 ameliorates disease in murine lupus models, in part, through the lack of pathogenic autoantibody secretion. It remains unclear, however, whether IRF5 is contributing to autoantibody production through a B cell-intrinsic function. To date, IRF5 function in healthy human B cells has not been characterized. Using human primary naive B cells, we define a critical intrinsic role for IRF5 in B cell activation, proliferation, and plasmablast differentiation. Targeted IRF5 knockdown resulted in significant immunoglobulin (Ig) D retention, reduced proliferation, plasmablast differentiation, and IgG secretion. The observed decreases were due to impaired B cell activation and clonal expansion. Distinct from murine studies, we identify and confirm new IRF5 target genes, IRF4, ERK1, and MYC, and pathways that mediate IRF5 B cell-intrinsic function. Together, these results identify IRF5 as an early regulator of human B cell activation and provide the first dataset in human primary B cells to map IRF5 dysfunction in SLE.

Keywords: interferon regulatory factor 5, human primary B cells, plasmablasts, autoantibodies, immunoglobulin G, differentiation, toll-like receptor

INTRODUCTION

Antibody secreting cells (ASCs), referred to as plasmablasts and plasma cells, are critical mediators of adaptive immunity and are often found elevated in the circulation of patients with systemic lupus erythematosus (SLE) (1–4). ASCs release high titers of antibody capable of neutralizing invading antigen and stem from naive and memory B cells that have been activated through antigen recognition. Differentiation of naive B cells to ASCs requires multiple B cell activation pathways,
including B cell receptor (BCR), T cell-mediated cytokine signaling, and toll-like receptors (TLRs) (5, 6). BCR activation occurs through binding of cognate antigen, leading to activation of distal signaling cascades, including the Ras-Raf-MEK-ERK1/2 kinase cascade and the Btk-PI3K-PLC-NFκB cascade, ultimately culminating in transcriptional activation of proliferation and prosurvival genes (7, 8). Complimentary T cell activation allows for interaction between B and T cells. Engagement of the respective B and T cell surface proteins, CD40 and CD40 ligand (CD40L), further enhances transcription of B cell survival and proliferation genes. T cell-mediated cytokine signaling acts to drive ASC differentiation, as well as to specify the antibody subclass secreted. TLR signaling can act synergistically with both T cell-dependent and -independent B cell activation pathways and represents a unique bridge between innate and adaptive immune responses (9).

These various B cell activation pathways utilize a network of transcription factors to drive proliferation, survival and differentiation (3). Immediately following activation, B cells rapidly proliferate to ensure sufficient numbers of antigen-specific B cells to undergo ASC differentiation. This initial burst of proliferation is dependent on the transcription factor MYC (10, 11). Following several rounds of proliferation, B cells upregulate interferon regulatory factor 4 (IRF4), BLIMP1, and XBP1, which control ASC differentiation and prepare the cell for antibody secretion (3, 12–14). In response to IRF4 upregulation, B cells undergo terminal chromosomal rearrangement of the immunoglobulin (Ig) locus known as class switch recombination (CSR) (12). CSR results in the switching of antibody subtype from IgM to IgG, IgA, or IgE, and is dependent on the enzyme activation-induced deaminase (AID). The majority of work on ASC differentiation has occurred in mice as human primary B cells are notoriously difficult to manipulate (by knockdown or overexpression) for functional analyses.

Genetic variants in or near the transcription factor interferon regulatory factor 5 (IRF5) have been robustly associated with SLE risk and elevated IRF5 expression and activation have been reported in SLE immune cells (15–19). Mice lacking Irf5 are protected from murine lupus disease onset and severity (20–23). A common finding between the different models of murine lupus that lack Irf5 is the significant decrease in pathogenic autoantibody secretion suggesting a role for IRF5 in B cells. In mice, Irf5 was found to regulate IL6, PRDM1, and IgG2a expression (22, 24–26). SLE pathogenesis is associated with polyclonal B cell hyperreactivity resulting in an autoreactive B cell repertoire, elevated circulating ASCs and autoantibodies (2, 27). Whether IRF5 contributes to ASC differentiation or antibody production in human primary B cells is not known.

Here, we developed a method of targeted gene knockdown in human primary naive B cells. While IRF5 expression and activity have been well-characterized in human monocytes and dendritic cells, its role in B cells remains to be defined (19, 28–35). We show that IRF5 is required in the early stages of B cell activation and proliferation in response to TLR9/BCR-induced ASC differentiation. IRF5 knockdown resulted in a significant increase in the number of IgD+ B cells, reduced activation, clonal expansion, plasmablast differentiation, and IgG1/3 secretion. Distinct from murine studies, we identify and confirm new IRF5 target genes, IRF4, ERK1, and MYC, that mediate IRF5 B cell-intrinsic function.

**MATERIALS AND METHODS**

**Naive B Cell Isolation**

Blood was drawn via peripheral phlebotomy and PBMC isolated by Ficoll centrifugation (18). PBMC were diluted to a concentration of 5 × 10^7 cells/mL and naive B cells isolated using Stem Cell Technologies Kit (Cat#: 19254). Magnetic separation was performed to achieve a >95% enriched population of naive B cells (CD19^+CD20^+IgD^+CD27^-), as determined by flow cytometry (Figure S1A in Supplementary Material). This study was carried out in accordance with the recommendations of the Rutgers Biomedical and Health Sciences IRB and the Feinstein Institute for Medical Research IRB with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Rutgers Biomedical and Health Sciences IRB and the Feinstein Institute for Medical Research IRB. The Ramos lymphoblastic B cell line was purchased from ATCC® and cultured in RPMI-1640 with 10% fetal bovine serum.

**Imaging Flow Cytometry Analysis of IRF5 Activation**

Isolated PBMC were stained for CD19 (BD Biosciences #562847) and fixed overnight in 1% paraformaldehyde. Cells were permeabilized the following day in 0.01% Triton-X-100 and stained for intracellular IRF5 (Abcam #ab193245) (19). Images were acquired on the Amnis ImageStream X Mark II imaging flow cytometer using the 40x objective. Nuclear translocation was quantified in the Amnis IDEAS software suite using the similarity score feature (Figure S1B in Supplementary Material).

**IRF5 siRNA Nucleofection**

Isolated naive B cells (3 × 10^6) were resuspended in Amxma buffer P3 (Lonza: #V4XP-3032) and distributed to Amxma 100 μL cuvettes. B cells were nucleofected with 500 nM of mock, ON-TARGETplus non-targeting control pool (GE Dharmacon: #D-001810-10-05), or SMARTpool ON-TARGETplus human IRF5 siRNA (GE Dharmacon: #L-011706-00-0010). Cells were nucleofected on the Amxma 4D Nucleofector using program EO-117 and then immediately added to 1 mL of RPMI 1640 (+10% FBS, 1x glutamine, 1x non-essential amino acids) and cultured for 24 h, pelleted and re-nucleofected with siRNA. For GFP co-nucleofection, pmagFP™ Vector (Lonza) or GFP mRNA (Trinity Biotech: #L6101) was titrated over a concentration range with 500 nM IRF5 siRNA; 15 μg GFP mRNA gave the best results.

**qRT-PCR and Western Blotting**

RNA was isolated with Trizol® and qRT-PCR performed as described (18) with primer sets: MYC 5′-CCTGTTGCTCCCATGAGGAGAC, 3′-CAGACTCTGACCTTTTGCCAGG; IRF4 5′-G
AAGGAGAAGAGACTTCC, 3′-CGATGCCCTTCTGGACCATTCC; IL65′-AGACAGCCTACTCACCCTTCCAG, 3′-TTCTGCGAAGGCCCTTTGCTG; PRDM1 5′-AGAAGGGCTCGAAGCTCAGGACTCCC; IL65′-TGCTGTTAGGTCTGCTGAGA. Threshold values (Ct) were averaged over each sample replicate, followed by normalization via the ΔΔCt method to β-actin. For Western blot analysis, 2 days postnucleofection, naive B cells were stimulated with mock or anti-IgM+ CpG-B for 24 h and then harvested for lysate preparation in RIPA buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, and 140 mM NaCl) (36).

**In Vitro B Cell Activation and Plasmablast Differentiation**

Isolated naive B cells were cultured in 96-well U-bottom plates at a minimal density of 1 × 10^6 with either 150 ng/mL CD40L (Peprotech #310-02) alone or with 100 ng/mL IL21 (Peprotech #200-21), 10 µg/mL anti-IgM antibody (Southern Biotech #2020-01), and 2.5 µg/mL CpG-B (Hycult Tech #HC4039). For plasmablast differentiation, isolated naive B cells were cultured for 7 days in the presence of stimulating cocktail.

**Flow Cytometry Analysis**

Isolated B cells were washed and stained with Live/Dead viability discrimination dye (Life Tech #L34968). Cells were subsequently blocked in 2% BSA supplemented with Fc Blocker (BioLegend) for 15 min and then stained with antibodies against B cell surface markers for 1 h [all antibodies were from BD Biosciences except CD38-PE/Texas Red (Life Technologies #MHCD3817); CD19-BV510, #562847; CD20-BUV396, #563782; CD27-BV421, #560448; IgD-APC, #348222; IgM-PerCP/Cy5.5, #314512; CD138-PE, #552026; IgG-PE/Cy7, #409316; CD45-APC/Cy7, #368516]. After staining, cells were washed twice in PBS without Mg^2+ or Ca^2+ and then fixed in 2% PFA before analysis on a BD Fortessa or BD LSR flow cytometer. Plasmablasts were defined as CD19^+CD20^+IgM^D^CD27^-CD38^+ B cells. B cell activation was determined with CD86 surface staining (#562432). Ig production was determined by intracellular staining with anti-IgA (Life Tech #Z25002), anti-IgE (Biolegend #325510), and anti-IgG antibodies in plasmablasts; AID was detected with antibody #Z25302 (Life Tech). For intracellular IRF5 staining, after overnight fixation, cells were permeabilized the following day in 0.1% Triton X-100 and rinsed in PBS 2x before blocking in 2% BSA solution. IRF5 staining was performed using anti-IRF5 antibody conjugated to Alexa Fluor 488 (Abcam Catalog#: ab124792) (34). A 40% protein a/g bead slurry was added to each IP for 3 h to allow conjugation of IRF5 antibody to beads. IP bead samples were then washed 3x in each of the following buffers in order—RIPA lysis buffer, LiCl wash buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate), and TE buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, EDTA). IP beads were incubated overnight in elution buffer (1% SDS/0.1 M NaHCO₃) at 65°C to reverse cross-links. Eluted DNA was then treated with RNase A (0.5 mg/mL) and Proteinase K (10 mg/mL) for 2 h each before isolation using phenol chloroform extraction. Briefly, an equal volume of phenol chloroform was added to each sample, followed by vigorous vortexing. The mixture was then transferred to a phase lock tube, spun for 5 min at max speed, and the aqueous phase removed. Repeated ethanol precipitation was used to isolate final DNA. Resulting ChIP DNA was quantified by bioanalyzer to ensure sufficient yield and proper fragmentation. Samples were submitted to the New York University (NYU) Genome Center for library preparation and single-end sequencing on Illumina HiSeq to a read depth of 50 million reads. Ramos B cell samples were submitted to the Rutgers NJMS Genomic Sequencing Core for ChIP-Seq.

**RNA-Seq of Primary Human B Cells**

Isolated primary B cells nucleofected with mock, scrambled, or IRF5 siRNA were mock or anti-IgM+ CpG-B stimulated for 6 h at 37°C. RNA was purified using the Qiagen RNeasy isolation kit and on-column DNA digestion performed to remove genomic DNA. Final RNA was eluted in 30 µL RNAse-free water and submitted to the NYU Genome Center for single end sequencing on Illumina HiSeq to a depth of 20 million reads in the case of primary B cells and 40 million reads for Ramos B cells.

**Bioinformatics Analysis of ChIP-Seq and RNA-Seq Data**

ChIP-Seq data was processed by HiChIP (37). Reads were mapped to the Hg19 human reference sequences using BWA.
at default parameters (38). Peaks were called using the MACS2 algorithm at the following parameters \((p = 0.0001, m = 10.30)\) and using IgG isotype controls as input against IRF5 ChIP treated samples. Motif enrichment analysis was performed using the TomTom Motif Comparison suite (MEME Suite 4.8) (39). Peaks were visualized using IGV genome browser (40). RNA-Seq data QC, read mapping by STAR (41), read counting by featureCounts (42) were handled through the QuickRNAseq pipeline developed at Pfizer (43). Subsequently, EdgeR was used to determine differentially expressed genes; a \(p\)-value \(\leq 0.05\) and a false discovery rate (FDR) \(<0.05\) after Benjamini-Hochberg correction was used for determining significant differential gene expression (44). Normalized RNA-Seq data are presented as reads per kilobase of transcript per million reads mapped (RPKM) (45). The QC report, processed read count table, RPKM table, and interactive data exploring tool generated by QuickRNaseq is available at https://baohongz.github.io/IRF5_knockdown. We used HOMER (46) for pathway analysis as it contains a program for performing functional enrichment analysis from a list of genes (http://homer.ucsd.edu/homer/microarray/go.html) HOMER uses a one-sided Fisher's exact test to determine the significance of over-representation of a gene set in the input list. We focused our analysis on enriched pathway gene sets from WikiPathways (47) as it is the most comprehensive open source pathway database. A more stringent cutoff of log2 fold-change \(\geq 1\) and FDR \(\leq 0.001\) was applied to select differentially expressed genes before performing the enrichment analysis to minimize the impact of false positives due to the small sample size.

### Statistical Analyses

Unless otherwise stated, one-way ANOVA or two-way ANOVA were used to compare means among three or more independent groups. Bonferroni posttest to compare all pairs of data sets was determined when overall \(p\)-value was \(<0.05\). All statistical analyses were performed using GraphPad Prism (version 7.0). Data are reported as mean \(\pm\) SD. In each figure legend, the number (n) of biological repeats included in the final statistical analysis is indicated. \(p\)-value \(<0.05\) was considered significant.

### RESULTS

#### TLR9/BCR Stimulation Induces IRF5 Nuclear Translocation

Among the pathways known to play a role in ASC differentiation is TLR signaling (48). IRF5 acts downstream of TLRs in monocytes and dendritic cells, but characterization of IRF5 activation in human B cells has not been shown. IRF5 resides in the cytoplasm of unstimulated cells and upon activation translocates to the nucleus (49–51). We examined IRF5 activation following treatment of healthy CD19+ B cells with activating stimuli using imaging flow cytometry (gating in Figure S1B in Supplementary Material). We failed to detect significant IRF5 nuclear translocation with BCR activating anti-IgM antibody or T cell-dependent CD40L but detected significant activation with the TLR9 agonist CpG-B (Figures 1A, B). As the majority of peripheral B cells are antigen naive and express low levels of TLR9, we combined BCR stimulation, known to upregulate TLR9, with CpG-B and examined IRF5 activation (52). Anti-IgM plus CpG-B also provided a significant increase in IRF5 nuclear translocation over mock (Figures 1A, B). Since ASC differentiation can utilize T cell-mediated signals, we examined IRF5 activation following stimulation with CD40L and IL21. Although we did not detect an increase in IRF5 activation with CD40L and IL21, the combination of CD40L, anti-IgM, IL21, and CpG-B significantly increased IRF5 nuclear translocation to levels seen with anti-IgM and CpG-B (Figures 1A, B).

In murine monocytes, IRF5 nuclear translocation was shown to increase linearly over time following stimulation (24). We measured IRF5 activation kinetics in human B cells following anti-IgM+ CpG-B stimulation. Significant IRF5 nuclear translocation was first observed at 1 h, with peak translocation occurring at 4 h (Figure 1C). To determine if activation is a byproduct of increased expression, we measured IRF5 protein levels in total B cells following 2 h stimulation; no significant difference in IRF5 levels was detected (Figures S1C,D in Supplementary Material). Although IRF5 expression was unchanged at this early time point of activation, IRF5 expression across B cell subsets may differ and has not previously been measured. In monocytes, increased IRF5 expression is known to be deterministic of subset fate, with higher IRF5 levels seen in inflammatory M1 macrophages (33). To determine if IRF5 expression has similar traits in B cells, we quantified expression in B cell subsets from healthy donors. Routine vaccination is known to increase the occurrence of plasmablasts and plasma cells in the periphery 7 days post-immunization (53). We therefore utilized peripheral blood from individuals immunized with the flu vaccine to define IRF5 protein levels in CD19+CD20+ naïve (IgD+CD38−CD27−CD24+), transitional (IgD+CD38−CD27+CD24+), non-switched memory (IgD+CD38+CD27−CD24−), switched memory (IgD−CD27+CD24+), plasmablasts (IgD−CD38−CD27+CD24−CD138+), and plasma cells (IgD−CD38−CD27−CD24−CD138+) (Figure 1D). Naive and transitional B cells expressed lower levels of IRF5 protein, whereas both non-switched and switched memory had increased expression. Plasmablasts and plasma cells had the highest IRF5 expression (Figures 1E,F). Similar findings were made in blood from healthy, non-vaccinated individuals (Figures S1E,F in Supplementary Material). These data suggest that IRF5 may regulate both B cell subset fate and effector function in mature B cell populations.
FIGURE 1 | Toll-like receptor 9/B cell receptor stimulation induces interferon regulatory factor 5 (IRF5) nuclear translocation. (A) Representative images of IRF5 cellular localization in human CD19+ B cells from a single healthy donor that were stimulated with mock, anti-immunoglobulin (Ig) M antibody, CD40 ligand (CD40L), CpG-B, or CD40L and IL21, and the combination of anti-IgM antibody, CD40L, IL21, and CpG-B for 2 h. PBMC were surface-stained with anti-CD19 antibodies, fixed and permeabilized, then stained for intracellular IRF5 and nuclear DRAQ5. Samples were then subjected to imaging flow cytometry followed by analysis in the IDEAS software suite. (B) Frequency of cells in (A) with IRF5 nuclear translocation, which was defined by an IRF5 and DRAQ5 similarity score ≥2 (one-way ANOVA with Tukey’s post hoc test; n = 4 independent donors). (C) IRF5 nuclear translocation was quantified over 12 h in isolated B cells following stimulation with anti-IgM + CpG-B. Data was normalized to mock to minimize donor variability (one-way ANOVA with Tukey’s post hoc test; n = 4 independent donors). (D) Representative gating strategy for human B cell subsets in peripheral blood of healthy donors who received the influenza vaccine 7 days prior to phlebotomy. B cell populations were defined as CD19+CD20+ naïve (IgD+CD38−CD27−CD24−), transitional (IgD+CD27−CD38+CD24+), non-switched memory (NSM; IgD+CD27+CD38−CD24−), switched memory (SM; IgD−CD27+CD24+), plasma blasts (PB; IgD−CD38−CD27−CD24−CD138+), and plasma cells (PC; IgD−CD38−CD27−CD24−CD138+). (E) Representative histograms of IRF5 protein expression in B cell subsets gated in (D). (F) Average MFI of IRF5 expression in gated B cell subsets (two-way ANOVA with Tukey’s multiple comparison post hoc test; n = 5 independent donors). Error bars represent SD. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

prepared at 72 h postnucleofection (with 24 h anti-IgM plus CpG-B stimulation) and analyzed by Western blot and flow cytometry (Figures 2B–D). Costimulation provided an increase in IRF5 expression levels over mock unstimulated, and these levels were significantly reduced following nucleofection with IRF5 siRNA, while IRF5 levels remained steady in mock and scrambled controls supporting the specificity of knockdown. Notably, analysis by flow cytometry showed reduced IRF5 expression amongst the total population of viable B cells in comparison to scrambled control. Stimulation with anti-IgM and CpG-B did not impact IRF5 knockdown efficacy (Figures 2A,B).

Interferon regulatory factor 5 is known to regulate the transcription of pro-apoptotic genes following DNA damage or death receptor signaling (56, 57). To quantify B cell viability following IRF5 knockdown, nucleofected naïve B cells were stained with trypan blue 24 h after mock or anti-IgM and CpG-B stimulation. Although nucleofection itself resulted in ~15% reduction in cell viability, no further decrease in viability was found after IRF5
knockdown (Figure 2E). As viability assays often fail to detect early stages of apoptosis, we assayed B cell apoptosis following IRF5 knockdown through Annexin V and 7-amino-actinomycin D (7-AAD) staining. CD40L treatment did not induce significant levels of apoptosis, whereas stimulation with CD40L, IL21, anti-IgM, and CpG-B induced a similar increase in apoptosis across all three siRNA conditions (Figures 2F,G). Upon exposure to antigen, only a small proportion of naive B cells will be selected to undergo ASC differentiation, thus the observed increase in apoptosis by the combination treatment is not surprising. However, IRF5 knockdown did not result in a significant decrease in apoptosis in comparison to control conditions (Figure 2G). This indicates that under the current experimental conditions, IRF5 does not have a significant impact on B cell viability or apoptosis.

Following stimulation, B cells rapidly proliferate and differentiate to ASCs. In an effort to measure ASC generation from the population of naive B cells showing IRF5 knockdown, naive B cells were conucleofected with GFP mRNA and IRF5 siRNA. Unfortunately, only ~20% of naive B cells expressed GFP and no correlation between GFP and IRF5 knockdown was found.
IRF5 knockdown, we determined whether there would be a consequential reduction in secreted antibody. We first measured percentages of nucleofected B cells expressing intracellular IgA, IgE, or IgG following stimulation for 5 days using flow cytometry. Intracellular Ig staining is indicative of the fraction of B cells that may secrete a specific antibody isotype. Representative flow gating for IgG in total CD19+CD20+ B cells is shown in Figure 3D. While few B cells expressed intracellular IgA or IgE, a large fraction of B cells stained positive for IgG. IRF5 knockdown resulted in a significant reduction in intracellular IgG staining (Figure 3E). IRF5 has previously been linked to IgG isotype secretion in mice, with Irf5−/− mice having reduced IgG2a levels following viral infection and in murine models of lupus (21, 22). As IL21 and CpG have both been shown to predominantly promote IgG secretion (59), we further measured IgG isotypes by ELISA. Following stimulation, IRF5 knockdown resulted in significantly decreased levels of IgG1, 2, and 3 but not 4 (Figure 3F). Since IgG2 and 4 were only secreted to a low extent, the largest impact was among the IgG1 and 3 isotypes. To determine if the reduction in IgG expression was due to a reduction in CSR, we assayed AID expression. AID expression is transcriptionally regulated, with expression quickly upregulated following B cell activation and proliferation. Interestingly, expression of AID was significantly decreased following IRF5 knockdown (Figure 3G). These data indicate that IRF5 contributes to early stages of ASC differentiation.

IRF5 Knockdown Impairs B Cell Proliferation and Activation

Stimulation of B cells results in a rapid proliferative burst, which is required for sufficient numbers of antigen-specific B cells to survive CSR. To investigate if a decrease in the number of B cells entering proliferation is responsible for the reduction in plasmablasts seen after IRF5 knockdown, we assayed B cell proliferation. Nucleofected B cells were labeled with the proliferation dye CFSE, stimulated with anti-IgM, CpG-B, CD40L, and IL21 for 5 days, and CFSE dilution determined through flow cytometry by gating on viable cells (Figure S3A in Supplementary Material). A significant reduction in the number of naive B cells undergoing proliferation was seen following IRF5 knockdown (Figures 3A,B). Compared to mock and scrambled controls, IRF5 knockdown resulted in a twofold reduction in proliferation.

B cell activation occurs immediately following antigen recognition, and can occur through each of the previously described activation pathways. CpG-B stimulation of naive B cells can upregulate expression of the activation marker CD86 (6). As B cell activation primes for proliferation, we assayed activation following IRF5 knockdown. Primary naive B cells were nucleofected and stimulated for 2 days. Stimulation induced the upregulation of CD86 expression in both mock- and scramble-nucleofected B cells, whereas IRF5 knockdown significantly reduced CD86 expression (Figure 3C). IRF5 is known to function downstream of TLR9, which has previously been shown to be involved in the upregulation of CD86 (52, 58). These data indicate that IRF5 acts downstream of TLR9/BCR to induce early B cell activation and proliferation.

IRF5 Knockdown Reduces Human IgG1 and 3 Secretion

Given the reduction in plasmablast differentiation seen after IRF5 knockdown, we determined whether there would be a
FIGURE 3 | Continued
Figure 4 | Interferon regulatory factor 5 (IRF5) binds promoter regions of genes associated with antibody secreting cell (ASC) differentiation. IRF5 ChIP-Seq was performed on isolated primary naive B cells from \( n = 2 \) independent donors. B cells were either mock or anti-IgM + CpG-B stimulated for 4 h. Reads were mapped through BWA and peaks called through MACs. (A) A pie chart showing representative IRF5 binding elements throughout the human primary B cell genome. (B) Common IRF5 binding motifs identified from ChIP-Seq. Motif sequences are shown in order of enrichment with associated transcription factor motif. (C) Representative peak distributions are shown for IRF4, ERK1, CASP5, and MYC. Peaks are boxed in red and were determined at a significance of \( p \leq 0.0001 \). (D) Same as (C) except IRF5 ChIP-Seq peaks are from the Ramos B cell line. Peaks were determined at \( p \leq 10^{-10} \). (E) Independent confirmation of IRF5 binding to IRF4, MYC, and ERK1 target sites through ChIP-qPCR in primary naive B cells mock stimulated or stimulated with anti-IgM + CpG-B for 4 h (two-way ANOVA with Tukey's post hoc test; \( n = 4 \) independent donors). Error bars represent SD. * \( p \leq 0.05 \); ** \( p \leq 0.01 \); *** \( p \leq 0.001 \).
Using motif discovery algorithms, we identified common motifs within 100 bp of peak centers. Among the represented motifs was the expected ISRE consensus sequence, as well as a newly identified IRF5/RUNX motif that was equally enriched for (Figure 4B). These data suggest cooperation between IRF5 and RUNX family members in the targeting of genes following B cell activation.

Interferon regulatory factor 5 was found to bind a wide range of genes, including several associated directly with ASC differentiation. Among these targets were IRF4, ERK1, and CASP5 (Figure 4C). While these genes are implicated in ASC differentiation and were targeted by IRF5 in human primary B cells following activation, none seemed to fully account for the early defects in B cell proliferation or activation (Figures 3A–C). As a result, there remained the possibility that several target genes were missing due to limitations, possibly, in obtaining sufficient numbers of primary naive B cells at the 4 h time point of IRF5 activation (Figure 1C). To ensure the identification of all possible IRF5 target genes, we performed IRF5 ChIP-Seq in Ramos B cells. Ramos are known to express high levels of TLR9, and thus recapitulate early stages of primary naive B cell activation following anti-IgM+ CpG-B stimulation (61). Enriched reads from Ramos B cells were similarly distributed as in primary
B cells (Figure S4A in Supplementary Material). Importantly, identical target genes, such as IRF4 and CASP5, were identified in both datasets (Figures 4C,D). Several additional target genes not seen in primary B cells were identified in Ramos. Among these was the proliferation gene MYC, and NFkB1 (Figure 4D).

To confirm that results from Ramos B cells were applicable to primary B cells, IRF5 ChIP-qPCR was performed on pooled primary naive B cells. Significant enrichment was seen on IRF4, MYC, and ERK1 following IRF5 ChIP (Figure 4E). Interestingly, MYC is downstream of ERK1 and an ERK1-Elk-Myc signaling pathway has been implicated in early B cell proliferation (8, 62).
These data support that IRF5 directly binds to promoters of genes important for early stages of B cell activation and proliferation.

### Identification of an IRF5-Dependent B Cell Transcriptome

Identification of IRF5 target genes suggested that IRF5 could influence the transcription of several genes important for ASC differentiation. We thus examined changes on the B cell transcriptome following IRF5 knockdown by high throughput cDNA sequencing (RNA-Seq). Nucleofected B cells were mock or anti-IgM+ CpG-B stimulated for 6 h; the 6 h timepoint was chosen to account for early IRF5-mediated gene transcription occurring in response to nuclear IRF5 at 4 h poststimulation (Figure 1C). RNA-Seq was performed on two independent biological replicates showing a strong correlation coefficient between gene expression (Figure 5A). Bioinformatics analysis found a dramatic shift in the transcriptome of B cells following knockdown (Figures S4B,C in Supplementary Material). After knockdown in mock-treated cells, 1,217 genes were downregulated while 865 genes were upregulated (Figure 5B). The most differentially expressed genes following IRF5 knockdown in the mock-treated group are listed in Table 1 (and shown in Figure S4B in Supplementary Material). Genes such as FcRL4 and SLC3A2, which were significantly upregulated following IRF5 knockdown, have been shown to regulate B cell activation and proliferation. FcRL4 is known to act as an inhibitor of BCR signaling (63). SLC3A2 was shown to be important for B cell proliferation and plasmablast differentiation in mice (64). Alternatively, the B cell cytokine LTB, as well as the proliferation marker Ki67, were found to be significantly downregulated after IRF5 knockdown (65). Following stimulation, IRF5 knockdown resulted in 2,755 downregulated and 2,995 upregulated genes (Figure 5C). Among the most differentially expressed genes within this group (Figure S4C in Supplementary Material; Table 2), which are relevant to B cell activation, included the upregulated genes ID3 and NR4A1 (66–68). Both have been shown to be involved in B cell activation and proliferation. In contrast, the B cell-associated cytokine LTA and the proliferation marker Ki67 were found both to be significantly downregulated.

Differential expression contrasts between stimulated mock-, scrambled-, and IRF5-nucleofected samples identified several genes uniquely modulated following IRF5 knockdown (Figure 5D). While several of these genes showed significant differential expression and have been previously implicated in B cell biology, they failed to intuitively explain the observed reduction in B cell proliferation and differentiation. As a result, we performed a further gene expression analyses focused on genes relevant to B cell activation, proliferation, and ASC differentiation (Figure 5E). Confirmation of our stimulation conditions was seen through the significant upregulation of genes involved in ASC differentiation (IRF4, XBP1, PRDM1), inflammatory cytokine expression (LTA, LTB), and proliferation (MYC, CDK4) (Figure 5E). Analysis of differential expression following IRF5 knockdown revealed a significant decrease in many of the genes (Figures 5E,F). As expected, expression of inflammatory cytokines such as IL6, LTA, LTB, and IL12A was significantly reduced following knockdown. Among the genes known to regulate ASC differentiation, expression of PRDM1, AICDA, and MYC were significantly downregulated whereas expression of IRF4 and XBP1 were not significantly impacted by IRF5 knockdown at this time point (Figure 5F). This may suggest that while IRF5 binds to the IRF4 promoter, it is dispensable in IRF4 transcriptional regulation. In contrast, IRF5 occupancy was found on the promoter region of MYC, and a corresponding significant decrease in MYC gene expression was seen after knockdown (Figure 5F). Reduction in MYC, and other genes identified by RNA-Seq, was confirmed by real-time qPCR.
in independent donors (Figure 5G). We next examined genes identified in Figure 5E for IRF-E or ISRE consensus binding sites in their 5′- and/or 3′-UTR. Results in Table 3 reveal that while many of the genes do contain consensus binding sites that IRF5 may recognize, not all do. Instead, IRF5 may utilize non-classical sequences, such as those identified in Figure 4B for direct regulation. Altogether, data indicate that one mechanism by which IRF5 regulates human ASC differentiation is through the early control of B cell proliferation via direct regulation of MYC expression followed by alterations in the expression of other ASC-associated genes.

To gain a further understanding of IRF5-regulated pathways in primary naive B cells, we performed an in-depth integrative analysis of ChIP-Seq and RNA-Seq datasets. Results in Figures 6A,B are from pathway analysis showing the top 20 most activated pathways when compared to mock untreated samples. Data in Figure 6A show a distinct upregulation in the unfolded protein response, adipogenesis, and translation factor pathways after IRF5 knockdown. Conversely, knockdown of IRF5 in untreated and treated samples resulted in the downregulation of cell cycle and BCR signaling pathways (Figure 6B). Downregulation of these pathways coincide with functional defects detected after IRF5 knockdown (Figures 2 and 3). Given that the NF-κB signaling pathway has been shown to play a role in murine ASC differentiation (69), we mapped out genes in this pathway that were affected by IRF5 knockdown (Figure 6C). We also mapped out cell cycle and DNA damage signaling genes affected by IRF5 knockdown since we detected an alteration in BCR/TLR9-induced cell proliferation (Figure 7). Last, given that early defects in B cell activation were detected after IRF5 knockdown, we mapped out the complex interaction of genes involved in the BCR signaling pathway that were differentially affected (Figure S5 in Supplementary Material). Together, these data implicate IRF5 in the regulation of multiple signaling pathways involved in ASC differentiation and B cell survival.

DISCUSSION

The differentiation of ASCs is reliant upon a large network of transcription factors, which are responsive to various B cell activation pathways. Among the B cell activation pathways known to play a role in ASC differentiation is the TLR signaling pathway...
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(6, 48). IRF5 is known to act downstream of TLR signaling in monocytes and dendritic cells, but characterization of IRF5 activation in human B cells has not been previously shown. Here we demonstrate that the transcription factor IRF5 acts downstream of TLR signaling to drive proliferation and differentiation to ASCs. However, unlike other transcription factors involved in ASC differentiation, such as \( \text{IRF4}, \text{PRDM1}, \text{XBP1}, \) and \( \text{MYC} \) that require early transcriptional upregulation (Figures 5E,F), IRF5 is uniquely poised for the rapid modulation of ASC-associated gene transcription in human B cells since its early regulation occurs through nuclear translocation (Figures 1A–C). Whether this effect is dependent on TLR9/BCR-induced B cell activation or would occur downstream of other B cell activating pathways is not currently known. However, since previous data in \( \text{Irf5}^{-/-} \) mice showed a direct transcriptional role for IRF5 in the regulation of \( \text{PRDM1} \) and the \( \gamma_2a \) locus (22, 25), it is intriguing to consider IRF5 as a global mediator of antibody responses. Our finding of significantly reduced plasmablast differentiation and IgG antibody production following IRF5 knockdown highlights the role of IRF5 in mediating antibody responses. Contrary

**FIGURE 6** Interferon regulatory factor 5 (IRF5) regulates numerous biologic signaling pathways in human primary B cells. (A) Data from ChIP-Seq and RNA-Seq were used to identify the top 20 upregulated pathways in which there were more upregulated genes than downregulated genes as compared to Mock untreated (NT). Heat map is shown from HOMER analysis with a cutoff of log2FC \( \geq \) 1 representing genes with at least a twofold change in expression and an false discovery rate (FDR) \( \leq \) 0.001. Pathways are ordered by negative log10 \( p \)-values of enrichment. (B) Same as (A) except top 20 downregulated pathways are shown. (C) Representative scheme showing enrichment of the upregulated NF-\( \kappa \)B survival signaling pathway after IRF5 knockdown. Boxes representing genes are color-coded based on the log2 fold-change values. Upregulated (red) or downregulated (blue) genes are shown as compared to Mock NT. Expression is shown by color code in four sections of each gene box. The gene box is divided into sections with one to one mapping comparisons of IRF5KD_NT, IRF5KD_anti-IgM+ CpG, Scr_anti-IgM+ CpG, and Mock_anti-IgM+ CpG vs. reference Mock_NT, as shown in the Legend.
to murine data, however, we were unable to detect IRF5 peak enrichment at PRDM1 or any IgG locus by ChIP-Seq. This may be due to the early time point of analysis (4 h post-stimulation) or that these genes do not contain regulatory sequences recognized by IRF5. Similar to Irf5−/− mice, though, data presented in Figure 3G suggest a role for IRF5 in controlling IgG isotype secretion; however, IgG2 and 4 levels were secreted at significantly lower levels than IgG1 and 3 in response to stimulation. Additional work will be necessary to determine the role of IRF5 in human CSR.

The finding of reduced plasmablast differentiation in isolated naive B cells nucleofected with IRF5 siRNA highlights a new B cell-intrinsic role for IRF5. These findings overlap with recent work in a murine model of lupus showing reduced ASCs in Irf5−/− MRL/lpr mice (20). However, it was not determined whether reduced ASCs was a result of IRF5 B cell-intrinsic or -extrinsic function. Data presented herein also point to IRF5 influences on late stage B cell effector functions. This is supported by the large difference in IgG isotype secretion as compared to intracellular IgG suggesting a potential role for IRF5 in secretion (Figures 3D–F).

Interferon regulatory factor 5 activity/function following nuclear translocation can occur through either transcriptional regulation or protein–protein interactions. We conclude from ChIP-Seq analysis that IRF5 regulates the transcriptional control of ASC differentiation. Through ChIP-Seq analysis, we found that IRF5 bound a wide range of target genes involved in proliferation, ASC differentiation, and cell cycle control. Among the most prominent targets were ERK1, IRF4, and MYC. Previous characterization of IRF5 transcriptional targets was in murine macrophages, revealing that IRF5 and NFκB bound promoters of inflammatory cytokines (60). Earlier ChIP-Seq work in human PBMC found IRF5 to have overlapping transcriptional targets with STAT4 (70). Results presented herein also pointed to IRF5 influences on late stage B cell effector functions. This is supported by the large difference in IgG isotype secretion as compared to intracellular IgG suggesting a potential role for IRF5 in secretion (Figures 3D–F)

Results from RNA-Seq further confirm an intrinsic role for IRF5 in ASC differentiation. Genes known to be critical in B cell proliferation and differentiation were drastically reduced following knockdown (Figure 5F). Our finding of decreased PRDM1 expression was similar to previous work in Irf5−/− mice carrying mutant DOCK2 (25, 26); however, we did not detect direct binding of IRF5 to the PRDM1 promoter even though ISRE and IRF-E sites were identified (Table 3). Rather, data indicate that decreased PRDM1 expression is a byproduct of other genes.
regulated by IRF5, such as ERK1 and MYC (8, 62). It is intriguing to propose from these data that IRF5 may be a gatekeeper for antigen-stimulated naïve B cells to enter the proliferation cycle. If true, then alterations in IRF5 activation during early stages of B cell activation may initiate the first break in self-tolerance. Current limitations in manipulating primary B cells, however, did not allow us to address whether the defect in B cell proliferation was due to reduced proliferation potential or reduced numbers of cells entering the cell cycle (Figures 3A,B). However, these data provide insight into other aspects of IRF5 function. For instance, the observed decrease in B cell proliferation suggests that the significant increase in IgD+ B cells detected after IRF5 knockdown is due to retention of IgD+ B cells rather than increased de novo synthesis since this would have been detected as an increase in proliferation rather than a decrease. Further, it implies that sufficient cell numbers were present for entering the cell cycle in response to BCR/TLR9 signaling. Instead, findings from pathway analysis of ChIP-Seq and RNA-Seq datasets support a key role for IRF5 in B cell proliferation via cell cycle regulation (Figure 7). Unfortunately, we were also unable to determine whether over-expression of MYC could restore ASC differentiation in IRF5 knockdown cells due to limitations in transfection and/or viral infection efficiency (54, 55, 71). Complementary experiments in mice or CRISPR/Cas9 in primary naïve B cells (72) will be required to further elucidate the mechanisms by which IRF5 controls early stages of ASC differentiation.

Last, results presented herein have direct implications for IRF5 function in SLE B cells. SLE patients carrying IRF5 risk polymorphisms show elevated IRF5 expression in B lymphoblastoid cell lines (15, 16). SLE patients have elevated numbers of circulating ASCs that contribute to pathogenic autoantibody production and elevated circulating autoantibodies are present years before clinical symptoms (2, 4). It is intriguing to propose that polymorphisms in IRF5 contribute to early skewing of B cell subset distribution and to breaks in self-tolerance which may ultimately lead to the observed increase in circulating ASCs and autoantibodies found in SLE patients.

REFERENCES


ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Rutgers Biomedical and Health Sciences IRB and the Feinstein Institute for Medical Research IRB with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Rutgers Biomedical and Health Sciences IRB and the Feinstein Institute for Medical Research IRB.

AUTHOR CONTRIBUTIONS

SD and BB planned and designed the study. SD and TS performed the experiments. SD and BZ analyzed bioinformatics data. SD, SS, AW, BZ, RD, TS, and BB analyzed experimental data. SD and BB prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01938/full#supplementary-material.


Conflict of Interest Statement: BZ and AW were employed by Pfizer Inc. All other authors declare not competing interests.

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