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Hannah L. Raczkowski
Schulich School of Medicine & Dentistry

Li S. Xu
Schulich School of Medicine & Dentistry

Wei Cen Wang
Schulich School of Medicine & Dentistry

Rodney P. DeKoter
Schulich School of Medicine & Dentistry, rdekoter@schulich.uwo.ca

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RESEARCH ARTICLE

Adaptive Immunity

The E26 Transformation–Specific Family Transcription Factor Spi-C Is Dynamically Regulated by External Signals in B Cells

Hannah L. Raczkowski, Li S. Xu, Wei Cen Wang, and Rodney P. DeKoter

Department of Microbiology & Immunology, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada; and Division of Genetics and Development, Children's Health Research Institute, Lawson Health Research Institute, London, Ontario, Canada

ABSTRACT

Spi-C is an E26 transformation-specific transcription factor closely related to PU.1 and Spi-B. Spi-C has lineage-instructive functions important in B cell development, Ab-generating responses, and red pulp macrophage generation. This research examined the regulation of Spi-C expression in mouse B cells. To determine the mechanism of *Spic* regulation, we identified the *Spic* promoter and upstream regulatory elements. The *Spic* promoter had unidirectional activity that was reduced by mutation of an NF-κB binding site. Reverse transcription-quantitative PCR analysis revealed that *Spic* expression was reduced in B cells following treatment with cytokines BAFF + IL-4 + IL-5, anti-IgM Ab, or LPS. Cytochalasin treatment partially prevented downregulation of *Spic*. Unstimulated B cells upregulated *Spic* on culture. *Spic* was repressed by an upstream regulatory region interacting with the heme-binding regulator Bach2. Taken together, these data indicate that Spi-C is dynamically regulated by external signals in B cells and provide insight into the mechanism of regulation. *ImmunoHorizons*, 2022, 6: 104–115.

INTRODUCTION

B cells express BCRs specific to Ags, and these same Ig molecules can be secreted as Abs. Ag-specific B cells can acquire the fate of a long-lived memory B cell responsible for rapid reactivation on secondary Ag challenge. Ag-specific B cells can also differentiate into Ab-secreting plasma cells (PCs) (1). B cell development is coordinated in stepwise fashion by a network of cell type– and developmental stage–specific transcription factors (2).

The E26 transformation-specific (ETS) transcription factors PU.1 (encoded by *Spi1*), Spi-B (encoded by *Spib*), and Spi-C (encoded by *Spic*) are significant contributors to cell fate decisions during hematopoiesis (3–6). PU.1 is required for generation of B cells and macrophages in mice (7–9) and for B cells in humans (10). *Spib*$^{-/-}$ mice show impairments in B cell development and function (4, 11). Mature B cells are not generated in mice lacking both PU.1 and Spi-B in the B cell lineage (12).

Spi-C is a lineage-instructive transcription factor that is important for the generation of multiple myeloid and lymphoid cell subsets. In the B cell compartment, Spi-C promotes the transition from large to small pre-B cells and regulates the generation of Ab-secreting cells (6, 13, 14). In the myeloid lineage, Spi-C is indispensable for the generation of splenic red pulp macrophages through a heme-dependent pathway (15, 16). Spi-C has recently been implicated in regulating the inflammatory profile of macrophages in response to NF-κB signaling, with evidence that it promotes a protective, anti-inflammatory phenotype (17). As well, altered expression of Spi-C has been noted

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Address correspondence and reprint requests to: Dr. Rodney P. DeKoter, Department of Microbiology & Immunology, Schulich School of Medicine & Dentistry, Western University, London, ON N6A 5C1, Canada. E-mail address: rdekoter@uwo.ca

ORCID: 0000-0002-9201-1983 (R.P.D.).

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H.L.R. conceptualized the study, performed experiments, and wrote the manuscript. L.S.X. and W.C.W. performed experiments. R.P.D. conceptualized the study, edited the manuscript, and acquired funding.

Abbreviations used in this article: BMDM, bone marrow–derived macrophage; ETS, E26 transformation-specific; PC, plasma cell; ROI, region of interest; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type.

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in inflammatory disease, both in mouse models and human patient cells (18, 19). In the B cell lineage, Spi-C is expressed at the pre-B, transitional, and mature B cell stages, with peak expression occurring in transitional B cells (20, 21). In mature and differentiated subsets, Spi-C expression is highest in Ab-secreting cells compared with all other populations, most notably in terminally differentiated PCs (6, 22).

Despite its important contributions to B cell fate decisions, the mechanisms underlying the regulation of Spi-C in B cells remain largely unexplored. There has been no work done at the molecular level to characterize regulatory elements of the *Spic* locus. This study aimed to investigate the regulation of Spi-C in B cells. Gene expression analysis showed that *Spic* expression was reduced in B cells following addition of a variety of proliferative signals. Cultured but unstimulated B cells upregulated *Spic* over time. At the molecular level, we found that the *Spic* promoter had unidirectional activity, which was reduced by mutation of an NF-κB binding site. *Spic* was repressed by two upstream regulatory regions interacting with the heme-binding regulator Bach2. Taken together, these data indicate that Spi-C is dynamically regulated by external signals in B cells and provide insight into the mechanism of regulation.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Pointe-Claire, QC, Canada). All animals were housed under specific pathogen-free conditions at the West Valley facility (London, ON, Canada) and were monitored in accordance with an animal use protocol approved by the Western University Council on Animal Care.

B cell enrichment

Spleens were removed from male and female mice aged 6–12 wk and dissociated into a single-cell suspension with ground-glass tissue homogenizers. RBCs were lysed with ammonium-chloride-potassium buffer, and B cells were enriched by negative selection using the Miltenyi system comprised of the QuadroMACS Separator magnet, LD depletion columns, streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and biotin-conjugated mouse anti-CD43 (clone S7; BD Biosciences). Effective enrichment was confirmed by flow cytometry with staining for CD19 (clone 6D5; BioLegend, San Diego, CA) gated on all viable cells.

Cell culture

Primary mouse B cells were cultured in RPMI-1640 (Wisent, St-Bruno, QC, Canada) containing 10% FBS, 10 U penicillin/1 mg/ml streptomycin/20 mM L-glutamine, and 10^{-5} M 2-ME. Additional reagents used for culture of primary B cells are listed in Supplemental Table I. Bone marrow was flushed from the femur and tibia of C57BL/6 wild-type (WT) mice aged 6–10 wk. Following erythrocyte lysis, bone marrow cells were plated at 2×10^5 in six-well plates and cultured for 6 d in IMDM + 10% FBS (Wisent) supplemented with 20 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ). Bone marrow–derived macrophages (BMDMs) were washed twice with D-PBS (Wisent) to remove nonadherent cells and cultured in fresh IMDM + 10% FBS alone or containing 1000 ng/ml LPS (List Biological Laboratories, Campbell, CA) or 40 μM hemin (Sigma-Aldrich, St. Louis, MO). After 48 h, BMDMs were harvested for RNA extraction. WEHI-279 B lymphoma cells were cultured in DMEM containing 4.5 g/l glucose, 10% FBS, 10 U penicillin, 1 mg/ml streptomycin, 20 mM L-glutamine, and 10^{-5} M 2-ME. 38B9 pre-B cells were cultured in RPMI-1640 containing the same supplements as for WEHI-279. Fetal liver-derived pro-B cells were cultured in IL-7–conditioned medium as previously described (23). All cells were cultured at 37°C and 5% CO_2. Images were taken with the Zeiss AxioObserver and A1 AxioCam ICM1 using ZEN 2 Pro software.

Plasmids and cloning

The *Spic* promoter, region of interest (ROI) 1, and ROI 2 were amplified from C57BL/6 genomic DNA by PCR using the Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA). PCR products were ligated into pSCB-Amp/Kan using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies, La Jolla, CA). The *Spic* promoter was cloned into pGL3-Basic (Promega, Madison, WI) using HindIII cut sites. Predicted NF-κB subunit binding sites within the *Spic* promoter were identified using CiiiDER (24) and ConTra v3 (25) software. Site-directed mutagenesis was performed on one common predicted site. *Spic* ROI 1 and ROI 2 were each ligated into the *Spic* promoter-containing pGL3-Basic vector using KpnI/SacI and XhoI/SacI sites. Site-directed mutagenesis was performed on one predicted Bach2 binding site in each construct. Predicted Spi-C binding sites were mutated by site-directed mutagenesis. Ligations were performed with T4 DNA Ligase (New England BioLabs). All PCR products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) prior to subsequent cloning. Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs). Constructs were verified by Sanger DNA Sequencing at the London Regional Genomics Center. Each cloned region was cloned and investigated in both the forward and reverse orientations. All restriction enzymes were purchased from New England Biolabs. Cloning and mutagenesis primers are listed in Supplemental Table II.

Transient transfection

WEHI-279 or 38B9 B cells in early log-phase growth were washed three times in serum-free DMEM (4.5 g/l glucose) or RPMI-1640 (Wisent). Cells were incubated for 10 min at room temperature with 0.35 μg of pRL-TK (Promega) and either 10 μg of each luciferase reporter vector or 5 μg of each reporter and 5 μg of an additional expression vector. Samples were electroporated at 220 V and 950 mF in 4-mm gap cuvettes (Thermo Fisher Scientific, Rochester, NY) using a GenePulser II with Capacitance Extender (Bio-Rad). Cells were recovered at room temperature for 10 min and plated in six-well culture plates in complete DMEM or RPMI for 24 h at 37°C, 5% CO_2.

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Luciferase assays

Cells were washed twice in D-PBS (Wisent), and lysates were collected using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured in 96-well opaque white plates using a Synergy H4 plate reader (BioTek, Winooski, VT). Data were collected using Gen5 software (BioTek).

Reverse transcription-quantitative PCR

Total RNA was extracted from fresh or cultured primary B cells using the RNeasy Minikit (Qiagen) or TRIzol reagent (Ambion, Austin, TX). cDNA synthesis (iScript cDNA Synthesis Kit; Bio-Rad, Mississauga, ON, Canada) was performed using equal starting RNA concentrations, followed by reverse transcription-quantitative PCR (RT-qPCR) analysis, which was conducted using the SensiFAST SYBR No-ROX Kit (Bioline, Singapore) on the QuantStudio 5 or QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). Relative transcript levels were normalized to TATA-binding protein (*Tbp*) and/or β-actin (*Actb*) and calculated as fold change using the comparative threshold cycle ($2-\Delta\Delta CT$) method. Primer sequences are listed in Supplemental Table III.

EMSA

Forward and reverse fully complementary oligonucleotides were synthesized with 5′ conjugation to IR700 dye to contain the predicted NF-κB site in the *Spic* promoter (5′-GCTGCAAAG**GGGATTTT**TTTT-3′, where bold indicated the consensus binding site) or a mutant predicted to be unable to bind NF-κB (5′-GCTGCAAAG**CCGATTTTT**TTT-3′, where underlined nucleotides represent changes). Recombinant GST-p50 subunit D434-969 was purchased from Sigma-Aldrich. Binding reactions were performed for 20 min at room temperature with 20 pmol annealed primers. Binding buffer contained 10 mM Tris–Cl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% Ficol-400, and 1 μg poly-dI-dC (LightShift; Thermo Fisher Scientific) in the presence of 1 μl of either control Ab (2.4G2; BD Biosciences) or anti–NF-κB p50 Ab (4D1; BioLegend). Protein–DNA complexes were run on 4% nondenaturing gels for 1 h using TGE running buffer and visualized using a Li-Cor Odyssey system at 700 nm.

Statistical analyses

All statistical analyses were performed using Prism 9.1.2 (GraphPad, La Jolla, CA). Statistical tests used are indicated in the figure legends. Each data point on the figures is a biological replicate representing cells enriched from a single mouse.

RESULTS

NF-κB activates transcription of Spic *through interaction with its promoter*

The canonical and noncanonical NF-κB signaling pathways have previously been implicated in regulation of *Spic* mRNA transcription. First, *Spic* was found to be activated by a pathway depending on noncanonical NF-κB signaling following induction of dsDNA breaks by RAG1/2 (13, 14). Second, Spic was found to be activated by LPS and TLR signaling in macrophages, activating canonical NF-κB signaling (17, 26). However, the *Spic* promoter has not been characterized, and no NF-κB binding sites were defined in previous studies. A region immediately upstream of the transcription start site encompassing 483 bp of sequence was found to be conserved across vertebrates (Fig. 1A). CiiiDER and ConTra v3 software packages were used to predict transcription factor binding sites within the conserved region of the promoter (Fig. 1B, 1C). One common predicted NF-κB binding site was identified by both programs. EMSA was used to determine the ability of rNF-κB p50 protein to bind to the predicted NF-κB site in the *Spic* promoter (Fig. 1D, 1E). P50 interacted with the WT, but not the mutant, NF-κB binding site, and the unmutated promoter sequence showed a supershifted complex in the presence of anti-p50 Ab (Fig. 1E, 1F). The 483-bp *Spic* promoter region was PCR amplified, cloned, and ligated into the pGL3-Basic luciferase reporter plasmid. We performed site-directed mutagenesis to mutate two crucial guanine nucleotides of the RGGRNN consensus sequence known to be required for NF-κB subunit binding (27) (Fig. 1D, 1E). Transient transfection of the *Spic* promoter reporter vectors into WEHI-279 B lymphoma cells, followed by luciferase assays, revealed that the promoter had activity in the forward, but not the reverse, orientation (Fig. 1G, 1H). Transfection of the mutant vector into WEHI-279 B cells resulted in reduced luciferase activity compared with the WT vector (Fig. 1H). In summary, these experiments identify the *Spic* promoter region and identify a functional NF-κB binding site that may be involved in *Spic* transcriptional regulation.

Proliferative signaling reduces Spic *expression in primary splenic B cells*

Our laboratory previously showed that *Spic* is expressed at high levels in transitional B cells (20). Transitional B cell survival requires BAFF signaling through the noncanonical NF-κB pathway (28). To determine whether BAFF or other cytokines can influence *Spic* expression, we enriched C57BL/6 B cells by CD43 column depletion to 97% as determined by flow cytometry (Fig. 2A), then treated with cytokines for various periods before *Spic* mRNA transcript levels were determined by RT-qPCR. Culture with 100 ng/ml BAFF neither increased nor decreased *Spic* expression relative to freshly isolated B cells (data not shown). Culture with IL-4 or IL-5 alone also had no effect on Spi-C expression. In B cells cultured with IL-4 and IL-5 in combination, a 5-fold decrease in *Spic* expression over time was observed (Fig. 2B), although there was no significant change in cell numbers (Fig. 2C). The combination of BAFF + IL-4 + IL-5 resulted in a 40-fold decrease in *Spic* expression over 72 h (Fig. 2D). To determine whether there was a link between B cell proliferation and *Spic* expression, we examined *Spic* expression following culture of B cells with BAFF + IL-4 + IL-5 for 72 h in the presence or absence of the actin polymerization inhibitor cytochalasin D (29). Addition of cytochalasin D reduced downregulation of *Spic*

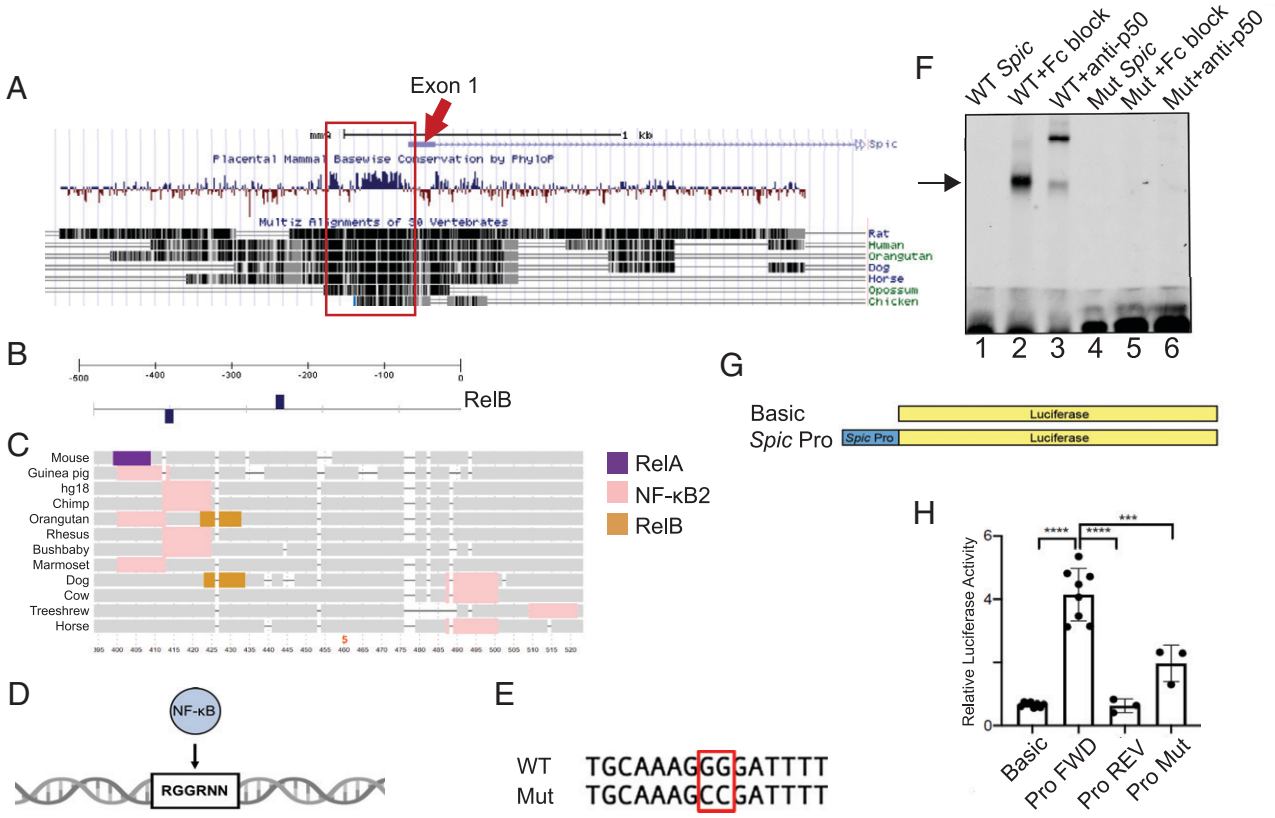

FIGURE 1. Identification of an NF-κB binding site in the *Spic* promoter.

(**A**) University of California Santa Cruz Genome Browser track showing *Spic* exon 1 and surrounding sequence. Arrow represents exon 1, and box denotes upstream region of conservation. (**B**) CiiiDER transcription factor binding site prediction within cloned promoter sequence including two possible RelB binding sites. (**C**) ConTra v3 cross-species transcription factor binding site prediction within one 110-bp region of the cloned promoter. Possible NF-κB subunit binding sites shown as colored segments. (**D**) Schematic of NF-κB consensus binding site. (**E**) DNA sequence of cloned region of Spic promoter showing WT sequence and mutant. (**F**) EMSA showing binding of the WT or mutant (Mut) *Spic* promoter oligonucleotide with p50 (lane 2) and supershift in the presence of anti-p50 (lane 3). The experiment shown is representative of five experiments. (**G**) Schematic of the Spic promoter luciferase reporter plasmids. (**H**) Transient transfection of WEHI-279 B cells and luciferase assays. Relative luciferase activity represents Renilla/Luciferase readings. Bars indicate mean ± SD. Significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Individual data points represent mean of triplicate wells for a single experiment. ***$p < 0.001$, ****$p < 0.0001$.

(Fig. 2E) and reduced cell counts at all time points, indicating that it blocked cell proliferation (Fig. 2F). These results suggest that downregulation of *Spic* by BAFF + IL-4 + IL-5 is dependent on actin polymerization and cell division.

Downregulation of Spic *expression in primary splenic B cells by CD40L, anti-IgM, or LPS*

To further examine the relationship between B cell proliferation and reduced *Spic* expression, we selected three additional molecules to investigate their effect on *Spic* expression in B cells. We examined the effect of CD40L by comparing *Spic* expression of stimulated cells to cultured but unstimulated B cells across three time points. *Spic* expression was reduced over time, with its lowest expression at 72 h, showing a 29-fold reduction in expression (Fig. 3A). B cells cultured with the addition of CD40L increased in number over time (Fig. 3B).

We next asked how BCR signaling influenced *Spic* expression. B cells were cultured with anti-IgM Abs for 24–72 h, and *Spic* expression was quantified by RT-qPCR relative to time-matched cultured but unstimulated cells. We found that BCR engagement reduced *Spic* expression in a time-dependent manner, with expression reducing by 50-fold (Fig. 3C). As expected, stimulation through the BCR also significantly increased the number of live cells in culture over time (Fig. 3D).

Alam et al. (17) recently reported that treatment of BMDMs with LPS activated *Spic* expression in an NF-κB–dependent manner. To evaluate whether a similar transcriptional program exists in B cells, we treated primary splenic B cells with LPS for 24–72 h. We observed that LPS treatment downregulated *Spic* expression by 225-fold in B cells (Fig. 3E). *Spic* expression was lowest at 48 h and recovered to an extent at 72 h. Corresponding cell count data displayed a considerable increase in

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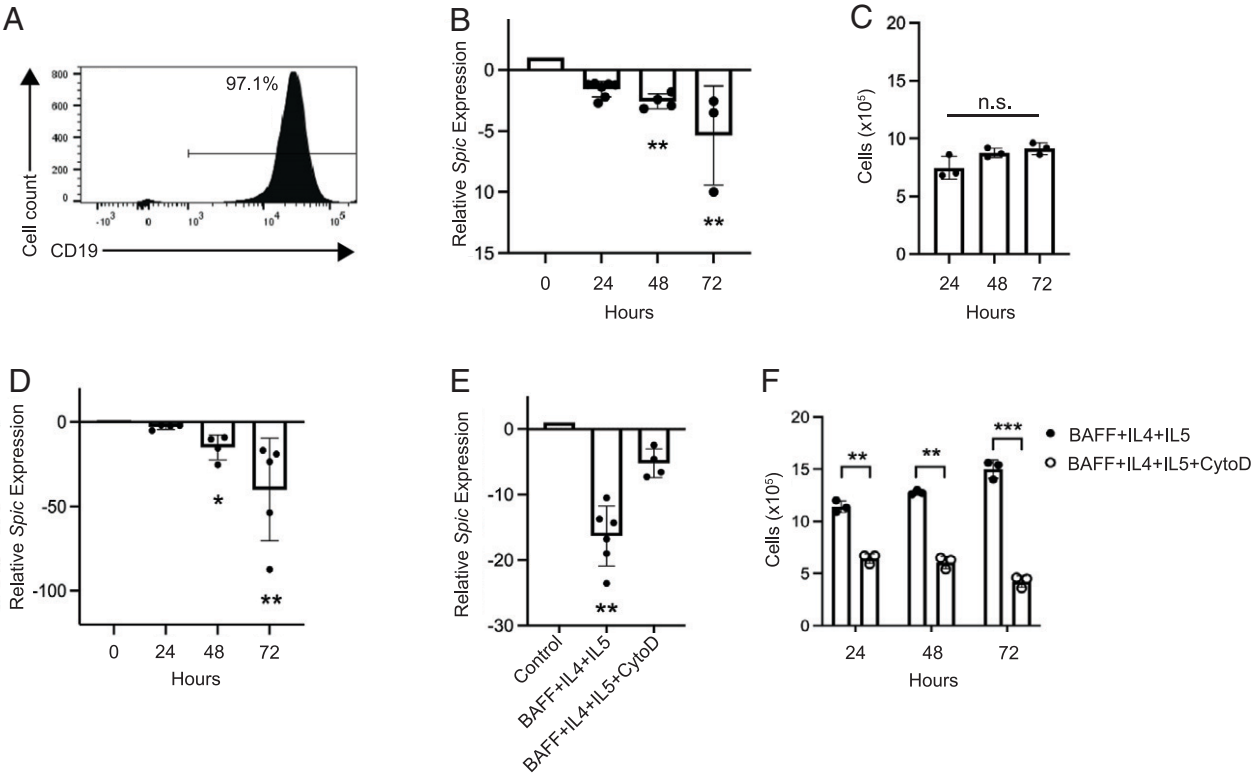

FIGURE 2. Combinations of IL-4, IL-5, and BAFF reduce *Spic* expression.

(**A**) Flow cytometry quantifying CD19$^+$ B cell frequency following CD43 depletion of spleen cells. (**B**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with IL-4 and IL-5. Bars indicate mean ± SD. Significance was determined by Kruskal–Wallis with Dunn's multiple comparisons test. (**C**) Corresponding viable cell counts for (B). (**D**) RT-qPCR analysis of *Spic* expression in B cells cultured in BAFF + IL-4 + IL-5 for the indicated times. (**E**) RT-qPCR analysis of *Spic* expression in B cells cultured with BAFF + IL-4 + IL-5 for 72 h in the presence or absence of cytochalasin D. Bars indicate mean ± SD. Significance was determined using Kruskal–Wallis with Dunn's multiple comparisons test. (**F**) Viable cell counts for B cells cultured in (D), with or without cytochalasin D. Bars indicate mean ± SD. Significance was determined by two-way ANOVA. Relative gene expression for all RT-qPCR was relative to freshly isolated B cells and normalized to *Tbp* as the reference gene. Each data point for qPCR experiments represents mean of duplicate wells for one biological replicate. Cell count data points indicate mean of triplicate counts for each biological replicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

live B cell counts over time, peaking at $>2.5 \times 10^6$ cells following 72 h in culture (Fig. 3F). In summary, stimulation with CD40L, anti-IgM, or LPS induced cell proliferation of B cells and strongly downregulated *Spic* expression.

Spic expression is increased by quiescence in primary splenic B cells

Based on the observations that various stimuli that induce proliferation downregulate *Spic* expression, we determined the effect of culture without cytokines on *Spic* expression in B cells. *Spic* expression was measured in cells cultured in complete RPMI media for 24–72 h, relative to freshly isolated B cells. We observed a time-dependent increase in *Spic* expression, culminating in a 20-fold increase by the 72-h time point (Fig. 4A). To determine whether the observed upregulation was specific to Spi-C among ETS transcription factors, we examined transcript levels of closely related family members *Spi1* and *Spib* using matched samples. Expression of *Spi1* and *Spib* increased over time, peaking at 11- and 4.3-fold increases, respectively (Fig. 4B, 4C). Cell counts showed stable numbers of live B cells over time after an initial decrease from 24 to 48 h (Fig. 4D). These results suggest that *Spic* expression is increased in unstimulated B cells, relative to the related ETS transcription factors *Spi1* and *Spib*.

To determine the upregulation of *Spic* in cultured B cells relative to other genes, we sought to examine the expression of genes with known patterns of expression during nutrient starvation and/or apoptosis. We selected *Tp53* as a gene that is expected to be upregulated in unstimulated B cells because of its well-documented increase in expression during apoptosis (30). *Acly* was chosen as a gene expected to be downregulated because of its role in fatty acid synthesis during cell division (31). We found that *Tp53* was upregulated over time by 12-fold in unstimulated B cells (Fig. 4E), whereas *Acly* expression increased by 3-fold following 72 h in culture (Fig. 4F). To additionally verify our findings, we used β-actin as a reference

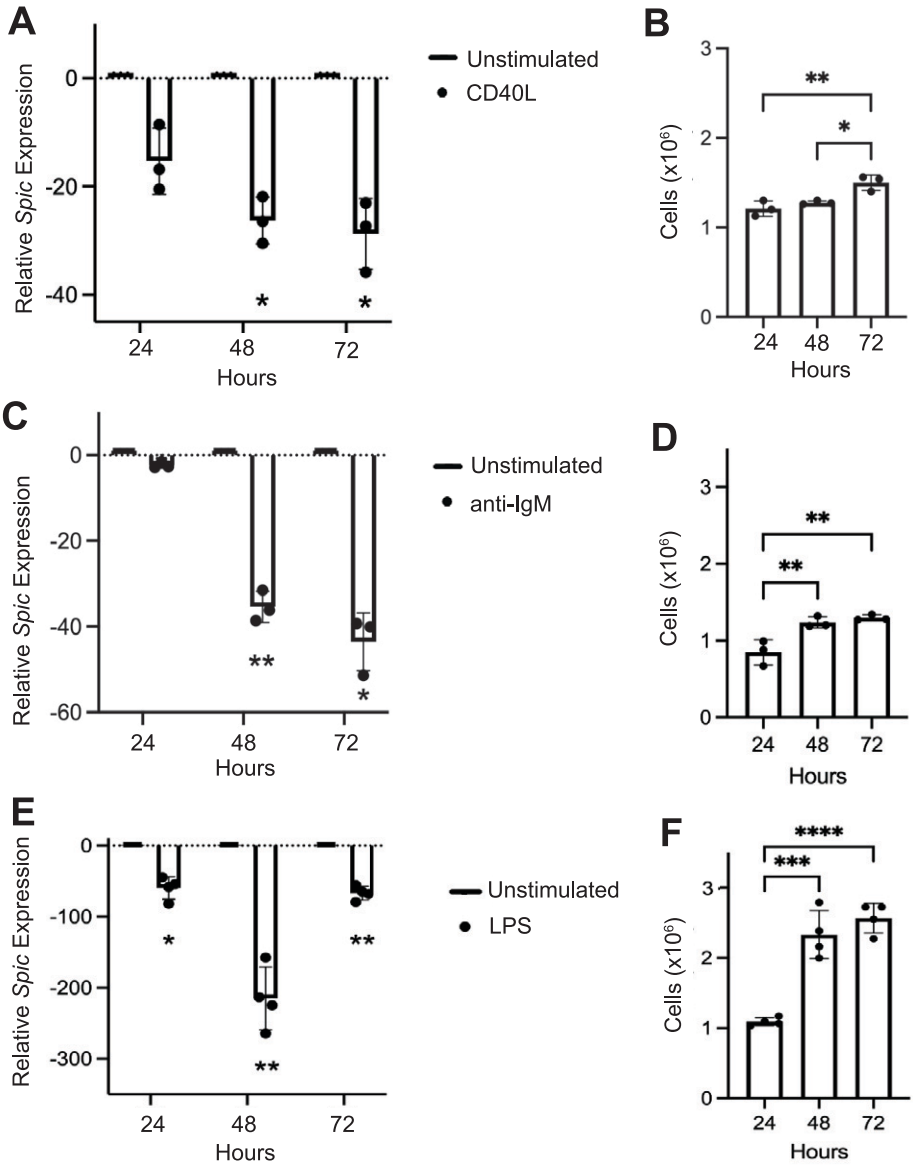

FIGURE 3. Stimulation with CD40L, anti-IgM, or LPS downregulates *Spic* expression in B cells.

(**A**) RT-qPCR analysis showing *Spic* expression in B cells cultured with or without CD40L for the indicated times. Bars indicate mean ± SD. (**B**) Viable cell counts for B cells cultured with CD40L for 72 h. Bars indicate mean ± SD. (**C**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with anti-IgM Abs for the indicated times. Bars indicate mean ± SD. (**D**) Corresponding viable cell counts for (C). Bars indicate mean ± SD. (**E**) RT-qPCR analysis showing *Spic* expression in B cells cultured with or without LPS for the indicated times. (**F**) Viable cell counts from (E). Relative gene expression for all RT-qPCRs was relative to time-matched cultured cells, using *Tbp* as the reference gene. Significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Each data point for qPCR experiments represents mean of duplicate wells for one biological replicate experiment. Cell count data points indicate mean of triplicate counts for each biological replicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

gene. We found that relative to *Actb*, *Spic* and *Tp53* transcript levels increased in a similar time-dependent manner and to the same extent, peaking at ~4-fold (Fig. 4G, 4H). Conversely, *Acly* expression decreased slightly and remained low throughout the assessed time period (Fig. 4I). Overall, these findings support the notion that *Spic* expression is increased in quiescent B cells, and to a higher degree than the related ETS transcription factors *Spi1* and *Spib*.

Imatinib (also known as Gleevec) is an Abl kinase inhibitor that blocks proliferation of v-Abl–transformed B cell lines (32). We treated the v-Abl–transformed pro-B cell line 38B9, and as a negative control for Abl transformation, IL-7–dependent fetal

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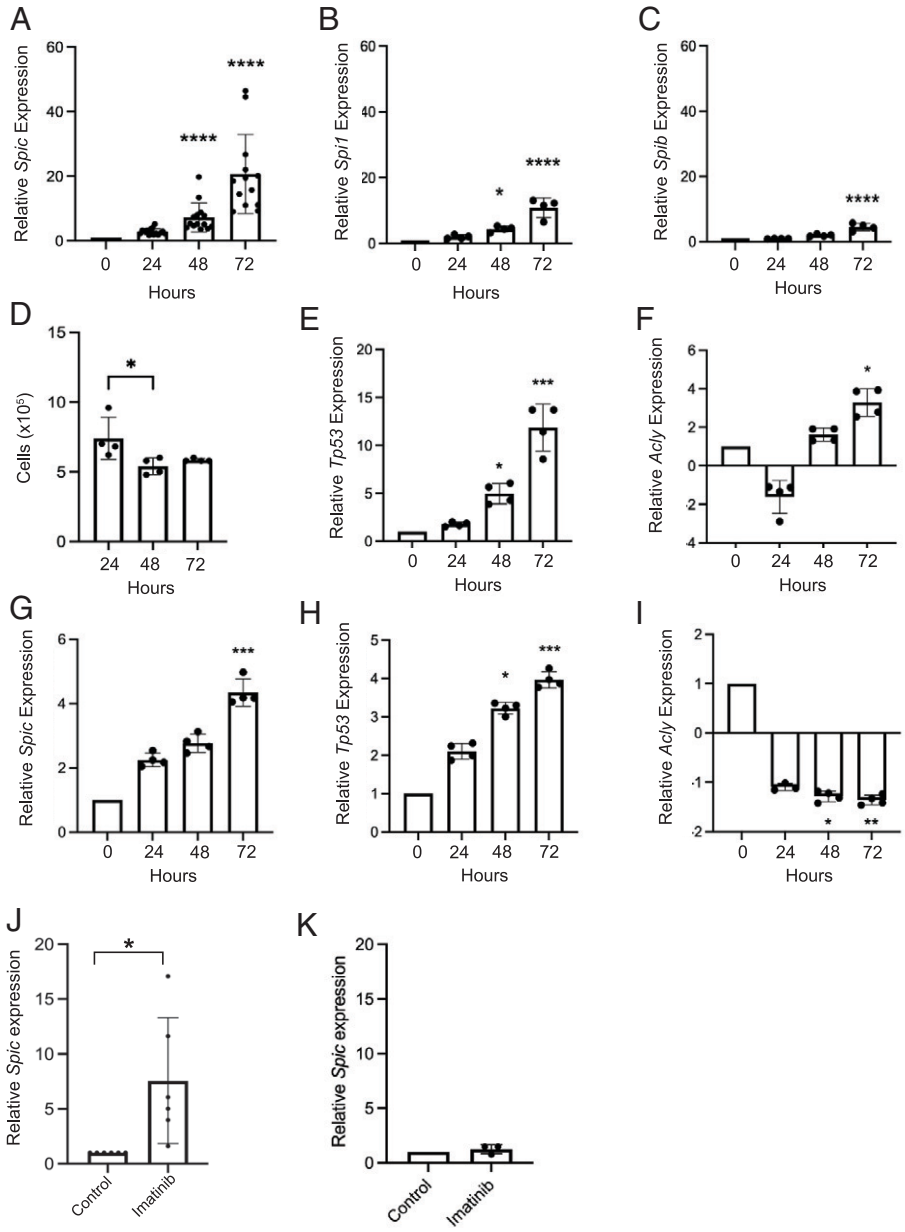

FIGURE 4. Induction of *Spic* by culture of B cells.

(**A–C**) RT-qPCR analysis of mRNA transcript levels of *Spic*, *Spi1*, and *Spib*. WT primary splenic B cells were cultured in complete RPMI for the indicated times. Bars indicate mean ± SD. Significance was determined using Kruskal–Wallis with Dunn's multiple comparisons test. (**D**) Live cell counts for cultured B cells. Bars indicate mean ± SD. Significance was determined using one-way ANOVA with Tukey's multiple comparison test. (**E** and **F**) RT-qPCR analysis quantifying expression of *Tp53* (E) and *Acly* (F) in cultured B cells. Data are shown as mean ± SD. For (A)–(F), *Tbp* was used as the reference gene. Significance was determined using Kruskal–Wallis with Dunn's multiple comparisons test. (**G**) RT-qPCR analysis of *Spic* expression in WT primary splenic B cells cultured with cytochalasin D for the indicated times. (**H**) RT-qPCR analysis of *Tp53* expression in WT primary splenic B cells cultured with cytochalasin D for the indicated times. (**I**) RT-qPCR analysis of *Acly* expression in WT primary splenic B cells cultured with cytochalasin D for the indicated times. For (G)–(I), data are relative to freshly isolated B cells and relative to *Actb*. Data are shown as mean ± SD. Significance was determined using Kruskal–Wallis with Dunn's multiple comparisons test. (**J**) RT-qPCR analysis of *Spic* expression in v-Abl–transformed 38B9 pro-B cells or (**K**) IL-7–withdrawn fetal liver-derived WT pro-B cells treated with imatinib for 24 h. Data represent mean ± SD. Significance was determined using one-sample Wilcoxon test. Expression data for (J) and (K) are relative to untreated cells on day 0 and normalized to *Actb* expression. Individual data points for qPCR experiments represent mean of duplicate wells for one biological replicate. Cell count data points indicate mean of triplicate counts for each biological replicate. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

liver-derived WT pro-B cells, with 10 mM imatinib for 48–72 h and assessed *Spic* expression. Imatinib treatment induced *Spic* expression by an average of 11-fold in 38B9 pro-B cells (Fig. 4J), but not in WT pro-B cells (Fig. 4K). These data further support the idea that *Spic* expression in B cells can be induced by quiescence, but reduced by agents that stimulate cell proliferation. We conclude that Spi-C is downregulated by cytokines, whether relative to levels in freshly isolated cells or to cells cultured without cytokines. These results suggest that Spi-C is regulated in a cell-cycle-dependent manner.

Activation of Spic *in BMDMs or in B cells by LPS and heme*

Because of the strong effect of LPS downregulating *Spic* in B cells, we sought to confirm the previously described reports of LPS treatment activating *Spic* expression in macrophages (17, 26). Bone marrow was isolated from WT mice and cultured for 6 d in the presence of GM-CSF to generate BMDMs (Fig. 5A). Culture of BMDMs with LPS for 48 h increased *Spic* expression by 5-fold (Fig. 5B). We also sought to confirm upregulation of *Spic* in BMDMs cultured with heme (15). Corroborating previous findings, we found that heme-treated BMDMs upregulated *Spic* expression by 5-fold compared with unstimulated cells (Fig. 5C).

To evaluate whether *Spic* is inducible by heme in B cells, we cultured enriched B cells for 48 or 72 h in the presence of 20 or 40 μM heme, and *Spic* expression was assessed by RT-qPCR. As a control, *Spic* expression was determined in RNA prepared from freshly isolated B cells. *Spic* expression was upregulated by 3.5-fold in response to heme at 72 h (Fig. 5D). Cell counts indicated that the viability of B cells cultured in the presence of heme was stable over 72 h (Fig. 5E). In summary, *Spic* is inducible by heme in both macrophages and B cells and is inducible by LPS in macrophages.

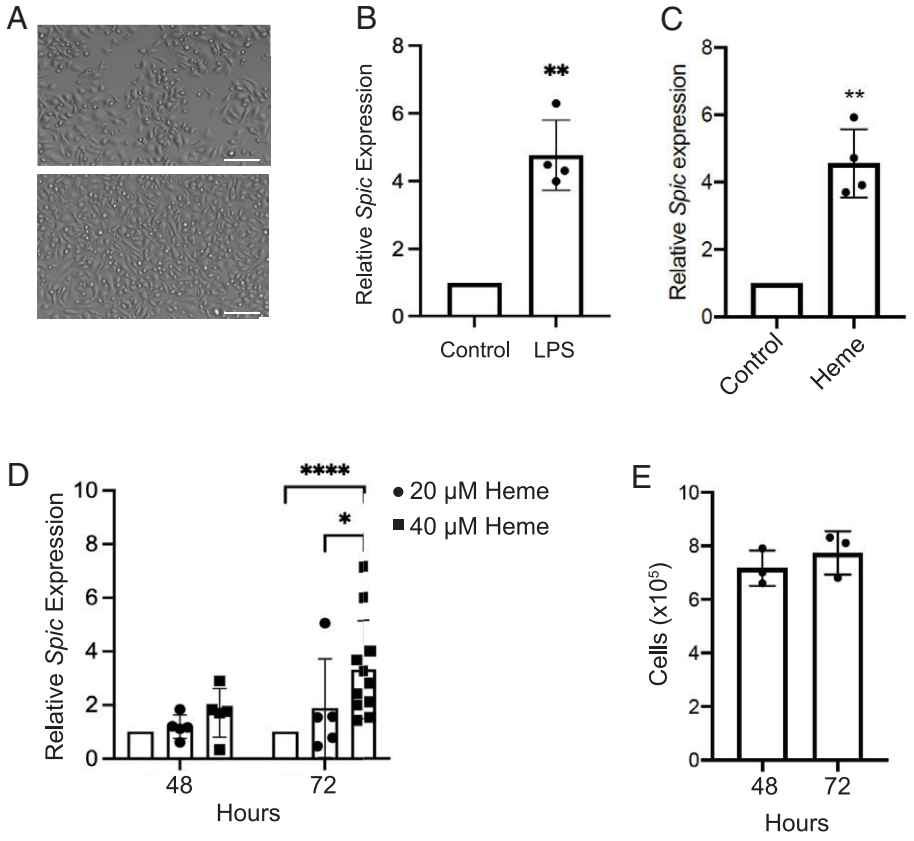

FIGURE 5. Activation of *Spic* in BMDMs or in B cells.

(**A**) Representative photomicrographs of BMDMs cultured in complete media alone (top) or with 40 μM heme (bottom). Original magnification ×20. Scale bars: 50 μm. (**B**) RT-qPCR analysis of *Spic* expression in primary BMDMs obtained from WT mice and cultured with LPS for 48 h. Bars indicate mean ± SD. Significance was determined using one-sample Wilcoxon test. (**C**) RT-qPCR analysis of *Spic* expression in BMDMs cultured with heme for 48 h. Bars indicate mean ± SD. Significance was determined using one sample and Wilcoxon test. (**D**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with or without heme. Bars indicate mean ± SD. Significance was calculated by one-way ANOVA with Tukey's test. Expression was determined relative to freshly enriched B cells. (**E**) Viable cell counts for B cells cultured in (D). Data points indicate mean of triplicates for each biological replicate. Expression was determined relative to unstimulated cells cultured for the indicated amounts of time. Relative gene expression for all RT-qPCR was normalized to *Tbp*. Each data point indicates mean of duplicate wells for one biological replicate. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

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Bach2 represses transcription of Spic *in B cells*

Heme induces *Spic* in macrophages by a mechanism involving de-repression by the heme-sensing transcription factor Bach1 (15). However, B cells express Bach2 and not Bach1 (33). Previous studies suggested that *Spic* expression is repressed at the transcriptional level by Bach2 in B cells (6, 34, 35). To determine whether Bach2 is involved in repression of Spic in B cells, we reanalyzed published anti-Bach2 chromatin immunoprecipitation sequencing data (36) and identified two binding peaks ~40 kb upstream of the *Spic* transcription start site (Fig. 6A, upper panel). These two regions, termed here ROI 1 and ROI 2, are in accordance with previous reports of *Spic* regulatory sequences interacting with Bach1 (35). To determine whether Bach2 regulates expression of *Spic* by interacting with the identified regulatory regions, we PCR amplified ~200-bp regions encompassing *Spic* ROI 1 or ROI 2, cloned them, and ligated them into the pGL3 luciferase reporter vector containing the *Spic* promoter described in Fig. 1 (Fig. 6A, lower panel). WEHI-279 B cells were transiently transfected with luciferase constructs, and luciferase activity was quantified. We found that transfection of ROI 1– or ROI 2–containing vectors alone did not enhance or repress luciferase expression relative to the vector containing only the *Spic* promoter in WEHI-279 B cells (Fig. 6B).

We then asked whether WEHI-279 or 38B9 cell lines expressed sufficiently high levels of endogenous Bach2 to observe the effects of its interaction with the ROIs. RT-qPCR showed that both 38B9 pro-B cells and WEHI-279 mature B cells expressed low levels of *Bach2* compared with primary splenic B cells (Fig. 6C). Therefore, we obtained a MIG-Bach2 retroviral vector allowing for cotransfection to enforce high levels of

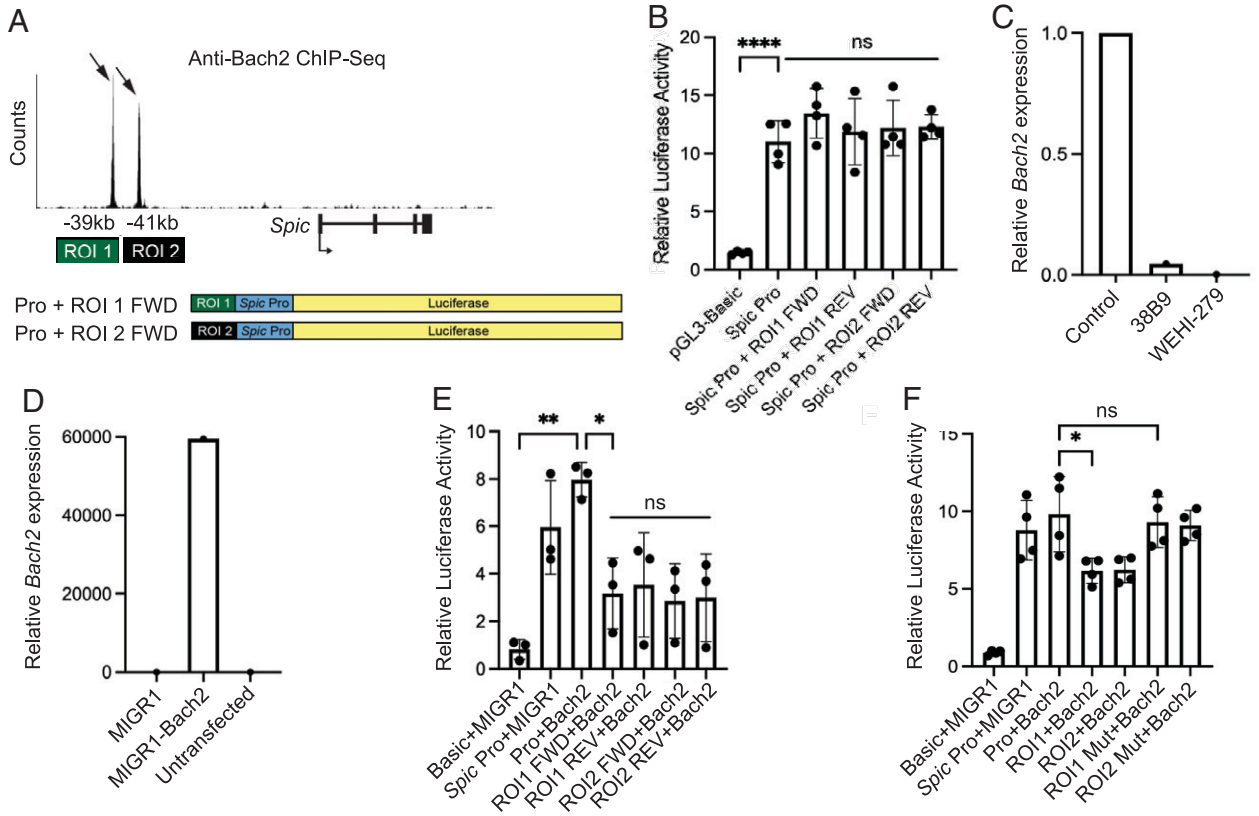

FIGURE 6. Bach2 represses *Spic* expression in B cells.

(**A**) Interaction of Bach2 with regulatory regions in the *Spic* locus (top). Chromatin immunoprecipitation sequencing data were reanalyzed to show interaction of Bach2 with a putative regulatory element located −39 and −41 kb upstream of the Spic transcription start site. Black arrows indicate locations of Bach2 binding sites. Schematic of luciferase reporters *Spic* promoter + ROI 1 and *Spic* promoter + ROI 2 (bottom). (**B**) *Spic* ROI 1 and ROI 2 have no significant activity in WEHI-279 B cells. Relative luciferase activity represents Renilla/Luciferase readings. Significance was determined using one-way ANOVA with Tukey's multiple comparisons test. (**C**) RT-qPCR analysis of *Bach2* expression in 38B9 pro-B cells and WEHI-279 cells compared with primary splenic B cells. Data represent one representative experiment of three performed. (**D**) RT-qPCR analysis of *Bach2* expression in WEHI-279 B cells transfected with MIGR1, MIGR1-Bach2, or untransfected. Data represent a single representative experiment. (**E**) Relative luciferase activity of ROI 1 and ROI 2 in WEHI-279 B cells cotransfected with MIGR1-Bach2. Bars indicate mean ± SD. Significance was determined using one-way ANOVA with Tukey's multiple comparisons test. (**F**) Relative luciferase activity of ROI 1 and ROI 2 in WEHI-279 B cells cotransfected with MIGR1-Bach2 following mutation of one Bach2 consensus binding site. Bars indicate mean ± SD. Significance was determined using one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

expression (37) (Fig. 6D). Transfection of vectors containing ROI 1 or ROI 2 in either orientation caused a significant reduction in relative luciferase activity when cotransfected with MIG-Bach2 (Fig. 6E). We next performed site-directed mutagenesis on one Bach2 consensus binding site within each ROI. Bach transcription factors interact with a Maf recognition element containing the consensus sequence 5′-TGACTCA-3′ or 5′-TGAGTCA-3′ (38). A predicted MAF recognition element within ROI 1 or ROI 2 was mutated to replace TG with CA. Cotransfection experiments showed that vectors containing mutated Bach2 sites were not repressed by cotransfection with MIG-Bach2 (Fig. 6F). In summary, these data suggest that Bach2 represses transcription of *Spic* by interacting with ROI 1 and ROI 2, and that this function is lost on mutation of one Bach2 binding site.

DISCUSSION

This study aimed to investigate the regulation of *Spic* by external signals and to characterize the molecular mechanisms responsible for its dynamic pattern of expression. We provided evidence that expression of Spi-C is highly sensitive to the presence of various stimuli in B cells. We characterized the Spic promoter and identified an NF-κB binding site that may be involved in regulation by NF-κB signaling. We showed that agents that induce cellular proliferation, including BAFF + IL-4 + IL5, CD40L, anti-IgM, or LPS, strongly downregulate *Spic* expression at the mRNA transcript level. In contrast, culture with heme or with complete media containing no stimulating agents induces upregulation of *Spic* expression in B cells. Finally, we found that Bach2, a key factor involved in processes including germinal center formation and memory B cell differentiation, represses *Spic* transcription through interaction with two upstream regulatory regions. Taken together, our findings show that the lineage-determining transcription factor Spi-C is highly responsive to regulation by external stimuli in B cells.

In general, we found that factors that induced cellular proliferation also downregulated *Spic* mRNA transcription, while factors that induced quiescence could upregulate *Spic*. *Spic* was downregulated in cytokine-stimulated cells, whether measured relative to freshly isolated B cells or cultured B cells. CD40L (CD154) and anti-IgM strongly downregulated *Spic* expression in splenic murine B cells. However, LPS downregulated *Spic* expression to the greatest extent, >200-fold, whereas LPS treatment upregulated *Spic* by ∼5-fold in BMDMs.

It is known that the NF-κB pathway becomes activated in both B cells and macrophages following TLR4 engagement by LPS (39, 40). However, a distinction between macrophages and B cells treated with LPS is in their proliferative response. LPS-activated macrophages experience cell-cycle arrest and instead respond with abundant production of proinflammatory cytokines and NO (39, 41). In contrast, B cells activated by LPS initiate a response characterized by robust proliferation and differentiation into Ab-secreting cells (42). Therefore, although both cell types activate the NF-κB pathway in response to LPS treatment, additional signaling events linked to the cell cycle may be responsible for differences in *Spic* expression. We found evidence that NF-κB regulates transcription of *Spic* through a key site located in the promoter. During B cell development, activation of the noncanonical NF-κB pathway was found to be crucial for activation of Spi-C expression during B cell development (13, 14).

Spi-C is highly expressed in transitional B cells and PCs, stages of B cell development that are nondividing and respond to BCR engagement without inducing proliferation (6, 20, 22). Spi-C directly represses genes that induce proliferation of B cells during the pre-B cell stage of development (13, 14). In our study, *Spic* was upregulated in cultured but unstimulated B cells and was upregulated to a greater extent than the related *Spi1* and *Spib* transcription factors. Taken together, these findings suggest that *Spic* expression is associated with quiescence in B cells.

Spi-C was induced in B cells in response to the metabolite heme. Although the molecular mechanism for its upregulation due to degradation of its repressor Bach1 is now well studied in macrophages, the biological relevance of Spi-C induction by heme in B cells remains unknown (36, 43–45). Although there is evidence that treatment of B cells with heme increases transcription of the endosomal transporter HRG-1, the mechanism of heme transport across the plasma membrane is not known (44). Despite early studies reporting that phagocytosis and pinocytosis occur rarely in lymphocytes, there is growing evidence that B cells may have a higher capacity for nonspecific uptake than previously thought (46, 47). Therefore, it is plausible that B cells pinocytose free heme. Alternatively, heme may be sensed externally as a danger-associated molecular pattern that drives a Spi-C–mediated immune response (6, 48). Although not thought to strongly activate PRRs, there is evidence that heme may be able to signal weakly through TLRs such as TLR4 (49). Finally, differentiating B cells also synthesize intracellular heme (44). These possible mechanisms of detection of heme by B cells provide insight into how it may be sensed, but further investigation of the downstream signaling pathways is warranted.

Because free heme is a potent catalyst of reactive oxygen species generation, it is maintained almost exclusively complexed with hemoglobin (48, 50). High levels of free heme are typically indicative of excessive hemolysis, which can arise because of disease or infection. It is conceivable that heme-dependent activation of Spi-C is one signaling pathway that promotes the generation of Ab-secreting cells in response to a nonspecific threat (44). Specifically, we propose that in the case of hemolytic infections, such as malaria or some forms of *Streptococcus*, B cells detect and become activated in response to free heme (48, 51). This leads to the proteosome-dependent degradation of Bach2, which frees *Spic* from constitutive repression (6). Spi-C may then act to counter its related factor Spi-B and promote B cell differentiation into Ab-secreting cells. In addition, we previously showed that Spi-C represses Bach2 transcription (6). Therefore, mutual cross-antagonism between Spi-C and Bach2 is a potential mechanism to promote the rapid generation of Abs to initiate immunity while the longer-term immune response begins to develop.

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In summary, this work characterized the regulation of the lineage-instructive transcription factor Spi-C in response to external signals in B cells. Although the downstream effects of Spi-C in B cell development and differentiation have been partly described, this study provides insight into the dynamic regulation of Spi-C in B cells. Understanding how Spi-C expression is regulated by external signals and downstream signaling pathways in B cells will enhance knowledge of adaptive immune responses.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES

1. Osmond, D. G., A. Rolink, and F. Melchers. 1998. Murine B lymphopoiesis: towards a unified model. *Immunol. Today* 19: 65–68.
2. Pang, S. H., S. Carotta, and S. L. Nutt. 2014. Transcriptional control of pre-B cell development and leukemia prevention. *Curr. Top. Microbiol. Immunol.* 381: 189–213.
3. DeKoter, R. P., and H. Singh. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 288: 1439–1441.
4. DeKoter, R. P., M. Geadah, S. Khoosal, L. S. Xu, G. Thillainadesan, J. Torchia, S. S. Chin, and L. A. Garrett-Sinha. 2010. Regulation of follicular B cell differentiation by the related E26 transformation-specific transcription factors PU.1, Spi-B, and Spi-C. *J. Immunol.* 185: 7374–7384.
5. Li, S. K., L. A. Solomon, P. C. Fulkerson, and R. P. DeKoter. 2015. Identification of a negative regulatory role for spi-C in the murine B cell lineage. *J. Immunol.* 194: 3798–3807.
6. Laramée, A. S., H. Raczkowski, P. Shao, C. Batista, D. Shukla, L. Xu, S. M. M. Haeryfar, Y. Tesfagiorgis, S. Kerfoot, and R. DeKoter. 2020. Opposing roles for the related ETS-family transcription factors Spi-B and Spi-C in regulating B cell differentiation and function. *Front. Immunol.* 11: 841.
7. Houston, I. B., M. B. Kamath, B. L. Schweitzer, T. M. Chlon, and R. P. DeKoter. 2007. Reduction in PU.1 activity results in a block to B-cell development, abnormal myeloid proliferation, and neonatal lethality. *Exp. Hematol.* 35: 1056–1068.
8. McKercher, S. R., B. E. Torbett, K. L. Anderson, G. W. Henkel, D. J. Vestal, H. Baribault, M. Klemsz, A. J. Feeney, G. E. Wu, C. J. Paige, and R. A. Maki. 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.* 15: 5647–5658.
9. Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265: 1573–1577.
10. Le Coz, C., D. N. Nguyen, C. Su, B. E. Nolan, A. V. Albrecht, S. Xhani, D. Sun, B. Demaree, P. Pillarisetti, C. Khanna, et al. 2021. Constrained chromatin accessibility in PU.1-mutated agammaglobulinemia patients. *J. Exp. Med.* 218: e20201750.
11. Li, S. K., A. K. Abbas, L. A. Solomon, G. M. Groux, and R. P. DeKoter. 2015. Nfkb1 activation by the E26 transformation-specific transcription factors PU.1 and Spi-B promotes Toll-like receptor-mediated splenic B cell proliferation. *Mol. Cell. Biol.* 35: 1619–1632.
12. Batista, C. R., S. K. H. Li, L. S. Xu, L. A. Solomon, and R. P. DeKoter. 2017. PU.1 regulates Ig light chain transcription and rearrangement in pre-B cells during B cell development. *J. Immunol.* 198: 1565–1574.
13. Bednarski, J. J., R. Pandey, E. Schulte, L. S. White, B. R. Chen, G. J. Sandoval, M. Kohyama, M. Haldar, A. Nickless, A. Trott, et al. 2016. RAG-mediated DNA double-strand breaks activate a cell type-specific checkpoint to inhibit pre-B cell receptor signals. *J. Exp. Med.* 213: 209–223.
14. Soodgupta, D., L. S. White, W. Yang, R. Johnston, J. M. Andrews, M. Kohyama, K. M. Murphy, N. Mosammaparast, J. E. Payton, and J. J. Bednarski. 2019. RAG-mediated DNA breaks attenuate PU.1 activity in early B cells through activation of a SPIC-BCLAF1 complex. *Cell Rep.* 29: 829–843.e5.
15. Haldar, M., M. Kohyama, A. Y. L. So, W. Kc, X. Wu, C. G. Briseño, A. T. Satpathy, N. M. Kretzer, H. Arase, N. S. Rajasekaran, et al. 2014. Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. *Cell* 156: 1223–1234.
16. Kohyama, M., W. Ise, B. T. Edelson, P. R. Wilker, K. Hildner, C. Mejia, W. A. Frazier, T. L. Murphy, and K. M. Murphy. 2009. Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature* 457: 318–321.
17. Alam, Z., S. Devalaraja, M. Li, T. K. J. To, I. W. Folkert, E. Mitchell-Velasquez, M. T. Dang, P. Young, C. J. Wilbur, M. A. Silverman, et al. 2020. Counter regulation of Spic by NF-κB and STAT signaling controls inflammation and iron metabolism in macrophages. *Cell Rep.* 31: 107825.
18. Kayama, H., M. Kohyama, D. Okuzaki, D. Motooka, S. Barman, R. Okumura, M. Muneta, K. Hoshino, I. Sasaki, W. Ise, et al. 2018. Heme ameliorates dextran sodium sulfate-induced colitis through providing intestinal macrophages with noninflammatory profiles. *Proc. Natl. Acad. Sci. USA* 115: 8418–8423.
19. Wang, A., S. D. Pope, J. S. Weinstein, S. Yu, C. Zhang, C. J. Booth, and R. Medzhitov. 2019. Specific sequences of infectious challenge lead to secondary hemophagocytic lymphohistiocytosis-like disease in mice. *Proc. Natl. Acad. Sci. USA* 116: 2200–2209.
20. Zhu, X., B. L. Schweitzer, E. J. Romer, C. E. W. Sulentic, and R. P. DeKoter. 2008. Transgenic expression of Spi-C impairs B-cell development and function by affecting genes associated with BCR signaling. *Eur. J. Immunol.* 38: 2587–2599.
21. Debnath, I., K. M. Roundy, D. M. Dunn, R. B. Weiss, J. J. Weis, and J. H. Weis. 2008. Defining a transcriptional fingerprint of murine splenic B-cell development. *Genes Immun.* 9: 706–720.
22. Shi, W., Y. Liao, S. N. Willis, N. Taubenheim, M. Inouye, D. M. Tarlinton, G. K. Smyth, P. D. Hodgkin, S. L. Nutt, and L. M. Corcoran. 2015. Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nat. Immunol.* 16: 663–673.
23. Schweitzer, B. L., K. J. Huang, M. B. Kamath, A. V. Emelyanov, B. K. Birshtein, and R. P. DeKoter. 2006. Spi-C has opposing effects to PU.1 on gene expression in progenitor B cells. *J. Immunol.* 177: 2195–2207.
24. Gearing, L. J., H. E. Cumming, R. Chapman, A. M. Finkel, I. B. Woodhouse, K. Luu, J. A. Gould, S. C. Forster, and P. J. Hertzog. 2019. CiiiDER: a tool for predicting and analysing transcription factor binding sites. *PLoS One* 14: e0215495.
25. Kreft, L., A. Soete, P. Hulpiau, A. Botzki, Y. Saeys, and P. De Bleser. 2017. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. *Nucleic Acids Res.* 45(W1): W490–W494.
26. Akilesh, H. M., M. B. Buechler, J. M. Duggan, W. O. Hahn, B. Matta, X. Sun, G. Gessay, E. Whalen, M. Mason, S. R. Presnell, et al. 2019. Chronic TLR7 and TLR9 signaling drives anemia via differentiation of specialized hemophagocytes. *Science* 363: eaao5213.

27. Wong, D., A. Teixeira, S. Oikonomopoulos, P. Humburg, I. N. Lone, D. Saliba, T. Siggers, M. Bulyk, D. Angelov, S. Dimitrov, et al. 2011. Extensive characterization of NF-κB binding uncovers non-canonical motifs and advances the interpretation of genetic functional traits. *Genome Biol*. 12: R70.
28. De Silva, N. S., K. Silva, M. M. Anderson, G. Bhagat, and U. Klein. 2016. Impairment of mature B cell maintenance upon combined deletion of the alternative NF-κB transcription factors RELB and NF-κB2 in B cells. *J. Immunol*. 196: 2591–2601.
29. Hao, S., and A. August. 2005. Actin depolymerization transduces the strength of B-cell receptor stimulation. *Mol. Biol. Cell* 16: 2275–2284.
30. Kiraz, Y., A. Adan, M. Kartal Yandim, and Y. Baran. 2016. Major apoptotic mechanisms and genes involved in apoptosis. *Tumour Biol*. 37: 8471–8486.
31. Rhee, J., L. A. Solomon, and R. P. DeKoter. 2019. A role for ATP citrate lyase in cell cycle regulation during myeloid differentiation. *Blood Cells Mol. Dis*. 76: 82–90.
32. Muljo, S. A., and M. S. Schlissel. 2003. A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines. *Nat. Immunol*. 4: 31–37.
33. Igarashi, K., and A. Itoh-Nakadai. 2016. Orchestration of B lymphoid cells and their inner myeloid by Bach. *Curr. Opin. Immunol*. 39: 136–142.
34. Miura, Y., M. Morooka, N. Sax, R. Roychoudhuri, A. Itoh-Nakadai, A. Brydun, R. Funayama, K. Nakayama, S. Satomi, M. Matsumoto, et al. 2018. Bach2 promotes B cell receptor-induced proliferation of B lymphocytes and represses cyclin-dependent kinase inhibitors. *J. Immunol*. 200: 2882–2893.
35. Itoh-Nakadai, A., R. Hikota, A. Muto, K. Kometani, M. Watanabe-Matsui, Y. Sato, M. Kobayashi, A. Nakamura, Y. Miura, Y. Yano, et al. 2014. The transcription repressors Bach2 and Bach1 promote B cell development by repressing the myeloid program. *Nat. Immunol*. 15: 1171–1180.
36. Itoh-Nakadai, A., M. Matsumoto, H. Kato, J. Sasaki, Y. Uehara, Y. Sato, R. Ebina-Shibuya, M. Morooka, R. Funayama, K. Nakayama, et al. 2017. A Bach2-Cebp gene regulatory network for the commitment of multipotent hematopoietic progenitors. *Cell Rep*. 18: 2401–2414.
37. Jang, E., H. R. Lee, G. H. Lee, A.-R. Oh, J.-Y. Cha, K. Igarashi, and J. Youn. 2017. Bach2 represses the AP-1-driven induction of interleukin-2 gene transcription in CD4+ T cells. *BMB Rep*. 50: 472–477.
38. Matsumoto, M., K. Kondo, T. Shiraki, A. Brydun, R. Funayama, K. Nakayama, N. Yaegashi, H. Katagiri, and K. Igarashi. 2016. Genomewide approaches for BACH1 target genes in mouse embryonic fibroblasts showed BACH1-Pparg pathway in adipogenesis. *Genes Cells* 21: 553–567.
39. Sharif, O., V. N. Bolshakov, S. Raines, P. Newham, and N. D. Perkins. 2007. Transcriptional profiling of the LPS induced NF-kappaB response in macrophages. *BMC Immunol*. 8: 1.
40. Sakai, J., E. Cammarota, J. A. Wright, P. Cicuta, R. A. Gottschalk, N. Li, I. D. C. Fraser, and C. E. Bryant. 2017. Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Sci. Rep*. 7: 1428.
41. Vadiveloo, P. K., E. Keramidaris, W. A. Morrison, and A. G. Stewart. 2001. Lipopolysaccharide-induced cell cycle arrest in macrophages occurs independently of nitric oxide synthase II induction. *Biochim. Biophys. Acta* 1539: 140–146.
42. Xu, H., L. N. Liew, I. C. Kuo, C. H. Huang, D. L. M. Goh, and K. Y. Chua. 2008. The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology* 125: 218–228.
43. Watanabe-Matsui, M., T. Matsumoto, T. Matsui, M. Ikeda-Saito, A. Muto, K. Murayama, and K. Igarashi. 2015. Heme binds to an intrinsically disordered region of Bach2 and alters its conformation. *Arch. Biochem. Biophys*. 565: 25–31.
44. Watanabe-Matsui, M., A. Muto, T. Matsui, A. Itoh-Nakadai, O. Nakajima, K. Murayama, M. Yamamoto, M. Ikeda-Saito, and K. Igarashi. 2011. Heme regulates B-cell differentiation, antibody class switch, and heme oxygenase-1 expression in B cells as a ligand of Bach2. *Blood* 117: 5438–5448.
45. Dutra, F. F., and M. T. Bozza. 2014. Heme on innate immunity and inflammation. *Front. Pharmacol*. 5: 115.
46. Martínez-Riaño, A., E. R. Bovolenta, P. Mendoza, C. L. Oeste, M. J. Martín-Bermejo, P. Bovolenta, M. Turner, N. Martínez-Martín, and B. Alarcón. 2018. Antigen phagocytosis by B cells is required for a potent humoral response. *EMBO Rep*. 19: e46016.
47. Goldmacher, V. S., N. L. Tinnel, and B. C. Nelson. 1986. Evidence that pinocytosis in lymphoid cells has a low capacity. *J. Cell Biol*. 102: 1312–1319.
48. Martins, R., and S. Knapp. 2018. Heme and hemolysis in innate immunity: adding insult to injury. *Curr. Opin. Immunol*. 50: 14–20.
49. Figueiredo, R. T., P. L. Fernandez, D. S. Mourao-Sa, B. N. Porto, F. F. Dutra, L. S. Alves, M. F. Oliveira, P. L. Oliveira, A. V. Graça-Souza, and M. T. Bozza. 2007. Characterization of heme as activator of Toll-like receptor 4. *J. Biol. Chem*. 282: 20221–20229.
50. Aich, A., M. Freundlich, and P. G. Vekilov. 2015. The free heme concentration in healthy human erythrocytes. *Blood Cells Mol. Dis*. 55: 402–409.
51. Orf, K., and A. J. Cunnington. 2015. Infection-related hemolysis and susceptibility to Gram-negative bacterial co-infection. *Front. Microbiol*. 6: 666.

https://doi.org/10.4049/immunohorizons.2100111