7-26-2021 10:00 AM

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

Hannah L. Raczkowski, *The University of Western Ontario*

Supervisor: DeKoter, Rodney P., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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 antibody-generating responses, B cell development, and red pulp macrophage generation. Spi-C is inducible by heme- and NF-κB-dependent pathways in macrophages. The present research aimed to examine the regulation of Spi-C in B cells. RT-qPCR revealed that *Spic* expression was reduced in B cells following addition of lipopolysaccharide, anti-IgM antibodies, CD40L, or cytokines BAFF+IL-4+IL-5. Blocking proliferative signaling partially prevented downregulation of *Spic*. Unstimulated B cells upregulated *Spic* over time. To determine the mechanism of *Spic* regulation, we examined the *Spic* promoter and regulatory elements. The *Spic* promoter had unidirectional activity, which was reduced by mutation of a predicted NF-κB binding site. Bach2 and Spi-C formed a negative regulatory loop, repressing transcription of one another. Taken together, these data indicate that Spi-C is dynamically regulated by external signals in B cells.

Keywords

PU.1, Spi-B, Spi-C, transcription factor, B cell, plasma cell, memory, cell fate decisions, differentiation, immunology.

Lay Summary

Immune cell fate decisions are important for generating the cells that protect the body from invading pathogens. One cell type that contributes to immune responses by detecting dangerous stimuli and producing antibodies is the B cell, which can aid in the elimination of threats such as bacteria, viruses, and parasites. The development and differentiation of B cells into antibody-secreting cells relies heavily on the actions of transcription factors, including the E26-transformation-specific factors PU.1, Spi-B, and Spi-C. While PU.1 and Spi-B have well-defined roles in B cell fate decisions, the contributions and regulation of Spi-C in B cells are largely unknown. The goal of my thesis was to investigate the regulation of Spi-C in B cells and determine the biological implications of its up- or downregulation.

Gene expression analysis revealed that *Spic* expression was decreased when B cells were treated with signals that cause cell division. Blocking cell division partially prevented the downregulation of *Spic*. B cells cultured without any growth factors upregulated *Spic* over time. To determine how *Spic* is regulated, we examined the gene sequences responsible for its up- or downregulation. We identified several transcription factors that altered the expression of Spi-C by interacting with its regulatory gene sequences, including one factor known to be important in B cell fate decisions. Overall, our findings indicate that Spi-C is dynamically regulated by external signals, acting to contribute to important B cell fate decisions necessary for a robust immune system.

Co-Authorship Statement

The following thesis is adapted from a manuscript in preparation, titled "The E26-transformation-specific-family transcription factor Spi-C is dynamically regulated by external signals in B cells." Sections of Chapters 1 and 4 are adapted from "Lineage-instructive functions of the E26-transformation-specific-family transcription factor Spi-C in immune cell development and disease" (Raczkowski & DeKoter, 2021). Experiments were designed by Rodney. P. DeKoter and Hannah Lily Raczkowski, with kind input from Steven M. Kerfoot and S. M. Mansour Haeryfar. All experiments were performed by Hannah Lily Raczkowski, with the exception of the following. RT-qPCR experiments examining *Spic* expression in pro-B cells and 38B9 cells treated with Imatinib (Figure 3–7C-D) were performed by Sherry Xu. Figure 3–10A-B and Figure 3–11A-B were adapted from "Opposing Roles for the Related ETS-Family Transcription Factors Spi-B and Spi-C in Regulating B Cell Differentiation and Function" and performed or reanalyzed by Anne-Sophie Laramée or Rodney P. DeKoter (Laramée, Raczkowski et al., 2020). The following work was written by Hannah Lily Raczkowski and reviewed by Rodney. P. DeKoter and Steven M. Kerfoot.

Acknowledgements

I thank Dr. Jeehee Youn (Hanyang University) for providing the MIGR1-Bach2 construct used in this study. Thank you to Drs. Yodit Tesfagiorgis, (Western University), Carolina Batista (Columbia University), and Kristin Chadwick (London Regional Flow Cytometry Facility) for their assistance with flow cytometry and cell sorting. I thank Jodi Dutz (West Valley Animal Facility) and Sherry Xu (Western University) for their help with animal care, husbandry, and genotyping. Thank you to Lee-Ann Briere for allowing me to use the Biomolecular Interactions & Conformations Facility.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab – antibody

ASC – antibody-secreting cell

Bach – BTB and CNC homology

BAFF – B cell activating factor

BCR – B cell receptor

BCLAF – Bcl2-associated factor

BM – bone marrow

BMDM – bone marrow-derived macrophage

cDNA – complementary deoxyribonucleic acid

CSR – class switch recombination

ETS – E26-transformation-specific

FO – follicular

GC – germinal centre

iHPC – inflammatory hemophagocyte

IL – interleukin

IRF – interferon regulatory factor

LPS – lipopolysaccharide

MAS – macrophage activation syndrome

MBC – memory B cell

mRNA – messenger ribonucleic acid

MZ – marginal zone

NP-KLH – 4-hydroxy-3-nitrophylacetyl conjugated to keyhole limpet hemocyanin

PRR – pattern recognition receptor

PB – plasmablast

PC – plasma cell

PE – phycoerythrin

RPM – red pulp macrophage

sHLH – secondary hemophagocytic lymphohistiocytosis

SHM – somatic hypermutation

TLR – toll-like receptor

WT – wildtype

1 Chapter 1: Background

1.1 The B cell

The immune system is essential to the survival, health, and homeostasis of most organisms. While the specifics of immune responses vary across species, the two main branches of immunity – innate and adaptive – act independently and synergistically to promote host survival in vertebrates. Both forms of immunity rely on the ability to detect threats and mount a protective immune response. Whether this is driven primarily by discriminating between 'self' and 'non-self' or 'dangerous' and 'non-dangerous' remains a debated topic in the field, with both theories possessing limitations (Matzinger, 1994; Pradeu & Cooper, 2012). Regardless, the healthy immune system must balance appropriate and controlled immune responses, while avoiding damage to host tissues and commensal bacteria. Innate immune cells are responsible for the earliest detection and action against perceived pathogens, primarily through the use of germline-encoded pattern recognition receptors (PRRs). Detection of pathogenic stimuli leads to a rapid and non-specific array of responses, which may include phagocytosis, cytokine secretion, and killing of cells or pathogens (Chaplin, 2010; Hoebe et al., 2004).

While innate immunity provides a key preliminary response to microbes, its ability to generate lasting immunological memory in preparation for subsequent exposures is limited. Traditionally speaking, the ability to generate immunological memory and fine-tuned specificity is reserved for B and T lymphocytes. This crucial capacity is made possible by somatic rearrangement of germline genes to form functional B and T cell receptors. Rearrangement of these gene elements allows for a nearly unlimited number of combinations that can recognize and mount an immune response against the plethora of antigens that may be encountered over a lifetime.

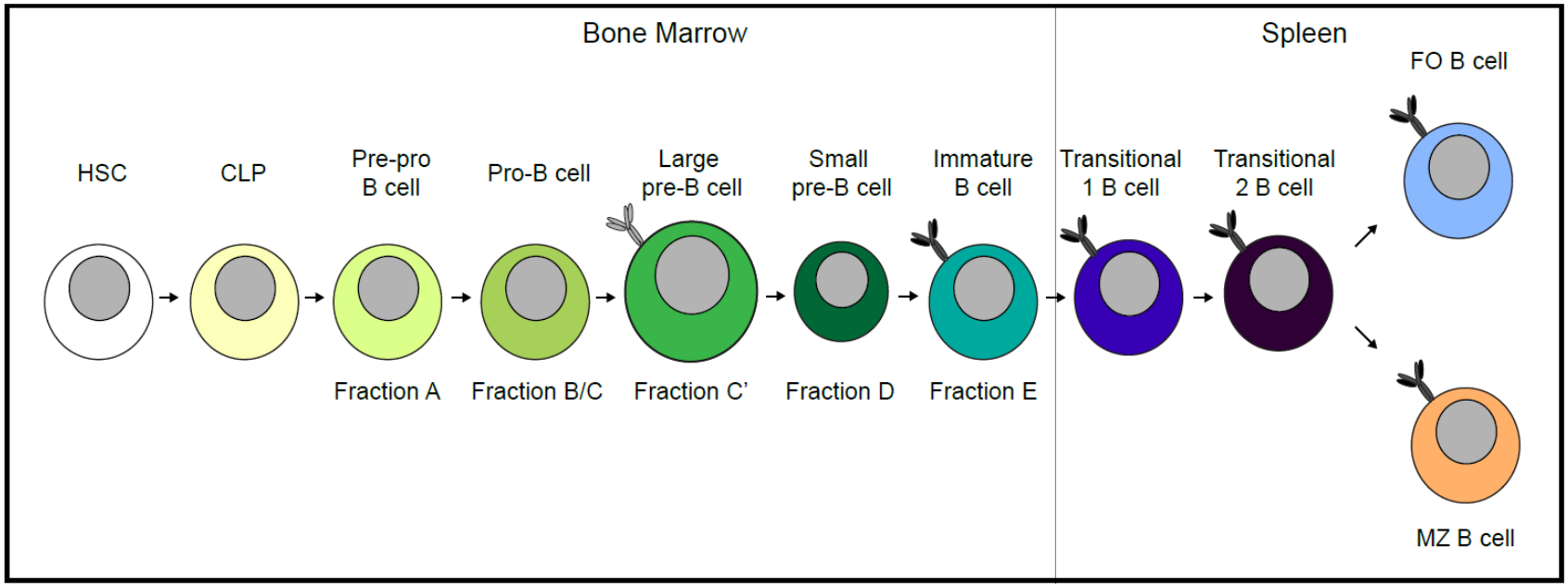
At the center of adaptive immunity is the B cell. The defining phenotype of this cell subset is the expression of membrane-bound B cell receptors (BCRs) specific to antigens, and upon differentiation into plasma cells (PCs), these same Immunoglobulin molecules are secreted as antibodies (Abs) (Chaplin, 2010; Cyster & Allen, 2019). Abs facilitate the resolution of infections by neutralization, opsonization and complement activation, or induction of

antibody-dependent cell-mediated cytotoxicity. In addition to the generation of Abs, activated B cells can acquire the fate of a long-lived memory B cell (MBC) responsible for rapid reactivation upon secondary antigen challenge. Naïve B cells and their differentiated counterparts are among the most critical immune cells involved in responses to natural infection and vaccination, and B cell biology remains a thriving field, despite comprehensive characterization of the subset since the early 1900s.

1.2 Murine B cell development

Murine B cell development in postnatal mice begins in the bone marrow (BM), where hematopoietic stem cells may initiate expression of lymphoid-restricted genes (Chaplin, 2010; Hardy & Hayakawa, 2001; Hardy et al., 2007; Lebien & Tedder, 2008). The current understanding of B cell development in mice is summarized in Figure 1–1. While there has been extensive debate regarding when lymphocyte precursors establish commitment to the lineage, the current consensus is that common lymphoid progenitors represent the point when alternate cell fates become highly unlikely (Hardy et al., 2007). These cells may then progress through the five main stages of intramedullary B cell development: pre-pro B, pro-B, large/early pre-B, small/late pre-B, and immature. Despite a wide range in nomenclature, these five terms are now well-accepted and in alignment with the more dated classification system known as Hardy fractions: A, B/C, C', D, and E (Hardy et al., 1991, 2007). Each stage is defined by phenotypic changes, namely expression of cell surface markers and rearrangement of the V (variable), D (diversity), and J (joining) gene segments to generate functional BCRs (Hardy & Hayakawa, 2001; Melchers, 2015). These strictly regulated stages give rise to immature B cells, which exit the BM to complete maturation and become naïve B cells ready for antigen encounter and activation.

Figure 1–1. Overview of murine B cell development. B cell development in postnatal mice begins in the bone marrow with hematopoietic stem cells (HSC). Common lymphoid progenitor (CLP) cells represent the earliest commitment to the lymphoid lineage. Pre-pro B cells (Hardy Fraction A) initiate recombinase activating gene (RAG) 1/2-mediated heavy chain immunoglobulin gene rearrangement at the D-J locus, followed by pro-B cells (Fraction B/C) joining a V segment to D-J_H. Large pre-B cells (Fraction C') signal through the pre-B cell receptor (BCR) consisting of rearranged heavy chains and surrogate light chains. Proliferation of large pre-B cells ceases and RAG 1/2 are re-expressed to mediate light chain rearrangement in small pre-B cells (Fraction D). Immature B cells (Fraction E) expressing successfully rearranged BCRs undergo selection. These cells exit the spleen and are termed transitional 1 or 2 B cells, depending on their location within the spleen. Transitional 2 B cells give rise to the B-2 subset: circulating follicular (FO) or non-circulating marginal zone (MZ) B cells.


Bone Marrow
Spleen
HSC
CLP
Pre-pro B cell
Pro-B cell
Large pre-B cell
Small pre-B cell
Immature B cell
Transitional 1 B cell
Transitional 2 B cell
FO B cell
MZ B cell
Fraction A
Fraction B/C
Fraction C'
Fraction D
Fraction E

Early B cell progenitors begin their complex series of signaling events in close proximity to BM stromal cells, which provide necessary signals to initiate B cell commitment (Melchers, 2015). Most notably, stromal cells secrete IL-7, which promotes pro-B cell survival, proliferation, and accessibility of heavy chain gene segments. The eventual expression of Pax5, which also promotes Ig loci accessibility and induces cell-surface expression of CD19, marks irreversible commitment to the B cell fate (Lebien & Tedder, 2008). Pre-pro B cells initiate BCR generation by rearrangement of the heavy chain D-J segments. Rearrangement is mediated by the recombinase activating genes (RAG1/2) and terminal deoxynucleotidyl transferase (Lebien & Tedder, 2008). Pro-B cells undergo joining of a heavy chain V segment to the $D\text{-}J_H$ segment, which marks the completion of the pro-B phase and entry into the pre-B stage of development.

Successfully rearranged μ heavy chains may then pair with surrogate light chains to form the pre-BCR. Signaling through the pre-BCR and IL-7Rα initiates an array of events including a proliferative burst characteristic of the large pre-B cell stage. However, proliferation is inherently self-limiting, as pre-BCR stimulation also results in the downregulation of surrogate light chains and IL-7Rα. As availability of surrogate light chains declines, proliferation ceases and RAG 1/2 are re-expressed in preparation for light chain rearrangement. Introduction of double-stranded DNA breaks solidifies the progression to the small pre-B cell stage, downregulating BLNK and SYK and halting cell cycle entry (Bednarski et al., 2016). Productive rearrangement of $V_L\text{-}J_L$ segments at either the Igκ or λ loci allows for cell surface expression of IgM on immature B cells and marks the completion of B cell development within the BM. In the case of autoreactivity, which may eliminate up to 90% of B cell precursors in mice, B cells are subjected to central tolerance (Pelanda & Torres, 2012). These self-recognizing cells undergo negative selection in the form of apoptosis, induction of anergy, and/or receptor editing by RAG 1/2.

Immature B cells expressing complete, non-autoreactive BCRs egress from the BM and enter circulation and secondary lymphoid tissues (Herzog et al., 2009). These migrating B cells, termed transitional B cells, primarily home to the spleen in response to chemotactic signals (Chung et al., 2003; Debnath et al., 2008; Sims et al., 2005). These cells are further divided into transitional 1 (T1) and 2 (T2) B cells, based on their phenotype, location, and functional

characteristics. T1 B cells travel through the splenic red pulp to the outer zone of the periarteriolar lymphoid sheath, whereas T2 B cells are restricted to B cell follicles. Additional rounds of negative selection occur during the transitional stage, where T1 B cells in particular are susceptible to apoptosis in response to BCR cross-linking. The maturation of T1 to T2 B cells relies heavily on the tumor necrosis factor (TNF) ligand family member known as B-cell activating factor (BAFF), as well as tonic BCR signaling and IL-4 (Debnath et al., 2008; Smulski & Eibel, 2018; Zhou et al., 2020). From the T2 stage, B cells may pursue one of two distinct B-2 fates: differentiation into circulating mature B cells known as follicular (FO) B cells, or non-circulating marginal zone (MZ) B cells (Allman & Pillai, 2008).

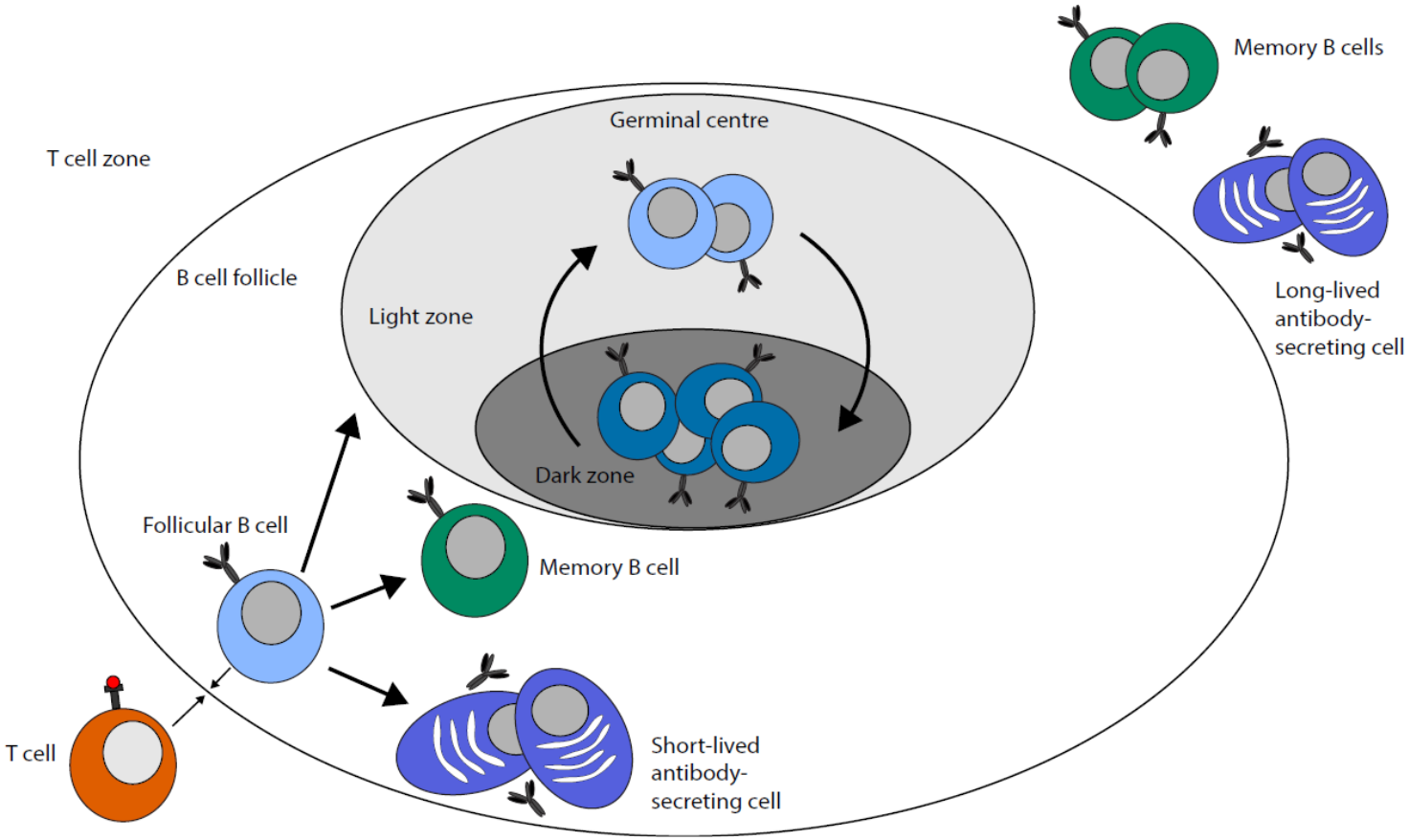
1.3 B cell activation and differentiation

Naïve, mature FO B cells freely circulate the blood and lymph, passing through secondary lymphoid organs, where they may be exposed to antigen (Pillai & Cariappa, 2009). Most of these cells are characterized by high expression of IgD and low expression of IgM. In contrast, MZ B cells express high levels of IgM and low levels of IgD and reside between the marginal sinus and red pulp. In addition to differences in surface phenotype and location, MZ and FO B cells differ in their function and primary methods of activation (Martin & Kearney, 2002). FO B cell activation and differentiation are summarized in Figure 1–2.

MZ B cells constitute roughly 15% of B cells in the spleen, and are unique in that they possess innate-like functions following antigen encounter (Cerutti et al., 2013; Martin & Kearney, 2002). They express BCRs that are often polyreactive, as well as high levels of TLRs. The location of MZ B cells within the spleen permits high exposure to blood-borne pathogens and activation by antigens presented by macrophages, dendritic cells, or neutrophils. MZ B cell activation is typically a T cell-independent process, both in response to carbohydrate and protein antigens. Despite a preference for T cell-independent activation, MZ B cells express higher levels of T cell co-signaling molecules than FO B cells (Oliver et al., 1999). In a classic example of activation, MZ B cells become activated in response to lipopolysaccharide (LPS), which may involve recognition by both BCRs and TLRs. Activated MZ B cells differentiate rapidly into short-lived antibody-secreting cells (ASCs), secreting predominantly IgM Abs. This swift response, while not tailored to the specific antigen to the extent of FO B cell responses, may provide early protection from pathogens in an innate-like manner.

FO B cells are typically activated in a T cell-dependent manner which initiates following binding of antigen to the BCR. BCR ligation induces cross-linking of BCRs, which initiates a signaling cascade involving proteins including spleen tyrosine kinase (Syk) and the scaffold protein B cell linker protein (Blnk). Many antigens engage other cell surface receptors such as TLRs or complement receptors, which may further amplify the signal. B cells will internalize the BCR and bound antigen through receptor-mediated endocytosis. Following enzymatic processing, antigen-derived peptides are presented on MHC class II molecules on the cell surface for presentation to $CD4^+$ T follicular helper cells. Chemotactic signals guide B cells to the interfollicular region, where T cells and activated B cells participate in prolonged cognate interactions (Kerfoot et al., 2011). CD40-CD40L co-stimulation triggers three significant events: transcription of Ig genes, B cell proliferation, and T cell secretion of cytokines including IL-4 and IL-21. Cytokines produced at this stage in activation drive further proliferation and differentiation, with additional implications for class switch recombination (CSR). CSR enables B cells to alter the isotype of their BCR from IgM and IgD to IgG, IgA, or IgE. Each Ab class has distinct functions, in part due to varying binding capabilities. Ab isotype dictates binding to Fc receptors that mediate key effector mechanisms such as opsonization and antibody-dependent cell-mediated cytotoxicity. As well, classes may differ in location, mechanisms of action, and biological roles. While originally considered a process occurring in germinal centres (GCs), CSR is now thought to occur after T cell priming but prior to GC formation (Roco et al., 2019).

Figure 1–2. Overview of follicular B cell fate decisions following T cell-dependent activation. B cells activated by antigen receive co-stimulatory signals from T cells in the interfollicular region. B cells can then immediately differentiate into short-lived antibody-secreting cells or memory B cells, or initiate germinal centre formation. Germinal centre B cells undergo somatic hypermutation and selection to generate high-affinity B cell receptors. These cells may once again differentiate into either long-lived antibody-secreting cells or memory B cells.


Memory B cells
Germinal centre
T cell zone
B cell follicle
Light zone
Long-lived
antibody-
secreting cell
Dark zone
Follicular B cell
Memory B cell
T cell
Short-lived
antibody-
secreting cell

In the first few days following antigen exposure but prior to the GC reaction, some activated FO B cells differentiate into ASCs (Cyster & Allen, 2019). As this early differentiation typically occurs before somatic hypermutation (SHM) and affinity maturation, BCRs have a limited capacity for binding to antigen. There has been some disagreement within the field about the definition of plasmablasts (PBs) vs. PCs, with the former term used somewhat interchangeably to describe both GC-independent ASCs and immature plasma cells retaining some capacity for proliferation (Cyster & Allen, 2019; MacLennan et al., 2003; Nutt et al., 2015). Regardless, ASCs derived from extrafollicular differentiation are defined by a short lifespan and maintenance in secondary lymphoid tissues. These cells are thought to provide an early protective Ab response to pathogens in the days between first detection and generation of GC-independent ASCs. In addition to ASCs, there is evidence of long-lived, but low-affinity, GC-independent MBCs (Takemori et al., 2014). This population allows cells to retain adaptability potential, while still generating an early memory response for use during primary or subsequent encounters (Palm & Henry, 2019).

FO B cells that do not pursue early differentiation go on to generate GCs, which are temporary biological niches within the B cell follicles of secondary lymphoid organs (Cyster & Allen, 2019; De Silva & Klein, 2015). Within GCs, B cells undergo intense clonal proliferation, targeted mutagenesis, and successive rounds of selection. GCs are split into two substructures known as the light zone and dark zone, which are defined on the basis of function and cell type. The dark zone contains densely packed B cells dividing rapidly and undergoing SHM to further diversify previously rearranged variable regions of Ig genes. Resulting GC B cells have a wide range of affinity for the target antigen, and thus cycle to the light zone to experience selection of clones with high-affinity BCRs (Kurosaki et al., 2015; Takemori et al., 2014). The light zone contains mainly follicular dendritic cells and T follicular helper cells, the latter of which mediates positive selection. GC B cells may recirculate through successive rounds of mutagenesis and selection to further increase BCR affinity for antigen, or proceed to differentiate into ASCs or MBCs. Antigen-experienced GC B cells ultimately give rise to long-lived, terminally differentiated PCs capable of secreting high levels of Abs with fine-tuned specificity (Nutt et al., 2015). GC-derived PCs home to the bone marrow, where they may be maintained for several months to years, constitutively producing Abs. Similar to their GC-independent counterpart, MBC generated from the GC circulate the periphery, patrolling for

antigen. MBCs, baring high-affinity, class-switched BCRs, are primed for rapid differentiation into PCs or formation of a GC upon antigen re-encounter (Kurosaki et al., 2015). This immunological memory is the foundation for natural immunity and vaccination (Nutt et al., 2015; Palm & Henry, 2019).

Many aspects of B cell differentiation may be recapitulated *in vitro*. To model T cell-dependent B cell activation, a culture system of CD40L, IL-4, and IL-5 is often utilized (Hasbold et al., 2004; Nutt et al., 2015). CD40L mimics T cell interaction, inducing activation and proliferation. IL-4 and IL-5 promote cell survival, CSR, and differentiation into ASCs (Franke et al., 2020; Hasbold et al., 2004; Horikawa & Takatsu, 2006). T cell-independent B cell activation and subsequent differentiation may also be achieved through the use of the Anti-IgM Abs or mitogens such as LPS (Nutt et al., 2015; Wortis et al., 1995). These culture conditions permit studies of B cell fate decisions of a highly reproducible nature.

1.4 Lineage-instructive transcription factors

Cell fate decisions occur over multiple steps to enable irreversible differentiation from a pluripotent progenitor into a specialized cell type (Gilbert, 2000). The first stage of cell fate commitment is known as specification, in which a cell is able to autonomously differentiate in a neutral environment, but its fate is not yet fully restricted (Gilbert, 2000). Following specification, cells may progress to a determined state, in which differentiation is irreversible, regardless of environmental changes. Cell type-specific transcription factors enforce the changes in gene expression that underly cell fate decisions.

While induction of differentiation of precursor cells into mature, specialized cell types typically involves hundreds of transcription factors, a relatively small cohort of transcription factors exert lineage-instructive effects on target cell types (Cole and & Young, 2008; Wontakal et al., 2012). The term “master regulator” was created to describe genes positioned at the top of a regulatory hierarchy, that function by regulating the transcription of tens to thousands of downstream genes, including those encoding other transcription factors (Ohno, 1979). Master regulators were first described in the context of sex determination, but were soon extended to fields including yeast cell specification, Drosophila development, and pluripotent stem cells (Boyer et al., 2005; Davis & Rebay, 2017). The term master regulator

has evolved to encompass any gene governing developmental lineage, which today is most commonly represented by genes necessary and sufficient to establish a particular lineage from a precursor cell (Chan & Kyba, 2013).

PU.1 (encoded by *Spi1*), an E26-transformation-specific (ETS)-family transcription factor, has been established as a lineage-instructive transcription factor in hematopoiesis (Batista & DeKoter, 2018; Burda et al., 2010; DeKoter & Singh, 2000). PU.1 is required for normal hematopoiesis, as evidenced by the failure of *Spi1*$^{-/-}$ to generate B cells and macrophages (Houston et al., 2007; McKercher et al., 1996; Scott et al., 1994). Using conditional knockout alleles of *Spi1*, multiple groups showed that PU.1 is required to generate myeloid and lymphoid progenitors (Iwasaki et al., 2005; Polli et al., 2005; Ye et al., 2005). PU.1 acts in a concentration-specific manner to enforce distinct dendritic cell fates (Chopin et al., 2019). PU.1 functions as a pioneer protein by accessing nucleosomal target sites to remodel chromatin, allowing recognition by unrelated, non-pioneer transcription factors (Minderjahn et al., 2020; Monticelli & Natoli, 2017). Taken together, these studies illustrate the indispensable role of PU.1's lineage-instructive function in hematopoiesis.

GATA-1, the first member of the GATA transcription factor family to be identified, represents another example of a lineage-instructive transcription factor in hematopoiesis (Ferreira et al., 2005). Its absence is marked by severe defects and early embryonic death due to a block in development of mature red blood cells (Pevny et al., 1991). In addition to its necessity in erythropoiesis, GATA-1 is also critical for normal generation of megakaryocytes and eosinophils. Multiple laboratories demonstrated that GATA-1 physically interacts with PU.1 to mutually antagonize functions in hematopoietic cells (Nerlov et al., 2000; Rekhtman et al., 1999; Zhang et al., 2000). One group utilized mice with GATA-1 and PU.1 transcriptional reporters to demonstrate that multipotent progenitors upregulating GATA-1 and PU.1 function as common myeloid progenitors and granulocyte/monocyte/lymphoid progenitors, respectively (Arinobu et al., 2007). GATA transcription factors, like PU.1, have pioneer activity that can remodel chromatin by interacting with nucleosomes (Zaret et al., 2008). Together, PU.1 and GATA-1 function as key lineage-instructive transcription factors in hematopoiesis and regulate the polarizing decision to pursue myeloid or lymphoid cell fates.

1.5 The E26-transformation-specific transcription factor Spi-C

1.5.1 Discovery of Spi-C

Spi-C (encoded by *Spic*) is a member of the ETS transcription factor family (Hollenhorst et al., 2011). The ETS family is comprised of 28 proteins in humans, each containing the characteristic and highly conserved ETS DNA-binding domain (Hollenhorst et al., 2011). ETS proteins are involved in crucial stages of development and differentiation in processes ranging from embryogenesis to adult immune responses (Hollenhorst et al., 2011). Spi-C was named for its high degree of similarity to two ETS transcription factors, PU.1 (encoded by *Spi1*) and Spi-B (encoded by *Spib*), together making up the SPI subfamily (Bemark et al., 1999). SPI factors have been identified as important regulators of hematopoiesis, with contributions to the generation of both myeloid- and lymphoid-lineage cell subsets (Turkistany & Dekoter, 2011). Originally identified by yeast one-hybrid screening of a cDNA library using LPS-stimulated murine splenic B cells, Spi-C was found to interact with the SP6 κ promoter κY element. The *Spic* gene encoding Spi-C is located on chromosome 10 and 12 of the mouse and human genomes, respectively (Carlsson et al., 2002). Months after its original discovery, a second group identified the same protein interacting with PU.1 binding motifs including the Immunoglobulin κ 3′ enhancer, but published their findings describing Spi-C as PU.1-related factor (Hashimoto et al., 1999).

1.5.2 Structure and function of Spi-C

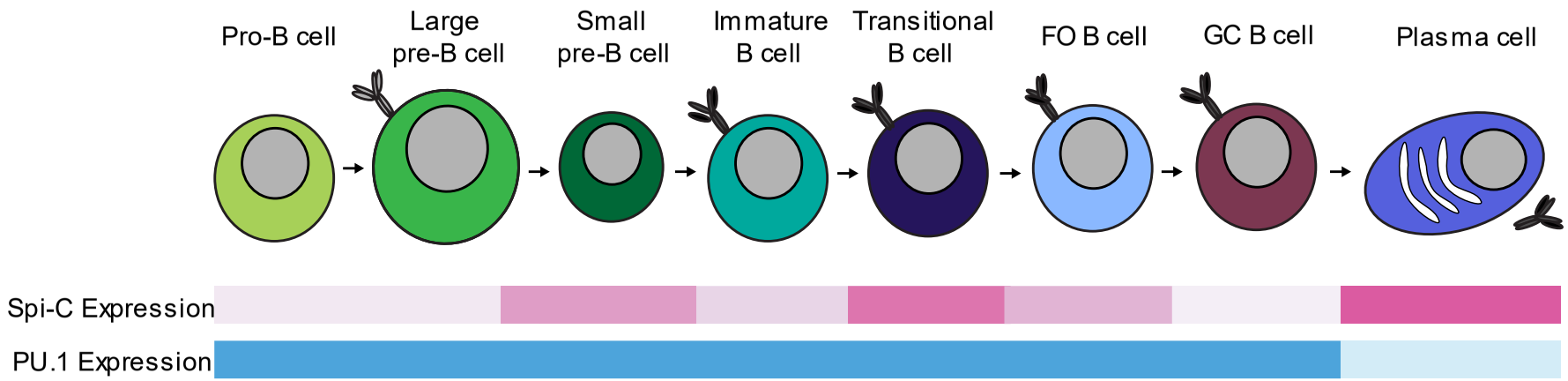
Murine Spi-C has 242 amino acids and has a mass of 29.2 kDa (Carlsson et al., 2002). The human protein is highly related, showing 65% amino acid identity to murine Spi-C. Both murine and human genes contain a 5′ non-coding exon preceding 5 coding exons. ETS family proteins including Spi-C contain a conserved DNA-binding domain, which allows protein interactions through a helix–turn–helix motif (Hollenhorst et al., 2011; Sharrocks, 2001). Similar to other ETS family transcription factors, Spi-C is able to bind to the 5′-GGA(A/T)-3′ core motif of purine-rich sequences (Bemark et al., 1999; Oikawa & Yamada, 2003). Whether Spi-C has pioneer activity like PU.1 has not yet been investigated. The murine Spi-C DNA-binding domain exhibits 57 and 60% sequence identity with that of PU.1 and Spi-B, respectively. In addition to a DNA-binding domain, Spi-C contains an acidic N-terminus transactivation domain, consistent with other SPI subfamily transcription factors (Carlsson et al., 2003). Spi-C lacks the proline/glutamic acid/serine/threonine domain present in both PU.1

and Spi-B, which is known to interact with IRF transcription factors (Brass et al., 1999). Spi-C lacks the critical S148 amino acid residue that is necessary for ternary complex formation with IRF4 and IRF8 (Brass et al., 1999; Li, et al., 2015).

1.5.3 Spi-C expression

The first studies of expression suggested that Spi-C was expressed at highest levels in the B cell compartment, with high expression in mature B cells (Bemark et al., 1999; Hashimoto et al., 1999). Soon after, Spi-C was found to be temporally expressed in B cells during development and differentiation into antibody-secreting cells, as well as in splenic macrophages (Carlsson et al., 2002, 2003). In the context of B cell development, Spi-C is expressed at the pre-B, transitional, and mature B cell stages, with peak expression occurring in transitional B cells (Debnath et al., 2008; Zhu et al., 2008). In mature and differentiated subsets, Spi-C expression is highest in antibody-secreting cells compared to all other populations, most notably in terminally differentiated ASCs (Shi et al., 2015). Expression of Spi-C and its highly related family member PU.1 during B cell development and differentiation is summarized in Figure 1–3. Spi-C is expressed at the very highest levels in red pulp macrophages (RPMs) (Kohyama et al., 2009). Spi-C has been detected in a number of other cell types, although with little insight into significance. For example, these studies showed that Spi-C is expressed in preimplantation embryos (Inoue et al., 2015; Kageyama et al., 2006), eosinophils (Fulkerson et al., 2006; Voehringer et al., 2007), and cells in inflammatory lung disease (Lian et al., 2005). As will be discussed below, Spi-C expression is induced in macrophages during inflammation in response to sterile injury (Bennett et al., 2019; Kayama et al., 2018b) or toll-like receptor (TLR) ligands (Akilesh et al., 2019; Z. Alam et al., 2020; Wang et al., 2019). High-throughput expression studies such as ImmGen have failed to detect high levels of Spi-C in cells other than RPM (Heng et al., 2008). These studies relied on expression of steady-state mRNA levels in cell subsets purified from disease-free mice, and therefore may have missed dynamic induction of Spi-C expression in response to specific stimuli.

Figure 1–3. Expression of Spi-C and PU.1 during B cell development and differentiation. Spi-C is dynamically regulated throughout B cell development and differentiation. Expression is low in early stages of B cell development and is elevated to drive commitment to the small pre-B cell stage. Expression is high during the transitional B cell stage and lower in follicular (FO) B cells. Spi-C expression drops during the germinal centre (GC) reaction and increases sharply in plasma cells. Expression of PU.1 is consistently high throughout B cell development and drops only during commitment to the plasma cell fate. Darker colours indicate higher levels of expression.


Pro-B cell
Large pre-B cell
Small pre-B cell
Immature B cell
Transitional B cell
FO B cell
GC B cell
Plasma cell
Spi-C Expression
PU.1 Expression

1.5.4 Interaction partners of Spi-C

ETS factors exhibit a high degree of similarity in DNA binding motifs, as evidenced by occupancy of similar purine-rich GGAA consensus motifs (Hollenhorst et al., 2011). PU.1 and Spi-B act as transcriptional activators, while Spi-C primarily represses target genes (Li et al., 2015; Schweitzer et al., 2006). Although PU.1 and Spi-B are functionally complementary, studies show that Spi-C antagonizes their actions at multiple stages of B cell development and function (Laramée et al., 2020; Soodgupta et al., 2019; Zhu et al., 2008). PU.1 and Spi-B activate genes including *Nfkb1*, *Bach2*, *Syk*, and *Blnk* during crucial stages of development and differentiation, whereas Spi-C represses these target genes (DeKoter et al., 2010; Laramée et al., 2020; Li, et al., 2015). Chromatin immunoprecipitation-sequencing studies demonstrated that PU.1, Spi-B, and Spi-C interact as monomers primarily with single GGAA consensus motifs (Laramée et al., 2020; Solomon et al., 2015). The binding sites occupied by PU.1, Spi-B, and Spi-C are largely overlapping, although not identical (Laramée et al., 2020; Solomon et al., 2015). Soodgupta et al. recently showed that ectopic expression of Spi-C in pre-B cells caused significant changes in genome-wide chromatin binding of PU.1, while PU.1 expression remained constant, suggesting that Spi-C displaces PU.1 from binding sites (Soodgupta et al., 2019). These results suggest that the mechanism of action of Spi-C repression of target genes is primarily by competition for PU.1 and/or Spi-B binding sites.

Although there is no direct evidence for a transcriptional repression function of Spi-C, it differs from PU.1 and Spi-B in proteins with which it can partner to regulate transcription. Spi-C lacks the Serine-148 amino acid residue in PU.1 (S149 in Spi-B) that is necessary for interaction with IRF transcription factors (Brass et al., 1999; Li et al., 2015). Therefore, Spi-C likely displaces not just PU.1 and Spi-B, but also PU.1/Spi-B/IRF4/IRF8 ternary complexes from ETS-IRF composite elements. Spi-C selectively forms complexes with BCLAF1 (Bcl2-associated factor 1), another transcription factor expressed in response to DNA damage, to exert transcriptional regulatory function distinct from PU.1/Spi-B (Soodgupta et al., 2019). This study was the first to identify a specific binding partner for Spi-C, and to provide evidence that Spi-C directly displaces PU.1 (complexed with IRF factors) throughout the genome to modify gene expression and influence cell fate decisions (Soodgupta et al., 2019).

1.6 Biological roles of Spi-C

1.6.1 Spi-C in monocyte and macrophage development

The role of Spi-C in RPM development represents a clear example of transcription factor lineage-instructive function in hematopoiesis (Kohyama et al., 2009). RPMs are critically important for degrading senescent erythrocytes and recycling heme-associated iron (Ganz, 2012). Kohyama et al. (2009) first discovered that RPMs have very high expression of *Spic* compared to other immune cell subsets including dendritic cells, bone marrow-derived macrophages, and B cells. Kohyama et al. went on to generate the first *Spic*$^{-/-}$ mice using gene targeting to investigate the contributions of Spi-C to RPM development. Initial observations of *Spic*$^{-/-}$ mice revealed that both sexes were viable, fertile, and had a normal lifespan. Despite the absence of any major abnormalities, breeding of mice heterozygous for *Spic* produced a frequency of only 9% *Spic*$^{-/-}$ mice, suggesting embryonic lethality. Flow cytometry experiments revealed that *Spic*$^{-/-}$ mice entirely lacked RPMs, resulting in an iron overload in splenic red pulp due to inefficient phagocytosis of red blood cells (Kohyama et al., 2009). Retroviral expression of Spi-C in BM cells was necessary and sufficient to promote the development of RPMs from *Spic*$^{-/-}$ BM cells. Taken together, these findings demonstrate that Spi-C has a lineage-instructive role in the generation of splenic RPMs.

The same group went on to explore the mechanism of induction of Spi-C in RPMs (Haldar et al., 2014). Expression of enhanced green fluorescent protein under control of the *Spic* locus revealed expression in RPMs and bone marrow macrophages (BMMs). Similar to RPMs, BMMs are involved in iron homeostasis and erythrocyte degradation (Ganz, 2012). Investigation by flow cytometry confirmed the absence of BMMs in *Spic*$^{-/-}$ mice, illustrating its key role in the generation of multiple macrophage subsets (Haldar et al., 2014). Iron-recycling macrophages in the liver also had notably high steady-state expression of *Spic*. Given the association of RPMs and BMMs with iron homeostasis and erythrophagocytosis, heme, a metabolite of erythrocyte degradation, was selected as a candidate inducer of Spi-C expression. Interestingly, heme rapidly upregulated *Spic* expression in bone-marrow derived macrophages (Haldar et al., 2014). Induction of *Spic* expression in myeloid cells by heme was confirmed in multiple *in vivo* systems. This unique process was the first described instance of a metabolite driving differentiation of a tissue-resident macrophage subset through a single lineage-specific transcription factor (Alam et al., 2017).

Haldar et al. (2014) determined the mechanism of Spi-C induction in RPMs by showing that *Spic* is constitutively repressed by the transcription factor Bach1 (BTB And CNC Homology 1). Bach1 is a member of the basic region leucine zipper family and is highly expressed in the myeloid lineage (Itoh-Nakadai et al., 2014; Oyake et al., 1996). Bach1 and the highly related factor Bach2, which conversely has lymphoid-restricted expression, repress target genes through a BTB protein–protein interaction motif (Igarashi et al., 2017). Bach1 requires dimerization with musculoaponeurotic fibrosarcoma (MAF) proteins in order to bind to target DNA sequences, while Bach2 may bind to DNA as a homodimer (Oyake et al., 1996). Binding of heme to Bach1 induced proteasome-dependent Bach1 degradation, in turn leading to the de-repression of *Spic*. De-repression of *Spic* by heme can drive the generation of RPMs and BMMs, respectively (Haldar et al., 2014). Recently, it was shown that IL-33 synergizes with heme to further upregulate *Spic* expression and drive the generation of RPMs through activation of MyD88 and ERK1/2 kinases (Lu et al., 2020). Overall, these studies were the first to describe differentiation of a tissue-resident macrophage subset driven by a metabolite, and to define the lineage-instructive role of Spi-C.

Akilesh et al. (2019) recently characterized a subset of macrophages generated in response to TLR7 activation that have high hemophagocytic capacity and Spi-C expression. These macrophages are similar to RPMs, but have a distinct phenotype and arise during inflammation, leading to the term inflammatory hemophagocytes (iHPCs). These cells were found to be responsible for anemia observed in mice overexpressing TLR7, which display a disease resembling the hyperinflammatory condition known as macrophage activation syndrome (MAS). While Spi-C was not required for iHPC development, high expression of the transcription factor was noted as a hallmark of the cell type. Additional exploration of TLR agonists demonstrated that iHPCs are also generated from Ly6C^{hi} monocytes following TLR9 activation. While LPS and IL-1β did not generate cells with the full iHPC transcriptional profile, both were sufficient to induce *Spic* expression in BM Ly6Chi monocytes. Taken together, this work suggests that MyD88 adaptor signaling upregulates *Spic* expression, which represents a novel pathway of *Spic* induction (Akilesh et al., 2019).

Alam et al. (2020) further explored the contributions of Spi-C to inflammatory states in monocytes and macrophages. LPS induced *Spic* expression in patrolling monocytes and macrophages in a heme-independent manner, which in turn altered the transcriptional profile of BMMs to an anti-inflammatory state. Interestingly, LPS treatment caused robust *Spic* induction in lung and peritoneal resident macrophages, which do not express *Spic* under normal conditions. *Spic* expression was found to be inhibited by IFNγ in a STAT-1-dependent mechanism both alone and following LPS treatment in BMMs. Finally, *Spic* was shown to be induced in lung and kidney models of sterile inflammation. Upregulation of Spi-C was shown to occur in a pathway dependent on canonical NF-κB signaling, which is of particular note, given *Nfkb1* transcription is repressed by Spi-C and activated by its family members PU.1 and Spi-B (Li et al., 2015a; Li et al., 2015b). Together with work by Akilesh et al., these studies demonstrate that Spi-C is inducible by inflammatory stimuli in macrophages in sterile and disease environments. While Spi-C induction in macrophages has been characterized, little is known about the pathways which regulate its expression in the B cell compartment.

1.6.2 Spi-C in B cell development

In B cells, Spi-C has been implicated in regulation of B cell development, antibody-generating responses, and suppression of pre-B cell receptor (BCR)-mediated proliferation (Bednarski et al., 2016; DeKoter et al., 2010; Laramée et al., 2020; Li et al., 2015; Zhu et al., 2008). Schweitzer et al. (2006) first set out to examine the biological function of Spi-C in B cells. Ectopic overexpression of Spi-C in cultured pro-B cells using a retroviral vector suggested that Spi-C functions as a negative regulator by opposing PU.1 and/or Spi-B activity on target gene expression (Schweitzer et al., 2006). Ectopic Spi-C expression in pro-B cells caused phenotypic changes indicating differentiation towards the pre-B cell stage, including increased CD25 and reduced c-Kit expression (Schweitzer et al., 2006). Spi-C downregulated expression of FcγRIIb and increased transcription of IgH, acting in contrast to PU.1. Overall, Schweitzer et al. demonstrated an opposing role for Spi-C in which it antagonizes PU.1 and promotes B cell differentiation.

To further explore the biological function of Spi-C, transgenic Eμ-Spic mice were generated that ectopically express Spi-C in developing B cells (Zhu et al., 2008). These mice had reduced numbers of total splenic B cells, impaired B cell development at the pro-B cell to pre-B cell

stage, and reduced frequencies of immature and transitional-1 B cells in the BM. Immunization of Eμ-Spic transgenic mice with haptenated keyhole limpet hemocyanin resulted in an increase in IgM titers, and decrease in IgG titers (Zhu et al., 2008). Splenic B cells from of Eμ-Spic transgenic mice treated with anti-IgM or anti-CD40 had reduced proliferation and increased cell death, illustrating impaired B cell signaling due to dysregulation of BCR-associated genes. In a follow-up study, transgenic expression of Spi-C was shown to rescue reduced CD23 expression observed in *Spi1*$^{+/-}$ *Spib*$^{-/-}$ mice (DeKoter et al., 2010). Experiments showed that Spi-C activated expression of CD23, reduced the frequency of marginal zone B cells, and increased the frequency of follicular and transitional 2 B cells (DeKoter et al., 2010). However, experiments involving ectopic or overexpression of a transcription factor must always be interpreted carefully and in conjunction with loss-of-function experiments. *Spic*$^{-/-}$ mice were generated in 2009, but B cell development and B cell function were not extensively analyzed in their initial publications (Haldar et al., 2014; Kohyama et al., 2009). To analyze the function of Spi-C in B cells using loss-of-function experiments, the DeKoter lab compared *Spib*$^{-/-}$ and *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice. Heterozygosity for *Spic* rescued impairments in B cell development and proliferation observed in Spi-B knockout mice (Laramée et al., 2020; Li et al., 2015). This phenotype was in part attributed to the opposing regulation of *Nfkb1* by PU.1/Spi-B and Spi-C, where PU.1 and Spi-B activated, and Spi-C repressed *Nfkb1* transcription (Li et al., 2015b). Upon immunization, heterozygosity for *Spic* also partially rescued frequencies of IgG_1 antibody-secreting cells relative to *Spib*$^{-/-}$ mice, suggesting that Spi-B and Spi-C may oppositely regulate B cell differentiation into antibody-secreting or MBCs. Taken together, these results demonstrate that Spi-C plays a role in B cell function.

As described above, de-repression of *Spic* by heme interaction with Bach1 in macrophages is an important mechanism for induction of RPM differentiation (Haldar et al., 2014). B cells express high levels of the related family member Bach2 rather than Bach1 (Igarashi & Itoh-Nakadai, 2016). Interestingly, the lymphoid-lineage repressor Bach2 also interacts directly with heme, indicating the possibility of a similar mechanism of induction of *Spic* in lymphocytes (Watanabe-Matsui et al., 2015). Whether heme induces Spi-C expression in B cells, as well as the biological consequences of its potential induction, remains unknown.

Bach2 is a key regulator at numerous stages of B cell development and differentiation during immune responses. At the pre-B cell stage of development, Bach2 opposes Bcl6 during negative selection to promote deletion of B cells with non-productive heavy chain rearrangements (Swaminathan et al., 2013). In the context of T cell-dependent immunization, *Bach2*$^{-/-}$ mice failed to form GCs, exhibited impaired CSR, and generated few high-affinity Abs (Muto et al., 2004, 2010). Shinnakasu and colleagues reported that haploid-insufficiency of Bach2 inhibited MBC differentiation in GC B cells, instead promoting the PC fate (Shinnakasu et al., 2016; Shinnakasu & Kurosaki, 2017). As well, repression of *Bach2* is an important step in the generation of ASCs (Kometani et al., 2013; Muto et al., 2010). Together, these findings demonstrate that Bach2 expression strongly promotes the MBC fate, while directly opposing ASC generation. We previously found that *Bach2* expression was reduced in *Spib*$^{-/-}$ mice, whereas *Spib*$^{-/-}$ *Spic*$^{+/-}$ mice had *Bach2* expression restored nearly to the level of the WT control (Laramée et al., 2020). *Bach2* and *Spib* were also concordantly downregulated following commitment to the ASC fate. These findings indicate that Spi-B may activate *Bach2* transcription to promote GC formation and MBC generation, while Spi-C represses *Bach2* and leads cells to pursue an ASC fate. However, no work has been done to directly examine the potential regulatory system involving Spi-B, Spi-C, and Bach2.

Bednarski et al. (2016) observed that Spi-C expression is induced in pre-B cells following the RAG-dependent generation of DNA double-stranded breaks in immunoglobulin light chain genes. Spi-C was shown to directly repress transcription of *Syk* and *Blnk*, which act as key transcription factors necessary for BCR signaling, including during the pre-B cell stage (Herzog et al., 2009). Conversely, PU.1 is required for expression of SYK and subsequent signaling through the BCR (Garrett-Sinha et al., 1999). RAG-mediated double-stranded breaks sequentially activate ATM and NF-κB2, leading to induction of *Spic*. Spi-C's repression of pre-BCR signaling prevented the cycling of small pre-B cells, therefore functioning to prevent the proliferation of cells during a period of severe genomic instability. Bednarski's group recently elaborated on the mechanism, solidifying a crucial role for Spi-C during B cell development. They found that once activated in response to double-stranded DNA breaks, Spi-C recruits BCLAF1 to chromatin and causes displacement of PU.1 chromatin binding throughout the genome (Soodgupta et al., 2019). While PU.1 activity promotes proliferation and expansion characteristic of large pre-B cells, DNA double-stranded break-driven expression of Spi-C

promotes transition to the small pre-B cell stage of development. These studies demonstrate that Spi-C has numerous important roles in the B cell compartment, including directing B cells as they progress through the final stages of intramedullary B cell development.

1.6.3 An emerging role for Spi-C in inflammation and disease

As described above, high levels of Spi-C expression were discovered to be a biomarker of hemophagocytic macrophages in inflammatory disease states (Akilesh et al., 2019; Alam et al., 2020; Haldar et al., 2014). Does Spi-C play a role in modulating cellular function in inflammatory disease? Kayama et al. (2018) utilized a mouse model of ulcerative colitis induced by dextran sodium sulfate. In this model, Spi-C is induced at high levels in intestinal CX3CR1^{high} macrophages (Kayama et al., 2018). These highly phagocytic cells take up residence in the large and small intestine and mediate innate immune responses to intestinal microorganisms. A myeloid-specific knockout for *Spic* was utilized to assess its function in this disease model. Mice lacking Spi-C exhibited exacerbated colitis and increased expression of TLR-responsive inflammatory genes such as *Il6* and *Il1a*, indicating an anti-inflammatory role for Spi-C in intestinal macrophages (Kayama et al., 2018). Finally, this study showed that Spi-C can interfere with formation of the IRF5-NF-κB p65 complex required for pro-inflammatory signaling, thus dampening the inflammatory environment (Kayama et al., 2018). Further, Harusato et al. reported that Bach1-deficient mice exhibit an improved phenotype in another model of ulcerative colitis (Harusato et al., 2013). While the authors attribute the improved disease state to increased expression of heme oxygenase-1, *Spic* expression in intestinal macrophages is presumably upregulated and likely contributes to the suppression of pathological inflammation. Indeed, another study reported that *Spic*$^{-/-}$*Bach1*$^{-/-}$ mice exposed to LPS lost the anti-inflammatory phenotype in BMDMs, illustrating the need for Spi-C in promoting the resolution of inflammation (Alam et al., 2020).

Spi-C was shown to be highly expressed in a mouse model of secondary hemophagocytic lymphohistiocytosis (sHLH) induced by repeated Poly I:C and LPS injection (Wang et al., 2019). sHLH is a hyperinflammatory condition characterized by activated, RBC-containing macrophages in the bone marrow, spleen, and liver, as well as impaired cytotoxicity and cytokine storm (Henderson & Cron, 2020). sHLH is known to develop secondary to infection, primary immunodeficiencies, or autoimmune disease. MAS, briefly described above, is

considered a form of sHLH in patients with pre-existing autoimmune disease (Crayne et al., 2019). Children with systemic juvenile idiopathic arthritis are thought to be particularly susceptible to MAS, with as many as 30-40% of patients developing the disease. As sHLH has an estimated mortality rate of approximately 80%, the disease continues to gain attention within the medical and research communities (Crayne et al., 2019; Henderson & Cron, 2020). Investigation of cultured sHLH macrophages found that they exhibit a unique transcriptional profile that included high expression of Spi-C (Wang et al., 2019). Interestingly, Spi-C transcription was also notably increased in bone marrow-derived macrophages collected from sHLH patients and the mouse model. Spi-C was found to be highly upregulated in macrophages stimulated with IC:LPS, and this upregulation correlated with upregulation of the target gene *Treml4*. However, neither Spi-C nor Treml4 were strictly required for the macrophage phenotype in this model (Wang et al., 2019). Based on these findings, it is plausible that Spi-C is a candidate for the maladaptive macrophage state of sHLH based on its coordination of the erythrophagocytic program.

Finally, the function of Spi-C in macrophages was studied in several different models of sterile inflammation induced by LPS and bleomycin (Alam et al., 2020) or zymosan (Bennett et al., 2019). In the Alam et al. study, absence of *Spic* in BMMs resulted in a shift to a pro-inflammatory cytokine profile. In addition to altering the inflammatory state of activated macrophages, *Spic* induced genes responsible for iron export and heme metabolism, particularly FpnI. Interestingly, Alam et al. demonstrated that *Spic* is an NF-κB-dependent secondary response gene in activated macrophages, suggesting a regulatory loop in which Spi-C downregulates NF-κB signaling to balance macrophages between a pro- and anti-inflammatory state (Alam et al., 2020). In zymosan-induced erythrophagocytosis, Spi-C was found to be highly induced through a TLR2-dependent pathway (Bennett et al., 2019). Absence of *Spic* led to a reduction in the ability of zymosan to induce stress erythropoiesis. The target gene *Gdf15* was found to be partially responsible for the Spi-C-dependent induction of stress erythropoiesis during inflammation (Bennett et al., 2019). In summary, there is an emerging role for Spi-C in regulation of the inflammatory state in macrophages in response to external stimuli. Spi-C functions to reduce inflammatory gene expression and cytokine production in macrophages, while activating hemophagocytic ability and iron recycling mechanisms.

1.7 Summary

In summary, Spi-C is a lineage-instructive transcription factor that is important in the generation of multiple myeloid and lymphoid cell subsets. In the B cell compartment, Spi-C is tightly regulated during development and differentiation, functioning to promote the transition from large to small pre-B cells and regulate antibody-generating responses. Despite its important contributions to B cell fate decisions, the regulation of Spi-C in B cells remains largely undetermined. Numerous signals capable of inducing Spi-C expression in macrophages have been identified, though none have been assessed in B cells. In addition, there has been no work done at the molecular level to characterize regulatory elements at the *Spic* locus. Understanding how Spi-C expression is regulated by external signals and downstream signaling pathways in B cells will enhance knowledge of adaptive immune responses.

1.8 Hypothesis & Specific Aims

The present study aimed to investigate the regulation of the lineage-instructive ETS transcription factor Spi-C in B cells. The governing hypothesis is that Spi-C expression is dynamically regulated by external signals in B cells.

We propose the following research aims:

I. To determine the effects of a diverse panel of external signals on *Spic* expression in primary splenic B cells.

II. To characterize regulatory elements at the *Spic* locus and investigate the molecular mechanisms underlining regulation of *Spic* transcription.

III. To determine the biological relevance of Spi-C's dynamic patterns of expression in B cells.

2 Chapter 2: Methods

2.1 Mice

Spib$^{-/-}$ and *Spib*$^{-/-}$*Spic*$^{+/-}$ mice were generated as previously described (Li et al., 2015). Wildtype (WT) 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), and were monitored in accordance with an animal use protocol approved by the Western University Council on Animal Care. Genotyping was performed by PCR, as previously described, using the primers outlined in Table 1 (DeKoter et al., 2010; Kohyama et al., 2009).

Table 1. Primer sequences for genotyping

Primer Name	DNA Sequence (5' → 3')	Source
Spib KO fwd	GGG CTC CTT GGC TTA TGC TCC	
rev	CAG AAA GCG AAG GAG CAA AGC TG	
Spib WT fwd	GGG CTC CTT GGC TTA TGC TCC	
Rev	CGC GCT GTC AAA CTG GTA GGT	
Spic KO fwd	AAG CTT GGC TGG ACG TAA ACT CCT	
rev	AGA ACC AAG ACT ACC AGC CCA GAT	
Spic WT	CGG CAG ACA CTT TGC TAT TTC	(Kohyama et al., 2009)
rev	CCT CAA CTG AAG CTC CTT TCT	

2.2 B Cell Enrichment

Spleens were removed from male and female mice aged 6-12 weeks and dissociated into a single cell suspension with ground glass tissue homogenizers. Red blood cells 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, Germany) and biotin-conjugated mouse anti-CD43 (BD Biosciences; clone S7). Effective enrichment was confirmed by flow cytometry with staining for CD19.

2.3 Cell Culture

Primary mouse B cells were cultured in RPMI-1640 (Wisent, St-Bruno, QC) containing 10% fetal bovine serum, 10 units penicillin/1 mg/mL streptomycin/20 mM L-glutamine, and 10^{-5} M β-mercaptoethanol (βME). Additional stimulants used for culture of primary B cells are listed in Table 2.

Mouse 38B9 pro-B cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 10 units penicillin/1 mg/mL streptomycin/20 mM L-glutamine, and 10^{-5} M βME. Mouse WEHI-279 cells were cultured in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, 10 units penicillin/1 mg/mL streptomycin/20 mM L-glutamine, and 10^{-5} M βME.

Bone marrow was flushed from the femur and tibia of C57BL/6 WT mice aged 6-10 weeks. Following erythrocyte lysis, BM cells were plated at 2 x 10^5 in 6-well plates and cultured for 6 days in IMDM + 10% fetal bovine serum (Wisent) supplemented with 20 ng/mL of GM-CSF (Peprotech, Rocky Hill, NJ). BMDMs were washed twice with D-PBS (Wisent) to remove non-adherent cells and cultured in fresh IMDM + 10% FBS alone or containing 1000 ng/mL LPS (List Biological Laboratories, Campbell, CA) or 40 uM hemin (Sigma-Aldrich, St. Louis, MO). After 48 hours, BMDMs were harvested for RNA extraction.
All cells were cultured at 37°C and 5% CO_2.

Table 2. Reagents used in primary B cell culture

Reagent	Concentration	Source
Anti-IgM Antibodies	20 µg/mL	Jackson ImmunoResearch, West Grove, PA
BAFF	100 ng/mL	Peprotech, Rocky Hill, NJ
Cytochalasin D	1 µg/mL	Sigma-Aldrich, St. Louis, MO
CD40L	50 ng/mL	R&D Systems, Minneapolis, NE
Hemin	20-40 µM	Sigma-Aldrich, St. Louis, MO
Imatinib	10 µM	Sigma-Aldrich
LPS	10 µg/mL	List Biological Laboratories, Campbell, CA
Recombinant murine IL-4	10 ng/mL	R&D Systems, Minneapolis, NE
Recombinant murine IL-5	10 ng/mL	R&D Systems

2.4. T Cell-Dependent Immunizations

Six- to eight-week-old mice were immunized intraperitoneally with 100 ug of NP_8-KLH (Biosearch Technologies, Novato, CA) adjuvanted with 50% (vol/vol) of Imject™ alum (ThermoFisher Scientific, Rochester, NY).

2.5 Plasmids and Cloning

The *Spic* promoter, ROI 1 and ROI 2, and *Bach2* ROI 1 and ROI 3 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 (Gearing et al., 2019) and ConTra v3 (Kreft et al., 2017) software packages. 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. *Bach2* ROI 1 and 3 were each cloned into the pGL3-Promoter vector using KpnI/SacI and XhoI/SacI sites. 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 Centre. 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 Table 3.

Table 3. Primer sequences for cloning and site-directed mutagenesis

Primer Name	DNA Sequence (5' → 3')
Bach2 ROI 1 cloning fwd	CCA CAT ATG TTA AAC ACC TCC TAT GT
rev	ATT CAA ATC TCC TGA GCC AGT TAA T
Bach2 ROI 1 SDM fwd	GAA AAA CAG GCC CTG TGC TTC GG
rev	GGA AGA GAC TTG CAT TCA AAG
Bach2 ROI 3 cloning fwd	TGC TTA GGA TAG AAG ACA CAA ATC T
rev	TGG ATC TCA TAG TCA TTT GGA GAA A
Bach2 ROI 3 SDM fwd	AAA AAC AGG ACC TGT GCT TCG
rev	CGG AAG AGA CTT GCA TTC
Spic promoter cloning fwd	GTT ATA AAG ACC CAC AGC CTC TAC
rev	CAA GCT TCA AGT GGC GAT ATC TGT ACT G
Spic promoter SDM fwd	CGA TTT TTT TTA AAA GGC ACA GTG
rev	GCT TTG CAG CAC TTC CTT AAA AAA TAG
Spic ROI 1 cloning fwd	TAA GTA ATA GGG AGG GAA ATA CCA AGC
rev	ATT CAT GTG ACT TTC CCA CGT C
Spic ROI 1 SDM fwd	AAA AAA CCC ACA ACT AAG CAA AAC TGG
rev	TTT TTT TTT TTC CAA AAG CTA TTT TG
Spic ROI 2 cloning fwd	CTC CTT TCC ATA TTG CTC ACT TAA ATC
rev	CCA AGT CTG AGC TTT CAA ATT CTA C
Spic ROI 2 SDM fwd	CTG AGA ACA ACA AGT CAG CAA ACT TGC
rev	AGG AAC ATC ACG AGC CAT

2.6 Transient Transfection

WEHI-279 or 38B9 B cells in early log-phase growth were washed three times in serum-free DMEM (4.5g/L glucose) or RPMI-1640 (Wisent, St-Bruno, QC). Cells were incubated for 10 minutes 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 (ThermoFisher Scientific, Rochester, NY) using a GenePulser II with Capacitance Extender (Bio-Rad). Cells were recovered at room temperature for 10 minutes and plated in 6-well culture plates in complete DMEM or RPMI for 24 hours at 37°C, 5% CO_2.

2.7 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 was collected using Gen5 software (BioTek).

2.8 Flow Cytometry

Cell-surface staining was performed using phycoerythrin (PE)-conjugated anti-CD19. Live/dead staining was performed using the Fixable Viability Dye eFluor 506 (eBioscience, San Diego, CA) according to manufacturer's instructions. Samples were run on a FACSCanto SORP or LSR II (BD Biosciences).

Cell sorting was performed on the FACS Aria III Cell Sorter. Anti-Fc-γ receptor blocking was performed using purified rat anti-mouse CD16/CD32 (BD Bioscience, Franklin Lakes, NJ). Cell-surface staining was performed using the following antibodies diluted in MACS buffer, purchased from eBioscience (San Diego, CA), BD Bioscience (Franklin Lakes, NJ) or Biolegend (San Diego, CA): Brilliant Violet 421-conjugated anti-CD138 (1:200), Brilliant Violet 711-conjugated anti-CD19 (1:200), fluorescein (FITC)-conjugated anti-CD4 (1:1000), R-phycoerythrin-Cyanin dye 7 (PE-Cy7)-conjugated anti-CD95 (1:100), and allophycocyanin (APC)-conjugated CD38 (1:500). Germinal centre B cells were defined as $CD19^{+}CD138^{-}CD95^{hi}CD38^{lo}$.

All data was acquired at the London Regional Flow Cytometry Facility using FACSDiva (Version 8.0.1) and analyzed with the FlowJo software (TreeStar, Ashland, OR) (Version 10.4.2).

2.9 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) was performed using equal starting RNA concentrations, followed by 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 (ThermoFisher 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-ΔΔCT) method (Pfaffl, 2004). Primer sequences are listed in Table 4.

Table 4. Primer sequences for RT-qPCR

Primer Name	DNA Sequence (5' → 3')	Source
Acly fwd	CCA GTG AAC AAC AGA CCT ATG A	(Rhee et al., 2019)
rev	AAT GCT GCC TCC AAT GAT G	
Actb fwd	TCA TCA CTA TTG GCA ACG AGC GGT TC	
rev	TAC CAC CAG ACA GCA CTG TGT TGG CA	
Bach2 fwd	CAT CTC TTC CTC TGC CCA GT	(Kometani et al.,
rev	AGA CAT GCC GTT CAA ACC AT	2013)
Spi1 fwd	ATT CGC CTG TAC CAG TTC CTG C	
rev	TGG ACG AGA ACT GGA AGG TAC C	
Spib fwd	GAG CGC TGC GCA ACT ATG C	
rev	GAC ATG CCG GGA GGC TG	
Spic fwd	AAA GGG AGG AAG AGG CAG GAG AAA	
rev	AAG TCT TTG GAG AAC AGC CTC GCT	
Tbp fwd	ACC GTG AAT CTT GGC TGT AAA C	(Eissa et al., 2016)
rev	GCA GCA AAT CGC TTG GGA TTA	
Tp53 fwd	CTC ACT CCA GCT ACC TGA AGA	(J. Li et al., 2008)
rev	AGA GGC AGT CAG TCA GTC TGA GTC A	

2.10 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.

3 Chapter 3: Results

3.1 Heme induces *Spic* in primary splenic B cells and bone-marrow derived macrophages

Spic expression has previously been found to be induced following signaling cascades initiated by heme or NF-κB in numerous myeloid and lymphoid cell subsets (Reviewed in Raczkowski & DeKoter, 2021). We sought to determine how *Spic* expression is influenced by external signals in primary B cells isolated from spleens of WT C57/BL6 mice. B cells were enriched by CD43 column depletion and efficient isolation was confirmed by flow cytometry (Figure 3–1A). Spi-C is known to be induced in macrophages following stimulation with heme to promote differentiation into RPMs (Haldar et al., 2014). To evaluate if *Spic* is inducible by heme in B cells, we cultured enriched B cells for 48 or 72 hours in the presence of 20 or 40 μM heme and assessed *Spic* expression by RT-qPCR. *Spic* expression was upregulated by 3.4-fold in response to 40 μM heme in B cells cultured for 72 hours (Figure 3–1B). As prolonged culture of primary B cells is limited in the absence of pro-survival signals, we performed viable cell counts to confirm adequate cell viability (Howard et al., 1981). Counts indicated that the viability of B cells cultured in the presence of heme was stable over time (Figure 3–1C). To validate our system of detecting *Spic* expression, we also sought to confirm upregulation of *Spic* in BMDMs cultured with heme reported in Haldar et al. (2014). BM was isolated from WT mice and cultured for 6 days in the presence of GM-CSF, after which adherent BMDMs were plated in the presence or absence of heme (Figure 3–1D). Corroborating previous findings, we found heme-treated BMDMs upregulated *Spic* expression by roughly 5-fold compared to untreated cells (Figure 3–1E). These results indicate that heme-mediated induction of Spi-C expression is not limited to myeloid-lineage cells and may play a role in B cell responses.

3.2 *Spic* expression remains stable in B cells cultured with IL-4, IL-5, or BAFF

Previous experiments in our lab investigating antibody-secreting cell generation involved *ex vivo* culture of murine B cells in the presence of IL-4 and IL-5, which act as strong pro-survival signals in biological environments and can promote survival and/or differentiation in longer-

term cultures (Laramée et al., 2020). We sought to characterize how these cytokines affect *Spic* expression and found that independently, IL-4 or IL-5 does not affect *Spic* expression compared to freshly enriched B cells (Figure 3–2A-B). Cell counting experiments revealed no differences in B cell counts following culture with IL-4 or IL-5 (data not shown).

B cell activating factor (BAFF) was previously identified as a cytokine that may increase *Spic* expression due to overlapping patterns of expression during B cell development, and activation of the non-canonical NF-κB pathway known to upregulate *Spic*. Of particular interest, *Spic* expression peaks during the transitional 1 B cell stage of development, where BAFF is known to be a key signaling molecule that promotes maturation and migration as B cells exit the BM (Smulski & Eibel, 2018; Zhu et al., 2008). We examined *Spic* expression in B cells cultured in the presence of BAFF but found that BAFF alone did not affect *Spic* expression over a 72-hour time course (Figure 3–2C). Expression of *Spic* exhibited a minor, albeit nonsignificant upward trend over time, with a maximum increase of approximately 2-fold. Corresponding cell counts showed a similar upward trend, but no significant differences over the same time period (Figure 3–2D). We next examined Spic expression following combinations of pro-survival signals IL-4 or IL-5 with BAFF to assess if the cytokines may exhibit synergistic effects. B cells cultured with BAFF + IL-4 or BAFF + IL-5 once again exhibited no differences in *Spic* expression (Figure 3–2E). These data indicate that *Spic* expression remains unchanged in B cells cultured with pro-survival cytokines IL-4 or IL-5, while BAFF may cause a limited increase in expression.

Figure 3–1. Heme induces *Spic* in B cells and bone-marrow derived macrophages. (**A**) Flow cytometry quantifying $CD19^+$ B cell frequency. (**B**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with heme under the indicated conditions. Bars indicate mean ± SD, *n* = at least 4 mice, two-way ANOVA, $*p < 0.05$, $****p < 0.0001$. Data points indicate mean of duplicate wells for each mouse. Expression was determined relative to freshly enriched B cells. (**C**) Viable cell counts for B cells cultured in (B). Data points indicate mean of triplicates for each mouse. (**D**) Representative photomicrographs of bone-marrow macrophages cultured in cIMDM alone (top) or with 40 µM heme (bottom). Original magnification 20X. Scale bar indicates 50 µM. Images were taken with the Zeiss AxioObserver and A1 AxioCam ICM1 using ZEN 2 Pro software. (**E**) RT-qPCR analysis of *Spic* expression in primary bone-marrow derived macrophages obtained from WT mice and cultured with heme for 48 hours. Bars indicate mean ± SD, *n* = at least 4 mice, one sample Wilcoxon test, $**p < 0.01$. Data points indicate mean of duplicate wells for each mouse. Expression was determined relative to unstimulated cells. Relative gene expression for all RT-qPCR was normalized to *Tbp*.

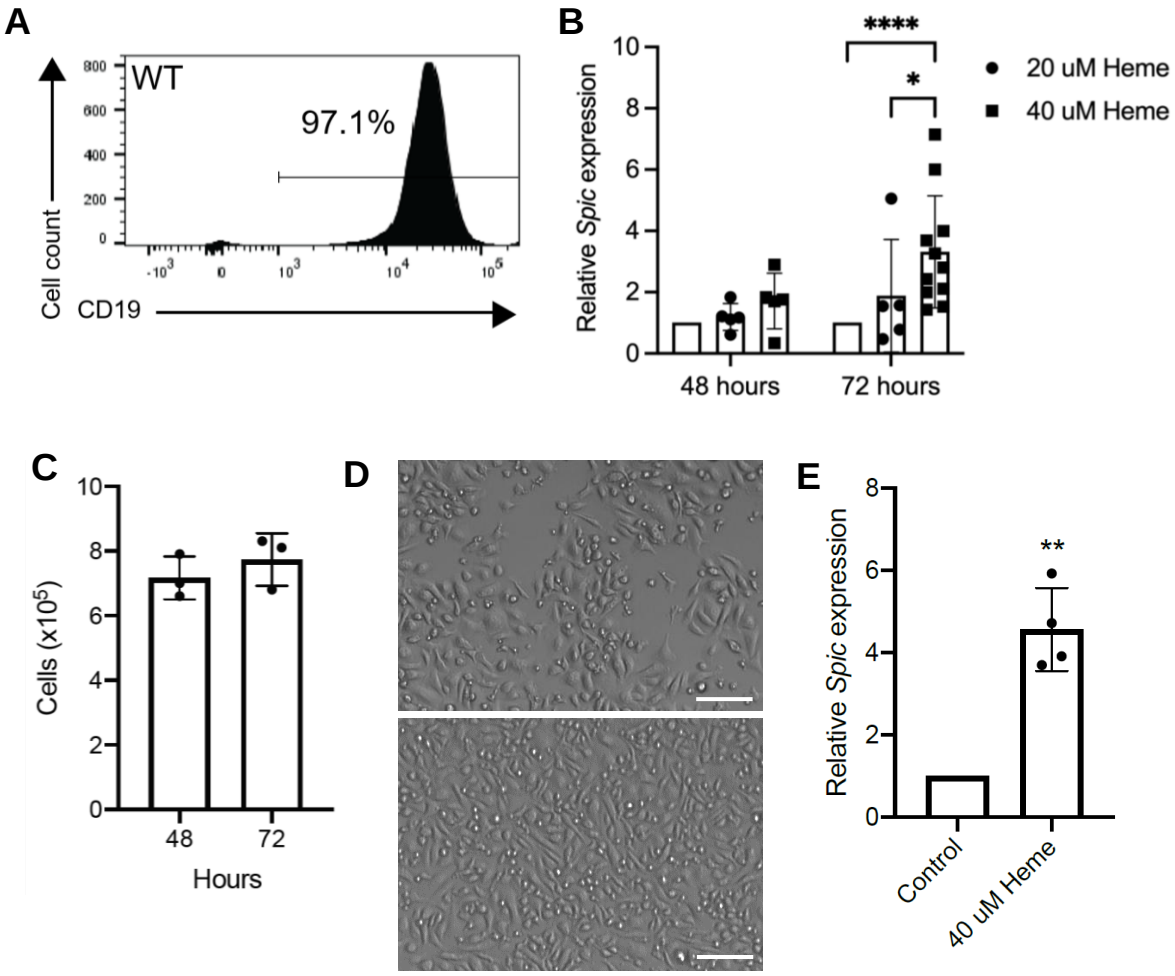
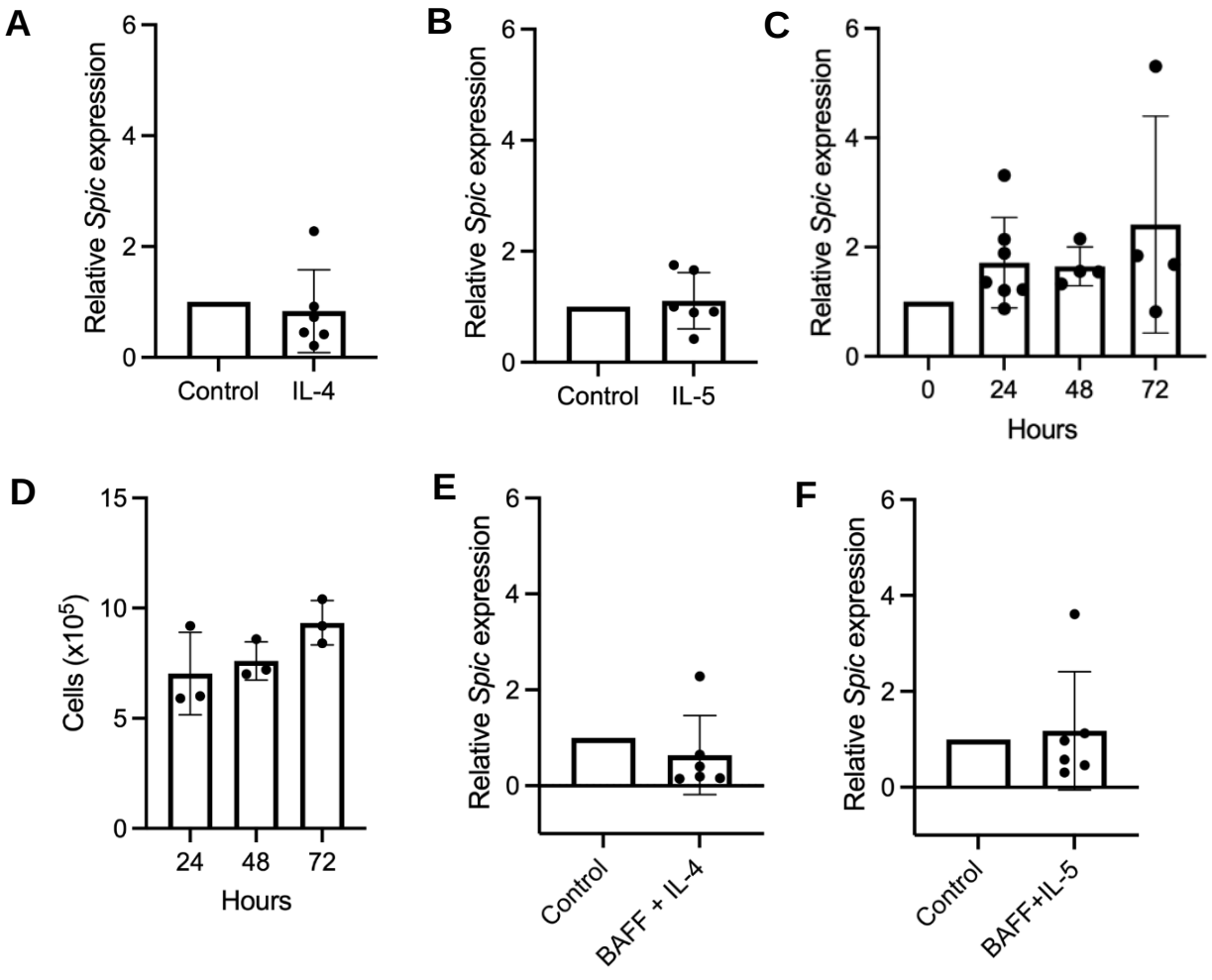

A
WT
97.1%
Cell count
CD19
B
Relative Spic expression
****
*
20 uM Heme
40 uM Heme
48 hours
72 hours
C
Cells (x10^5)
48
72
Hours
D
E
Relative Spic expression
**
Control
40 uM Heme

Figure 3–2. *Spic* expression remains constant in B cells cultured with IL-4, IL-5, or BAFF. (**A-B**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with IL-4 or IL-5, respectively for 24 hours. Bars indicate mean ± SD, *n* = at least 6 mice, one sample Wilcoxon test. (**C**) RT-qPCR analysis of *Spic* expression in B cells cultured in BAFF for the indicated times. Bars indicate mean ± SD, *n* = at least 4 mice, Kruskall-Wallis with Dunn's multiple comparisons test. (**D**) Viable cell counts for B cells cultured in (C). Bars indicate mean ± SD, *n* = 3 mice, one-way ANOVA. (**E-F**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with combinations of BAFF + IL-4 or BAFF + IL-5, respectively for 24 hours. Bars indicate mean ± SD, *n* = at least 6 mice, one sample Wilcoxon test. Relative gene expression for all RT-qPCR was relative to freshly isolated B cells and normalized to *Tbp*. All data points represent mean of duplicate wells for each mouse.


A
Relative Spic expression
0
2
4
6
Control
IL-4
B
Relative Spic expression
0
2
4
6
Control
IL-5
C
Relative Spic expression
0
2
4
6
0
24
48
72
Hours
D
Cells (x10^5)
0
5
10
15
24
48
72
Hours
E
Relative Spic expression
0
2
4
6
Control
BAFF + IL-4
F
Relative Spic expression
0
2
4
6
Control
BAFF+IL-5

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

We continued to screen combinations of stimuli to determine their impact on *Spic* expression in primary splenic B cells. We next cultured B cells with IL-4 and IL-5 in combination and observed a modest decrease in *Spic* expression over time (Figure 3–3A). Expression of *Spic* was lowest at 72 hours, decreasing by 5-fold compared to freshly isolated cells. Interestingly, we noted that decreased *Spic* expression appeared to correlate with a slight, but insignificant increase in B cell counts over time (Figure 3–3B). Finally, the combination of BAFF + IL-4 + IL-5 resulted in a robust decrease in *Spic* expression by 40-fold over 72 hours (Figure 3–3C). B cells cultured in this condition also increased substantially in number over the same time course, exceeding the 10^6 cells initially placed into culture on day zero (Figure 3–3D). To examine the apparent link between B cell proliferation and *Spic* expression, we utilized the drug Cytochalasin D, a potent inhibitor of actin polymerization that therefore prevents cell division (Figure 3–3D; Hao & August, 2005). We examined *Spic* expression following culture of B cells with BAFF + IL-4 + IL-5 for 72 hours in the presence or absence of Cytochalasin D and found that addition of Cytochalasin D partially blocked downregulation of *Spic* (Figure 3–3E). B cells cultured with the cytokine mixture downregulated *Spic* by approximately 15-fold, while those cultured in the presence of Cytochalasin D decreased *Spic* expression by ~5-fold. This indicates that downregulation of *Spic* by BAFF + IL-4 + IL-5 is dependent on actin polymerization and blocking cell division may impair *Spic* downregulation.

We previously reported that stimulation of B cell co-receptors with CD40L reduced *Spic* expression in B cells compared to freshly isolated cells (Figure 3–4A; Laramée et al., 2020). We further examined the effect of CD40L by comparing *Spic* expression of stimulated cells to unstimulated B cells across three time points. *Spic* expression was reduced over time, with its lowest expression at 72-hours, showing a 20-fold reduction in expression (Figure 3–4B). B cells cultured with the addition of CD40L increased in number over time (Figure 3–4C). We went on to examine how CD40L affects *Spic* expression in combination with the cytokines discussed above. We found that compared to freshly isolated B cells, *Spic* was downregulated in the presence of CD40L regardless of the presence of IL-4, IL-5, or BAFF in any combination, suggesting a strong transcriptional pull towards *Spic* repression by CD40L (Figure 3–4D-I). Of the combinations including CD40L assessed, CD40L + IL-4 + IL-5 caused

the greatest downregulation of *Spic*, reducing expression by over 20-fold (Figure 3–4F). B cells cultured in CD40L and BAFF reduced *Spic* expression by approximately 5-fold and was the condition in which *Spic* expression was downregulated the least (Figure 3–4G).

Figure 3–3. Certain combinations of IL-4, IL-5, and BAFF repress *Spic* expression. (**A**) 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, n = at least 3 mice, Kruskall-Wallis with Dunn's multiple comparisons test. ** $p < 0.01$ (**B**) Corresponding viable cell counts for (A). (**C**) RT-qPCR analysis of *Spic* expression in B cells cultured in BAFF + IL-4 + IL-5 for the indicated times. (**D**) Viable cell counts for B cells cultured in (C) or with the addition of Cytochalasin D. Bars indicate mean ± SD, $n = 3$, two-way ANOVA **$p < 0.01$, ***$p < 0.001$. (**E**) RT-qPCR analysis of *Spic* expression in B cells cultured with BAFF + IL-4 + IL-5 for 72 hours in the presence of absence of Cytochalasin D. Bars indicate mean ± SD, n = at least 4 mice, Kruskall-Wallis with Dunn's multiple comparisons test, **$p < 0.01$. Relative gene expression for all RT-qPCR was relative to freshly isolated B cells and normalized to *Tbp*. All data points for qPCR experiments represent mean of duplicate wells for each mouse. Cell count data points indicate mean of triplicate counts for each mouse.

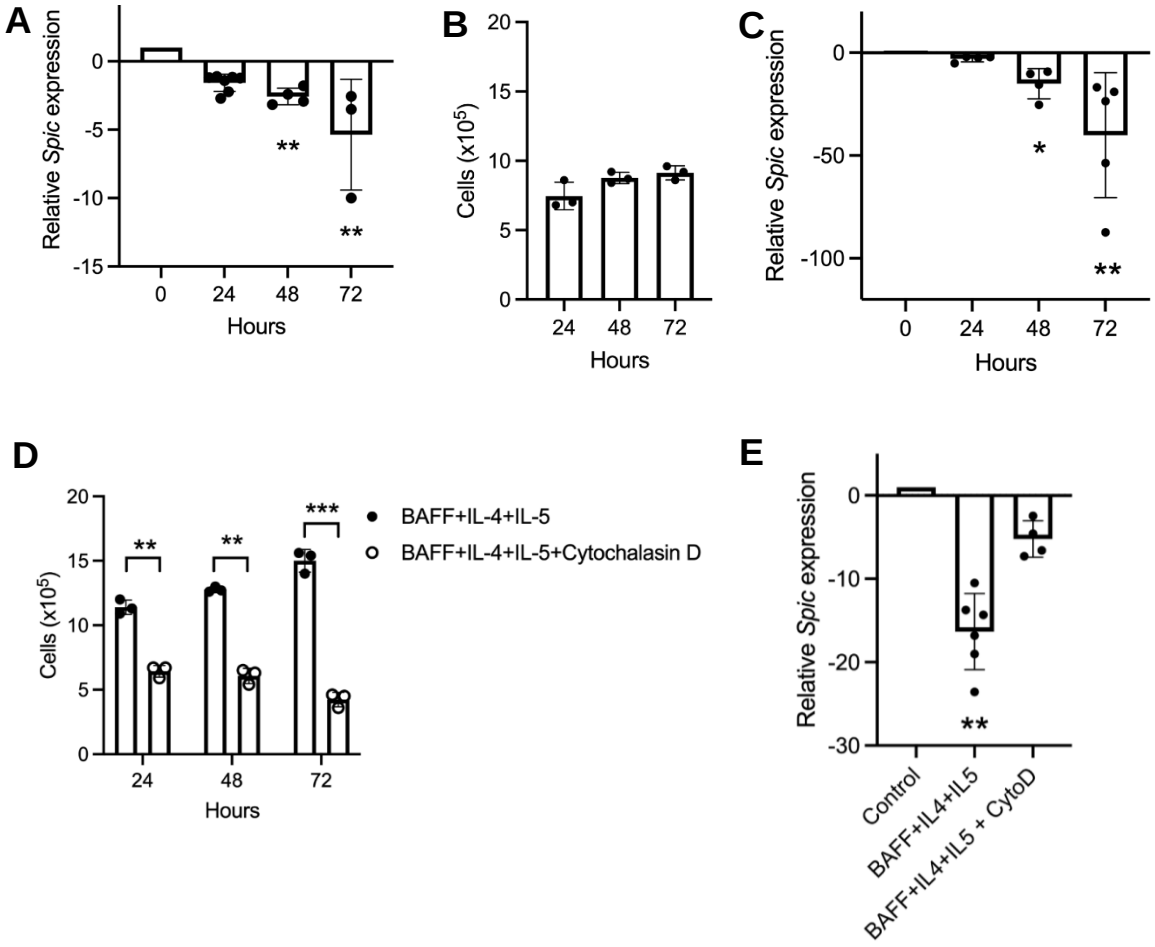

A
Relative Spic expression
0
-5
-10
-15
**
**
0
24
48
72
Hours
B
Cells (x10^5)
20
15
10
5
0
24
48
72
Hours
C
Relative Spic expression
0
-50
-100
*
**
0
24
48
72
Hours
D
Cells (x10^5)
20
15
10
5
0
**
**
***
24
48
72
Hours
BAFF+IL-4+IL-5
BAFF+IL-4+IL-5+Cytochalasin D
E
Relative Spic expression
0
-10
-20
-30
**
Control
BAFF+IL4+IL5
BAFF+IL4+IL5 + CytoD

Figure 3–4. CD40L downregulates *Spic* expression in B cells. (**A**) RT-qPCR analysis of *Spic* expression in primary B cells enriched from WT mouse spleens and cultured with CD40L for 24 hours. Bars indicate mean ± SD, *n* = 6 mice, one sample Wilcoxon test, **p < 0.01. (**B**) RT-qPCR analysis showing *Spic* expression in B cells cultured with or without CD40L for the indicated times. Bars indicate mean ± SD, n = 3 mice, two-way ANOVA, *p < 0.05. (**C**) Viable cell counts for B cells cultured with CD40L for 72 hours. Bars indicate mean ± SD, *n* = 3 mice, one-way ANOVA ***p* < 0.01, ****p* < 0.001. Data points indicate mean of triplicate counts for each mouse. (**D-I**) RT-qPCR analysis of *Spic* expression in B cells cultured with the indicated conditions for 24 hours. Bars indicate mean ± SD, *n* = at least 3 mice, one sample Wilcoxon, *p < 0.05, **p < 0.01. Relative gene expression for all RT-qPCR was relative to freshly isolated B cells, with the exception of (B), which was relative to time-matched unstimulated cells. All expression data was normalized to *Tbp*. All data points for qPCR experiments represent mean of duplicate wells for each mouse.

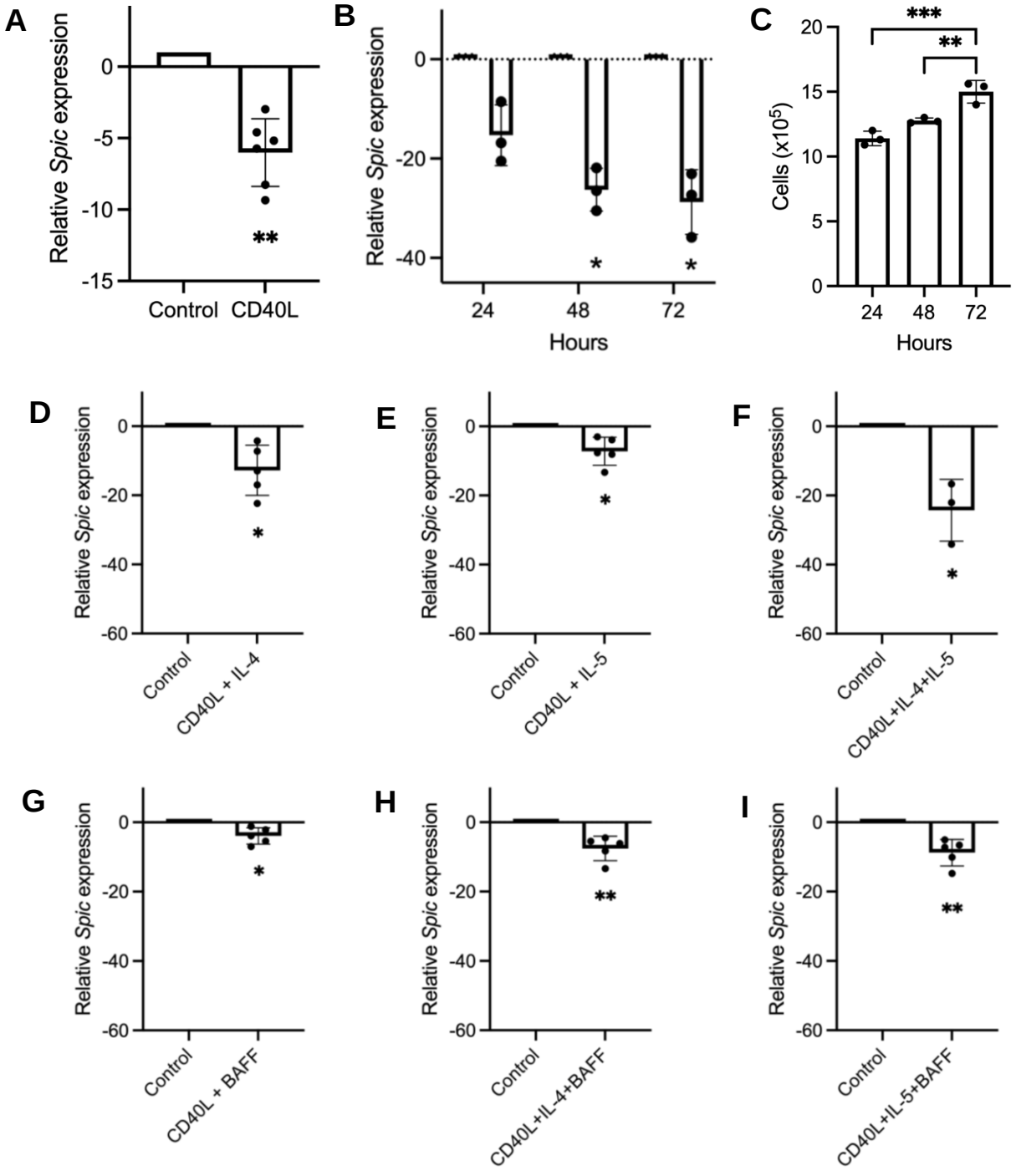
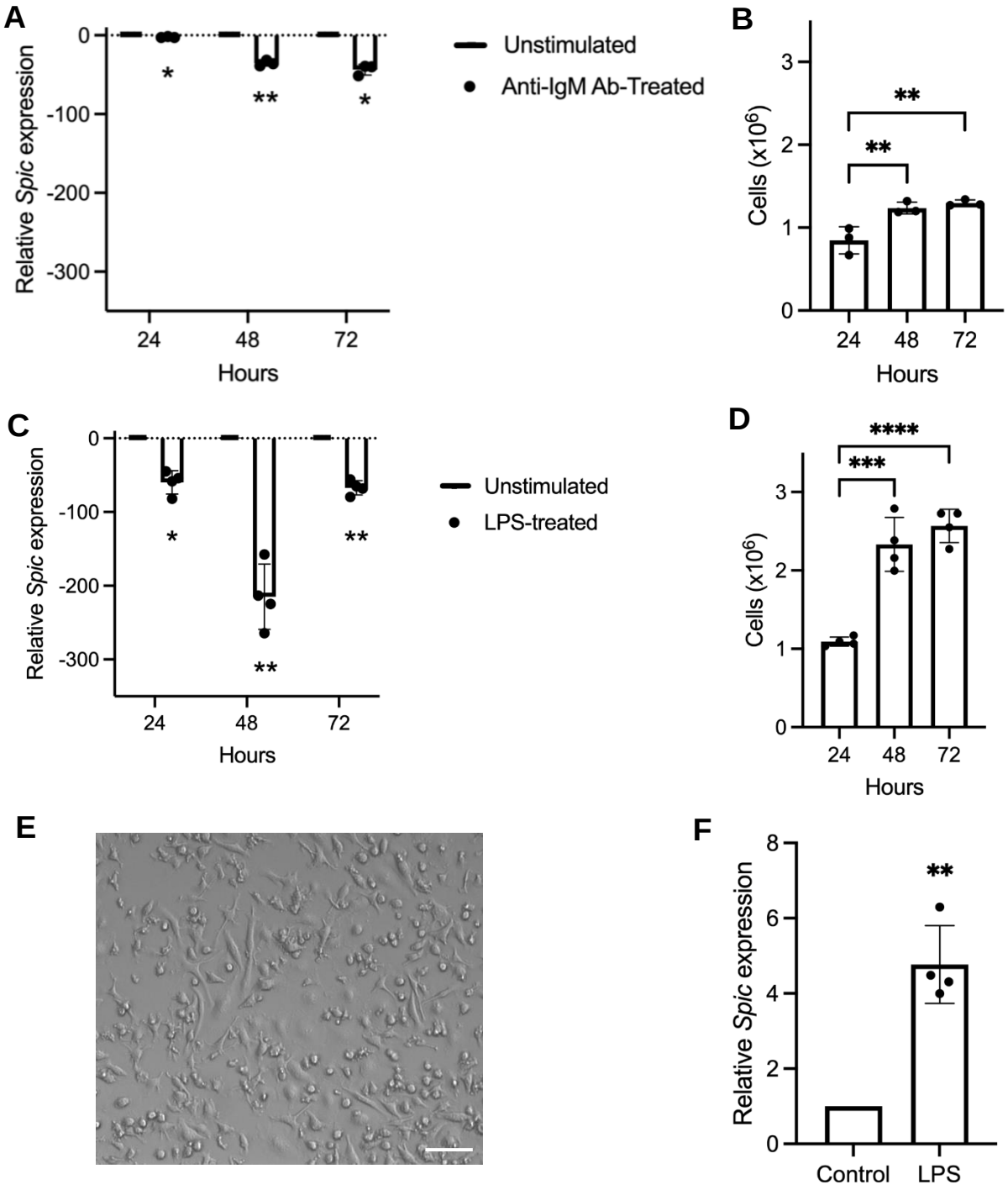

A
Relative Spic expression
0
-5
-10
-15
**
Control
CD40L
B
Relative Spic expression
0
-20
-40
*
*
24
48
72
Hours
C
***
**
Cells (x10^5)
20
15
10
5
0
24
48
72
Hours
D
Relative Spic expression
0
-20
-40
-60
*
Control
CD40L + IL-4
E
Relative Spic expression
0
-20
-40
-60
*
Control
CD40L + IL-5
F
Relative Spic expression
0
-20
-40
-60
*
Control
CD40L+IL-4+IL-5
G
Relative Spic expression
0
-20
-40
-60
*
Control
CD40L + BAFF
H
Relative Spic expression
0
-20
-40
-60
**
Control
CD40L+IL-4+BAFF
I
Relative Spic expression
0
-20
-40
-60
**
Control
CD40L+IL-5+BAFF

Figure 3–5. Treatment with anti-IgM Abs or LPS downregulates *Spic* expression in B cells. (**A**) 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, n = 3 mice, two-way ANOVA, *$p < 0.05$, **$p < 0.01$. (**B**) Corresponding viable cell counts for (A). Bars indicate mean ± SD, n = 3 mice, one-way ANOVA with Tukey's multiple comparison test. **$p < 0.01$. (**C**) RT-qPCR analysis showing *Spic* expression in B cells cultured with or without LPS for the indicated times. (**D**) Viable cell counts from (C). (**E**) Representative photomicrograph of bone-marrow derived macrophages cultured with LPS. Original magnification 20X. Scale bar indicates 50 μM. Images were taken with the Zeiss AxioObserver and A1 AxioCam ICM1 using ZEN 2 Pro software. (**F**) RT-qPCR analysis of *Spic* expression in primary bone-marrow derived macrophages obtained from WT mice and cultured with LPS for 48 hours. Bars indicate mean ± SD, n = 4 mice, one sample Wilcoxon test, **$p < 0.01$. Relative gene expression for all RT-qPCR was relative to time-matched unstimulated cells, with data was normalized to *Tbp*. All data points for qPCR experiments represent mean of duplicate wells for each mouse. Cell count data points indicate mean of triplicate counts for each mouse.


A
Relative Spic expression
0
-100
-200
-300
*
**
*
24
48
72
Hours
Unstimulated
Anti-IgM Ab-Treated
B
Cells (x10^6)
3
2
1
0
**
**
24
48
72
Hours
C
Relative Spic expression
0
-100
-200
-300
*
**
**
24
48
72
Hours
Unstimulated
LPS-treated
D
Cells (x10^6)
3
2
1
0
****
***
24
48
72
Hours
E
F
Relative Spic expression
8
6
4
2
0
**
Control
LPS

To determine the extent of the relationship between B cell proliferation and reduced *Spic* expression, we selected two additional signals to investigate their effect on *Spic* expression in B cells. We first asked how signaling through the BCR influenced *Spic* expression. B cells were cultured with anti-IgM Abs for 24-72 hours and *Spic* expression was quantified by RT-qPCR relative to time-matched unstimulated cells. We found that BCR engagement reduced *Spic* expression in a time-dependent manner, with expression decreasing by as much as 50-fold (Figure 3–5A). As expected, stimulation through the BCR also significantly increased the number of live cells in culture over time (Figure 3–5B). Alam and colleagues recently reported that treatment of BMDMs with LPS activated *Spic* in an NF-κB-dependent pathway to push macrophages towards an anti-inflammatory phenotype and control the immune response (Alam et al., 2020). To evaluate whether a similar transcriptional program exists in B cells, we treated primary splenic B cells with LPS for 24-72 hours. Despite activating transcription of *Spic* in BMDMs, we discovered that LPS treatment robustly downregulated *Spic* expression in B cells (Figure 3–5C). *Spic* expression was lowest at 48 hours with a negative fold change of over 200 compared to unstimulated cells. Corresponding cell count data displayed a considerable increase in live B cells over time, peaking at over 2.5 x 10^6 cells following 72 hours in culture (Figure 3–5D). To validate our findings, we sought to reproduce the previously described reports of LPS treatment activating *Spic* expression in macrophages (Alam et al., 2020). We utilized the aforementioned culture system to generate BMDMs and plated adherent cells in the presence or absence of LPS. Macrophages morphology for BMDMs treated with LPS is shown for a representative experiment (Figure 3–1D). We found that LPS increased *Spic* expression in BMDMs by approximately 5-fold, which confirmed the modest upregulation observed by the Haldar laboratory (Figure 3–5F; Alam et al., 2020). These data further support the correlation between B cell division and downregulation of *Spic*, including in the context of a signal that activates *Spic* expression in the myeloid lineage.

3.4 *Spic* expression is increased by quiescence or anti-proliferative signaling in primary splenic B cells

Based on our previous observations, we next asked how expression of SPI-family transcription factors changes over time in unstimulated B cells. To assess *Spic* expression in cells serving as an unstimulated control, we quantified expression in cells cultured in cRPMI alone for 24-72 hours relative to freshly isolated B cells. We observed a time-dependent increase in *Spic* expression, culminating in a 20-fold increase by the 72-hour timepoint (Figure 3–6A). To determine if the observed upregulation was an apoptosis-related artifact, 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 approximately 10- and 5-fold increases, respectively (Figure 3–6B-C). Cell count data showed stable numbers of live B cells over time after an initial decrease from 24 to 48 hours (Figure 3–6D). These results suggest that *Spic* expression is increased in unstimulated B cells, relative to the related ETS transcription factors *Spi1* and *Spib*.

To further explore the upregulation of *Spic* in unstimulated 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 due to its well-documented increase in expression during apoptosis (Kiraz et al., 2016). *Acly* was chosen as a gene expected to be downregulated because of its essential role in fatty acid synthesis during cell division (Rhee et al., 2019). We found that *Tp53* was upregulated over time by nearly 12-fold in unstimulated B cells, whereas *Acly* expression decreased at 24 hours and increased by up to 3-fold following 72 hours in culture (Figure 3–6E-F). To validate our findings, we selected β-actin as an additional reference 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 approximately 4-fold (Figure 3–6G-H). Conversely, *Acly* expression decreased slightly and remained low throughout the assessed time period (Figure 3–6I). Overall, these findings support the notion that *Spic* expression is increased in quiescent B cells.

Finally, we investigated *Spic* expression in B cells treated with drugs known to inhibit cell division. Primary splenic B cells were treated with Cytochalasin D, followed by quantification of *Spic* expression and cell counting. *Spic* expression increased modestly over 72 hours, with

a peak increase of approximately 4-fold observed at 48 and 72 hours (Figure 3–7A). Cell counts remained stable over the same time period (Figure 3–7B). We utilized Imatinib to evaluate how inhibition of Bcr-Abl Tyrosine-kinase signaling affects *Spic* expression in B cells. Imatinib (also known as Gleevec or Glivec) is an Abl kinase inhibitor that blocks proliferation of v-Abl-transformed B cell lines (Muljo & Schlissel, 2003). We treated the v-Abl-transformed pro-B cell line 38B9, and as a control treated IL-7-withdrawn fetal liver-derived WT pro-B cells – with 10 μM Imatinib for 48-72 hours and assessed *Spic* expression. Preliminary data indicated that Imatinib treatment induced *Spic* expression in 38B9 pro-B cells but not WT pro-B cells (Figure 3–7C-D). While not yet significant, *Spic* expression appears to increase by over 10-fold in 38B9 pro-B cells, while remaining unchanged in WT pro-B cells. These data further support the proposed model of quiescence or the absence of stimulation inducing *Spic* expression in B cells.

Figure 3–6. Expression of SPI family members in unstimulated 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, n = at least 4 mice, Kruskall-Wallis with Dunn's multiple comparisons test, *$p < 0.05$, ****$p < 0.0001$. (**D**) Live cell counts for unstimulated B cells. Bars indicate mean ± SD, n = 4 mice, one-way ANOVA with Tukey's multiple comparison test. (**E-F**) RT-qPCR analysis quantifying expression of the indicated genes in unstimulated B cells. Data shown as mean ± SD, n = 4 mice, Kruskall-Wallis with Dunn's multiple comparisons test, ***$p < 0.001$. (**G-I**) RT-qPCR analysis quantifying expression of the indicated genes in unstimulated B cells normalized to *Actb* expression. n = 4 mice, Kruskall-Wallis with Dunn's multiple comparisons test, **$p < 0.01$. (**F**) Relative gene expression for all RT-qPCR was relative to freshly isolated B cells. Expression was normalized to *Tbp* for all RT-qPCR, with the exception of panels G-I. Individual data points for qPCR experiments represent mean of duplicate wells for each mouse. Cell count data points indicate mean of triplicate counts for each mouse.

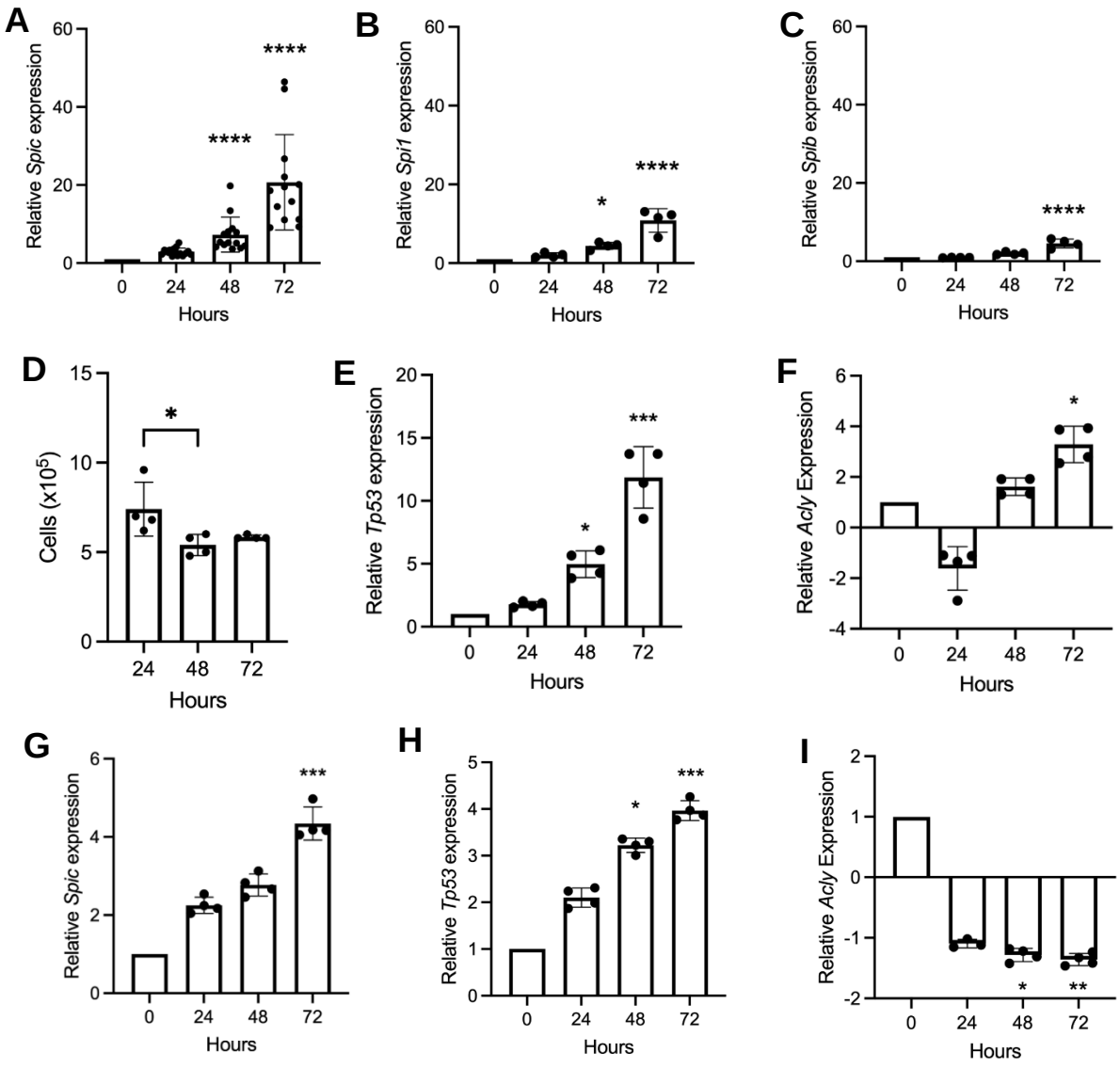
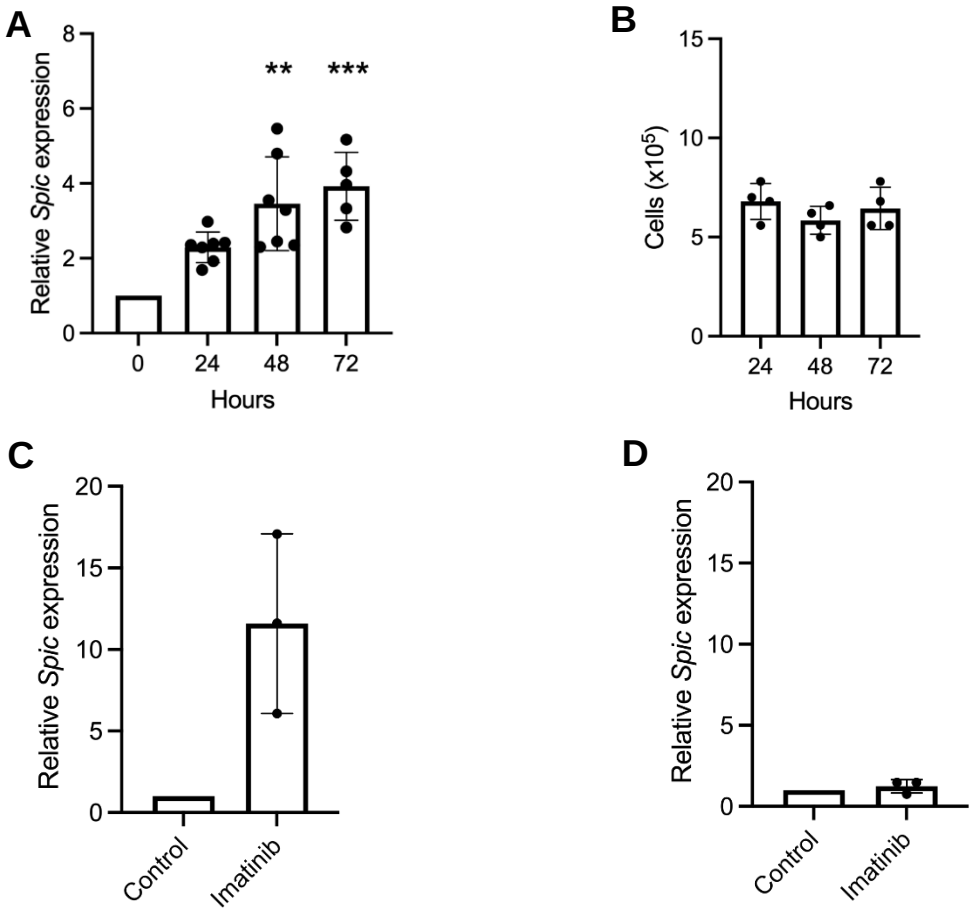

A
Relative Spic expression
60
40
20
0
****
****
0
24
48
72
Hours
B
Relative Spi1 expression
60
40
20
0
*
****
0
24
48
72
Hours
C
Relative Spib expression
60
40
20
0
****
0
24
48
72
Hours
D
Cells (x10^5)
15
10
5
0
*
24
48
72
Hours
E
Relative Tp53 expression
20
15
10
5
0
*
***
0
24
48
72
Hours
F
Relative Acly Expression
6
4
2
0
-2
-4
*
0
24
48
72
Hours
G
Relative Spic expression
6
4
2
0
***
0
24
48
72
Hours
H
Relative Tp53 expression
5
4
3
2
1
0
*
***
0
24
48
72
Hours
I
Relative Acly Expression
2
1
0
-1
-2
*
**
0
24
48
72
Hours

Figure 3–7. B cells treated with drugs that impair proliferation activate *Spic* expression. (**A**) RT-qPCR analysis of *Spic* expression in WT primary splenic B cells cultured with Cytochalasin D for the indicated times. Expression data is relative to freshly isolated B cells and relative to *Tbp*. Data is shown as mean ± SD, *n* = at least 5 mice, Kruskall-Wallis with Dunn's multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.001$. (**B**) Live cell counts for cells in (A). Bars indicate mean ± SD, *n* = 4 mice, one-way ANOVA with Tukey's multiple comparison test. (**C**) RT-qPCR analysis of *Spic* expression in v-Abl-transformed 38B9 pro-B cells or (**D**) IL-7-withdrawn fetal liver-derived WT pro-B cells treated with Imatinib for 24 hours. Data represents mean ± SD, *n* = 3 mice, one sample Wilcoxon test. Expression data for C-D is relative to untreated cells on Day 0 and normalized to *Actb* expression. Individual data points for qPCR experiments represent mean of duplicate wells for each mouse. Cell count data points indicate mean of triplicate counts for each mouse.


A
Relative Spic expression
8
6
4
2
0
**
***
0
24
48
72
Hours
B
Cells (x10^5)
15
10
5
0
24
48
72
Hours
C
Relative Spic expression
20
15
10
5
0
Control
Imatinib
D
Relative Spic expression
20
15
10
5
0
Control
Imatinib

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

Previous sections of this thesis investigate stimuli that influence expression of *Spic*. Following identification of numerous signals that can up- or downregulate *Spic* expression, we sought to characterize three important regulatory elements of *Spic*. The *Spic* promoter has not been previously characterized. We selected a region immediately upstream of the transcription start site spanning approximately 500 base pairs, amplified and cloned it using PCR, and finally ligated it into the pGL3-Basic vector compatible with the Dual-Luciferase Assay system (Figure 3–8A-B). Analysis of cross-species DNA sequence identity confirmed that the cloned region is highly conserved across multiple vertebrates (Figure 3–8A). We tested the activity of the promoter by transiently transfecting the promoter-containing vector into two B cell lines: 38B9 pro-B cells and WEHI-279 lymphoma cells. Luciferase assays showed that the promoter possesses activity in only the forward orientation in both cell lines, with higher relative luciferase activity observed in WEHI-279 B cells (Figure 3–8C-D). These data indicate that the *Spic* promoter has unidirectional activity in two B cell lines.

Based on evidence that signaling through the canonical or non-canonical NF-κB pathways can activate *Spic* expression in myeloid- and lymphoid-lineage cells, we asked whether NF-κB might mediate promoter activity (Alam et al., 2020; Soodgupta et al., 2019). CiiiDER and ConTra v3 software packages were used to identify predicted NF-κB subunit binding sites within the cloned region of the promoter (Figure 3–8A-B; Gearing et al., 2019; Kreft et al., 2017). One common predicted NF-κB binding site at approximately 400 bp into the cloned promoter region was identified by both programs. We next performed site-directed mutagenesis to mutate two crucial guanine nucleotides required for NF-κB subunit binding (Figure 3–8C-D). Transfection of the mutant vector caused a significant reduction in relative luciferase activity compared to the wildtype vector, suggesting that NF-κB activates transcription of *Spic* by binding to the identified site (Figure 3–8E).

Figure 3–8. Characterization of the *Spic* promoter. (**A**) UCSC Mouse Genome Track showing *Spic* exon 1 and surrounding sequence. Arrow represents exon 1 and box denotes upstream region of conservation. (**B**) Schematic of luciferase reporters, pGL3-basic (top) and *Spic* promoter (bottom). (**C**) Activity of the *Spic* promoter in 38B9 pro-B cells and (**D**) WEHI-279 B cells. Relative luciferase activity represents Renilla/Luciferase readings. (*n* = at least 3 independent experiments in triplicate, one-way ANOVA with Tukey's multiple comparisons test). Individual data points represent mean of triplicate wells for a single experiment. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

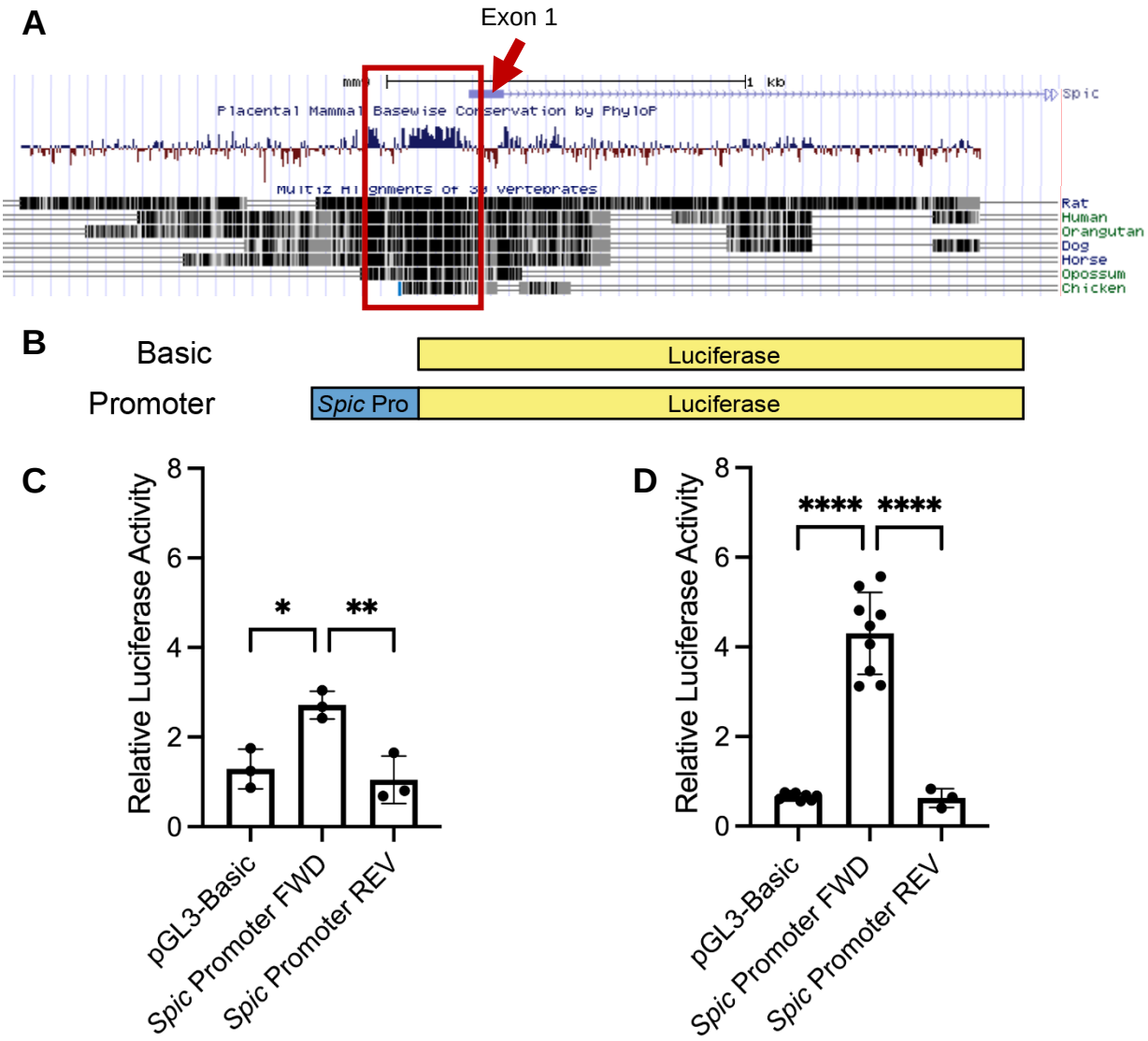
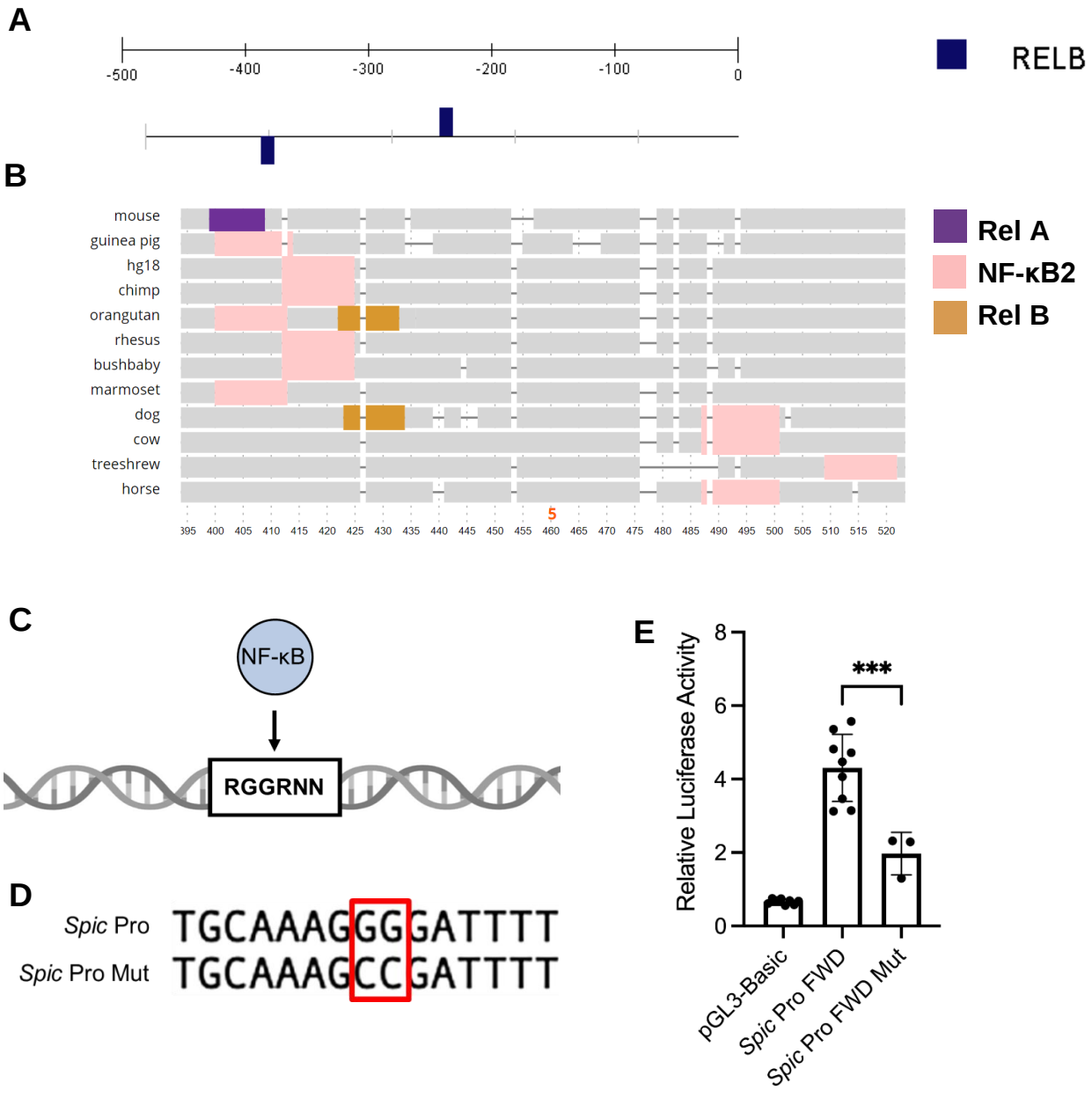

A
Exon 1
1 kb
Spic
Placental Mammal Basewise Conservation by PhyloP
Multiz Alignments of 30 vertebrates
Rat
Human
Orangutan
Dog
Horse
Opossum
Chicken
B
Basic
Promoter
Luciferase
Spic Pro
Luciferase
C
Relative Luciferase Activity
0
2
4
6
8
*
**
pGL3-Basic
Spic Promoter FWD
Spic Promoter REV
D
Relative Luciferase Activity
0
2
4
6
8
****
****
pGL3-Basic
Spic Promoter FWD
Spic Promoter REV

Figure 3–9. Activation of *Spic* transcription by NF-κB. (**A**) CiiiDER transcription factor binding site prediction within cloned promoter sequence including two possible RelB binding sites. (**B**) 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 coloured segments. (**C**) Schematic of NF-κB consensus binding site. (**D**) Sanger sequencing of cloned region of *Spic* promoter showing wildtype sequence and mutant. (**E**) Transient transfection of WEHI-279 B cells and luciferase assays indicated a reduction in luciferase activity following site-directed mutagenesis. Relative luciferase activity represents Renilla/Luciferase readings. Bars indicate mean ± SD, n = at least 3, one-way ANOVA with Tukey's multiple comparisons test. Individual data points represent mean of triplicate wells for a single experiment. ***$p < 0.001$. Data shown in (**E**) are derived from the same experiments as 3–8D and split up for clarity.

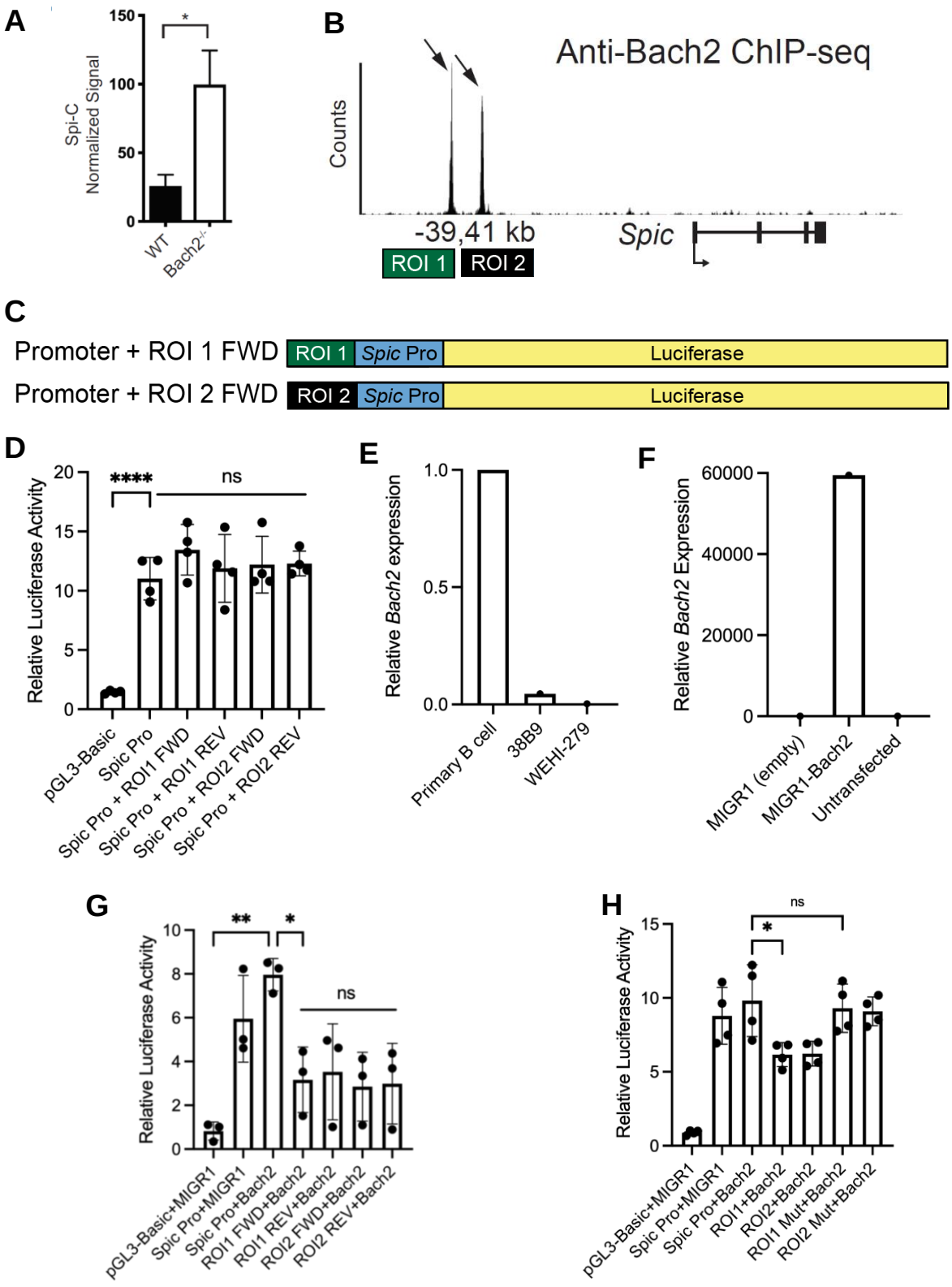

A
-500
-400
-300
-200
-100
0
RELB
B
mouse
guinea pig
hg18
chimp
orangutan
rhesus
bushbaby
marmoset
dog
cow
treeshrew
horse
Rel A
NF-κB2
Rel B
C
NF-κB
RGGRNN
D
Spic Pro TGCAAAGGGATTTT
Spic Pro Mut TGCAAAGCCGATTTT
E
Relative Luciferase Activity
***
pGL3-Basic
Spic Pro FWD
Spic Pro FWD Mut

3.6 Bach2 represses transcription of *Spic*

Previous studies showed that *Spic* expression is increased in B cells from Bach2 knockout mice (Figure 3–10A; Laramée et al., 2020; Miura et al., 2018). Reanalysis of published anti-Bach2 ChIP-Seq data led to the identification of two peaks approximately 40 kb upstream of the *Spic* transcription start site where Bach2 binds to DNA (Figure 3–10B; Itoh-Nakadai et al., 2014; Laramée et al., 2020). These two regions, arbitrarily named region of interest (ROI) 1 and 2 are in accordance with previous reports of *Spic* regulatory sequences (Itoh-Nakadai et al., 2014). To determine if Bach2 regulates expression of *Spic* by interacting with the identified regulatory regions, we used PCR to clone ROI 1 and 2, and ligated these potential regulatory regions upstream of the *Spic* promoter in a luciferase-containing vector (Figure 3–10C). WEHI-279 B cells were transiently transfected with luciferase constructs (Figures 3–8B, 3–10C) and luciferase activity was quantified. We found that transfection of ROI 1- or 2-containing vectors alone did not enhance or repress luciferase expression relative to the vector containing only the *Spic* promoter (Figure 3–10D).

We next asked whether our cell lines expressed sufficiently high levels of endogenous Bach2 to observe the effects of its interaction with the ROIs. We performed RT-qPCR to assess *Bach2* expression in WEHI-279 B cells and 38B9 pro-B cells. We found that both 38B9 pro-B cells and WEHI-279 mature B cells expressed low or undetectable levels of *Bach2* compared to primary splenic B cells (Figure 3–10E). Therefore, we obtained a MIGR1-Bach2 expression vector allowing for co-transfection and high levels of expression in electroporated cells (Figure 3–10F). Co-transfection of WEHI-279 cells with the Bach2 expression vector and the *Spic* promoter-containing vector appeared to modestly increase relative luciferase activity compared to the combination of the *Spic* promoter and empty MIGR1 vectors (Figure 3–10G). Transfection of vectors containing ROI 1 or 2 in either orientation caused a significant reduction in relative luciferase activity in the presence of Bach2 (Figure 3–10G). We next performed site-directed mutagenesis on one Bach2 consensus binding site within each ROI (Laramée et al., 2020). Co-transfection experiments showed that vectors containing mutated Bach2 sites increased relative luciferase activity to the level observed following transfection with the *Spic* promoter (Figure 3–10H). These data indicate that Bach2 represses transcription of *Spic* by interacting with ROI 1 and 2, and that this function is lost upon mutation of one Bach2 binding site.

Figure 3–10. Bach2 represses *Spic* expression in B cells. (**A**) Increased *Spic* mRNA expression in anti-IgM-stimulated splenic B cells lacking Bach2. Agilent microarray data from Miura et al., 2018 was re-analyzed; y-axis shows *Spic* normalized signal. Result is shown as mean ± SD (n = 3 biological replicates, unpaired t-test), $*p < 0.05$. (**B**) Interaction of Bach2 with regulatory regions in the *Spic* locus. ChIP-seq data from Itoh-Nakadai et al., 2017 was re-analyzed 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 (**C**) Schematic of luciferase reporters *Spic* promoter + ROI 1 (top) and *Spic* promoter + ROI 2 (bottom). (**D**) *Spic* ROI 1 and ROI 2 have no significant activity in WEHI-279 B cells. Relative luciferase activity represents Renilla/Luciferase readings. $n = 4$ independent experiments in triplicate, one-way ANOVA with Tukey's multiple comparisons test. $****p < 0.0001$. (**E**) RT-qPCR analysis of *Bach2* expression in 38B9 pro-B cells and WEHI-279 cells compared to primary splenic B cells. Data represents a single representative experiment. (**F**) RT-qPCR analysis of *Bach2* expression in WEHI-279 B cells transfected with MIGR1, MIGR1-Bach2, or untransfected. Data represents a single representative experiment. (**G**) Relative luciferase activity of ROI 1 and ROI 2 in WEHI-279 B cells co-transfected with MIGR1-Bach2. Bars indicate mean ± SD, $n = 3$ independent experiments in triplicate, one-way ANOVA with Tukey's multiple comparisons test. $*p < 0.05$ $**p < 0.01$. (**H**) Relative luciferase activity of ROI 1 and ROI 2 in WEHI-279 B cells co-transfected with MIGR1-Bach2 following mutation of one Bach2 consensus binding site. Bars indicate mean ± SD, $n = 4$ independent experiments in triplicate, one-way ANOVA with Tukey's multiple comparisons test. $*p < 0.05$.

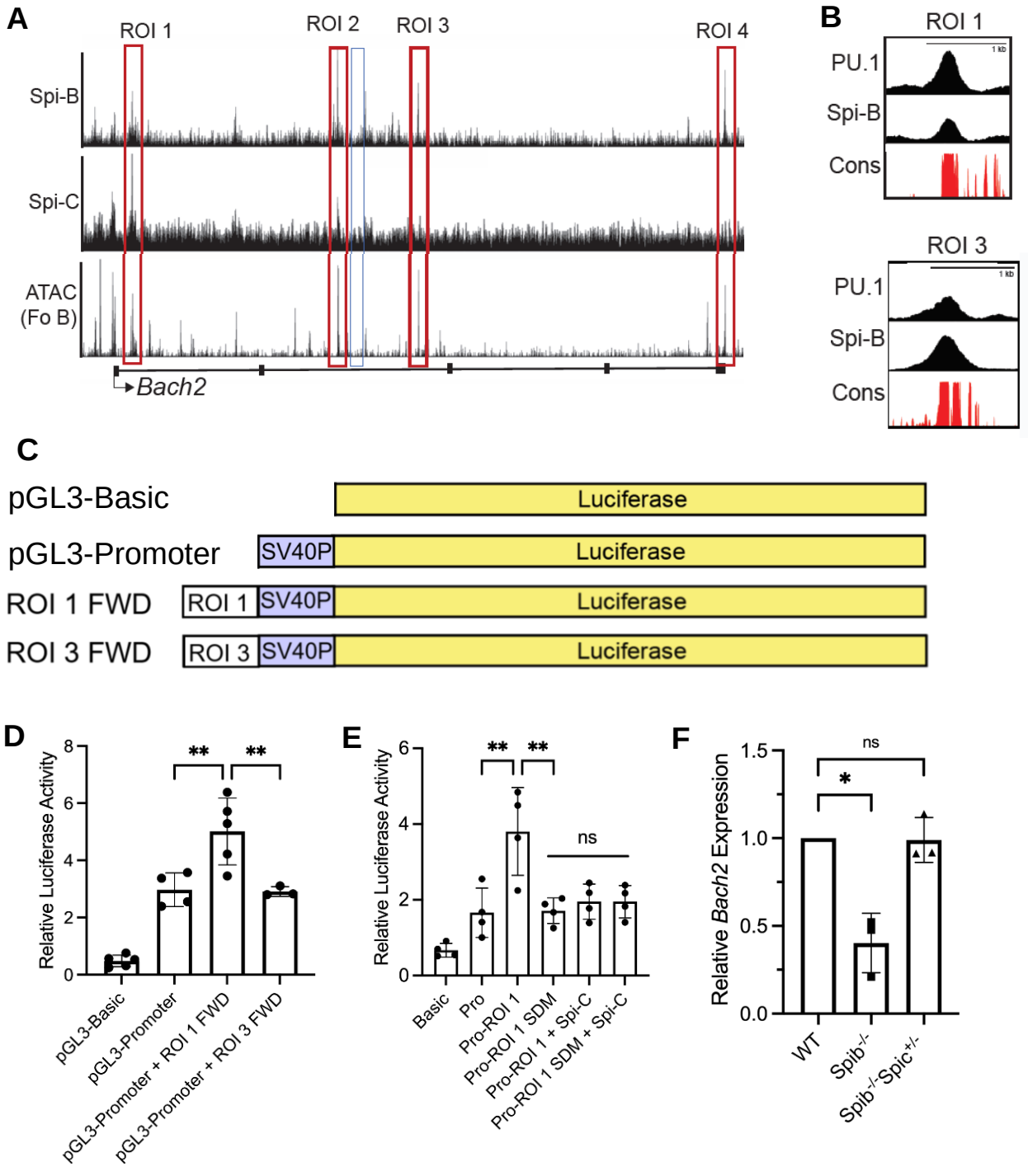

A
Spi-C Normalized Signal
150
100
50
0
*
WT
Bach2-/-
B
Anti-Bach2 ChIP-seq
Counts
-39,41 kb
ROI 1
ROI 2
Spic
C
Promoter + ROI 1 FWD
ROI 1
Spic Pro
Luciferase
Promoter + ROI 2 FWD
ROI 2
Spic Pro
Luciferase
D
Relative Luciferase Activity
20
15
10
5
0
****
ns
pGL3-Basic
Spic Pro
Spic Pro + ROI1 FWD
Spic Pro + ROI1 REV
Spic Pro + ROI2 FWD
Spic Pro + ROI2 REV
E
Relative Bach2 expression
1.0
0.5
0.0
Primary B cell
38B9
WEHI-279
F
Relative Bach2 Expression
60000
40000
20000
0
MIGR1 (empty)
MIGR1-Bach2
Untransfected
G
Relative Luciferase Activity
10
8
6
4
2
0
**
*
ns
pGL3-Basic+MIGR1
Spic Pro+MIGR1
Spic Pro+Bach2
ROI1 FWD+Bach2
ROI1 REV+Bach2
ROI2 FWD+Bach2
ROI2 REV+Bach2
H
Relative Luciferase Activity
15
10
5
0
ns
*
pGL3-Basic+MIGR1
Spic Pro+MIGR1
Spic Pro+Bach2
ROI1+Bach2
ROI2+Bach2
ROI1 Mut+Bach2
ROI2 Mut+Bach2

3.7 Spi-B and Spi-C oppositely regulate transcription of *Bach2*

We previously identified Bach2 as a candidate gene that is oppositely regulated by SPI factors PU.1, Spi-B, and Spi-C (Laramée et al., 2020). ChIP-Seq analysis revealed four peaks within the *Bach2* locus indicative of binding by all three members of the SPI subfamily (Figure 3–11A). Reanalysis of ATAC-Seq confirmed chromatin accessibility at all four ROI, further suggesting that these ROI act as regulatory regions to influence expression of Bach2 (Figure 3–11A). Cross-species analysis of evolutionary conservation revealed substantial conservation of ROI 1 and 3 in particular (Figure 3–11B). We constructed luciferase reporter vectors using the pGL3-Promoter construct and cloned ROI 1 and 3 upstream in both orientations. Transfection into WEHI-279 lymphoma cells revealed enhancer activity for ROI 1, but no activity in ROI 3 (Figure 3–11C). As WEHI-279 cells are not expected to express high levels of Spi-C, we performed co-transfection with a pcDNA3-Spi-C expression vector. As well, we mutated one ETS factor consensus binding site and evaluated luciferase activity in the presence or absence of the Spi-C expression vector. We found that mutation of one ETS binding site and/or co-transfection with pcDNA3-Spi-C quenched luciferase activity to the level of the promoter, indicating that Spi-B and Spi-C oppositely regulate transcription of Bach2 (Figure 3–11D).

Finally, we sought to further explore the regulation of Bach2 by Spi-B and Spi-C using WT, *Spib*$^{-/-}$, and *Spib*$^{-/-}$*Spic*$^{+/-}$ mouse models. We asked whether loss of one *Spic* allele could alter expression of *Bach2* compared to WT mice or those lacking Spi-B. We performed RT-qPCR to determine transcript levels of *Bach2* in GC B cells – where Bach2 is known to be highly expressed – derived from each of our genotypes of interest (Laramée et al., 2020). Mice were immunized with NP-KLH adjuvanted with alum and GC B cells were harvested by FACS on day 10 post-immunization. We found that Bach2 was significantly downregulated in GC B cells from *Spib*$^{-/-}$, while heterozygosity for *Spic* restored transcript levels to WT levels (Figure 3–11E). Taken together, these data show that Bach2 is oppositely regulated by Spi-B and Spi-C in B cells, with Bach2 and Spi-C forming a negative regulatory loop.

Figure 3–11. Regulation of *Bach2* by Spi-B and Spi-C. (**A**) UCSC genome browser tracks of ChIP-seq analysis of 3XFLAG-tagged Spi-B in WEHI-279 cells (top panel) and Spi-C in 38B9 cells (middle panel). Also shown is IMMGEN ATAC-seq analysis performed in enriched follicular B cells (Fo B) (bottom panel). *Bach2* gene structure is shown below, with black boxes denoting exons and lines representing introns. Red boxes indicate regions-of-interest (ROIs 1-4), while the blue box denotes the negative control region (NCR). (**B**) Multi-species conservation analysis (in red) visualized as superimposed UCSC tracks of ChIP-seq (ROI 1 and 3, in black) within the *Bach2* locus for PU.1 and Spi-B. (**C**) Schematic of luciferase reporters (top to bottom): pGL3-Basic, pGL3-Promoter (SV40), pGL3-Promoter-ROI 1, and pGL3-Promoter-ROI 3. (**D**) Bach2 ROI 1, but not ROI 3, displays enhancer activity in WEHI-279 B cells. Relative luciferase activity represents Renilla/Luciferase readings. (n = at least 3 independent experiments in triplicate, mixed-effects ANOVA with Tukey's multiple comparisons test). ** p <0.01. (**E**) *Bach2* ROI 1 enhancer activity in WEHI-279 cells is reduced by mutation of the ETS binding site or co-transfection with pcDNA3-Spi-C (n = 4 independent experiments in triplicates, mixed-effects ANOVA with Tukey's multiple comparisons test). ** $p < 0.01$. (**F**) Reduced *Bach2* mRNA transcript levels in $Spib^{-/-}$ GC B cells. RT-qPCR analysis was performed on RNA prepared from splenic GC B cells enriched from WT, $Spib^{-/-}$, and $Spib^{-/-}Spic^{+/-}$ mice by cell sorting, 10 days post-immunization with NP-KLH. Result is shown for representative experiment with triplicate technical replicates, Kruskal-Wallis with Dunn's multiple comparisons test, *$p < 0.05$.


A
ROI 1
ROI 2
ROI 3
ROI 4
Spi-B
Spi-C
ATAC
(Fo B)
Bach2
B
ROI 1
PU.1
Spi-B
Cons
ROI 3
PU.1
Spi-B
Cons
C
pGL3-Basic
Luciferase
pGL3-Promoter
SV40P
Luciferase
ROI 1 FWD
ROI 1
SV40P
Luciferase
ROI 3 FWD
ROI 3
SV40P
Luciferase
D
Relative Luciferase Activity
**
**
pGL3-Basic
pGL3-Promoter
pGL3-Promoter + ROI 1 FWD
pGL3-Promoter + ROI 3 FWD
E
Relative Luciferase Activity
**
**
ns
Basic
Pro
Pro-ROI 1
Pro-ROI 1 SDM
Pro-ROI 1 + Spi-C
Pro-ROI 1 SDM + Spi-C
F
Relative Bach2 Expression
ns
*
WT
Spib-/-
Spib-/-Spic+/-

4 Chapter 4: Discussion

4.1 General Discussion

The present study aimed to investigate the regulation of *Spic* by external signals and to characterize the molecular mechanisms responsible for its dynamic patterns of expression. We provided evidence that expression of Spi-C is highly sensitive to the presence of various stimulants in B cells, particularly following exposure to proliferative stimuli. We showed that the absence of stimuli or use of drugs that activate a quiescent-like state upregulate *Spic*, though the exact molecular basis for this remains unknown. We also showed that NF-κB activates transcription of *Spic* by interacting with its promoter, while Bach2 represses its transcription through interaction with two upstream regulatory regions. Finally, we showed that Spi-B and Spi-C oppositely regulate transcription of *Bach2*, a key factor involved in processes including germinal centre formation and MBC differentiation (Igarashi et al., 2017; Igarashi & Itoh-Nakadai, 2016). Taken together, our findings suggest that Spi-C is a tightly regulated factor opposing its related factor Spi-B.

Spi-C was induced in B cells in response to the metabolite heme. Though the molecular mechanism for its upregulation due to degradation of its constitutive repressor Bach2 is now well understood, the biological relevance of Spi-C induction by heme in B cells remains unknown (Dutra & Bozza, 2014; Itoh-Nakadai et al., 2014; Watanabe-Matsui et al., 2011, 2015). First, it is unclear if and how heme gains entry into B cells. While there is evidence that treatment of B cells with heme increases transcription of the endosomal transporter HRG-1, the mechanism of entry into the plasma membrane is not known (Watanabe-Matsui et al., 2011). 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 non-specific uptake than previously thought (Goldmacher et al., 1986; Martínez-Riaño et al., 2018). 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 (Laramée et al., 2020; Martins & Knapp, 2018). While not thought to strongly activate PRRs, there is evidence that heme may be able to signal weakly through TLRs such as TLR4 (Figueiredo et al., 2007). 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.

Since free heme is a potent catalyst of reactive oxygen species generation, it is maintained almost exclusively in the more stable form of hemoglobin (Aich et al., 2015; Martins & Knapp, 2018). High levels of free heme are typically indicative of excessive hemolysis, which can arise due to disease or infection. Together with evidence that heme accelerates antibody-secreting fate commitment, it is plausible that heme-dependent activation of Spi-C is one signaling pathway that promotes the generation of ASCs in response to a nonspecific threat (Watanabe-Matsui et al., 2011). 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 (Martins & Knapp, 2018; Orf & Cunnington, 2015). This leads to the proteosome-dependent degradation of Bach2, which frees Spi-C from constitutive repression (Laramée et al., 2020). Spi-C may then act to counter its related factor Spi-B and promote B cell differentiation into ASCs. This proposed mechanism would allow for the rapid generation of antibodies to initiate immunity while the longer-term immune response begins to develop.

Across all stages of B cell development and differentiation, *Spic* expression is highest during the transitional stage of development (Zhu et al., 2008). This pattern of expression, and the known signaling pathway of BAFF by the non-canonical NF-κB pathway, led to the investigation of BAFF as a possible activator of *Spic* transcription (Bednarski et al., 2016). Despite the nonsignificant increase in *Spic* expression, we observed a slight upward trend across time points. Specifically, we observed an approximately 2-fold increase in *Spic* expression in B cells cultured with BAFF. Based on our findings that *Spic* is readily downregulated, an important question is how high expression of *Spic* must be to effect its biological functions. Work from other groups in macrophages has showed that even during critical signaling events, *Spic* expression may only increase slightly (Alam et al., 2020; Haldar et al., 2014). Numerous experiments by Alam and colleagues in particular reported increases in *Spic* expression of as little as 1.75-fold, despite their noteworthy overall findings (Alam et al., 2020). As such, extremely tight regulation of the factor may lead to mere modest changes in expression that still result in biological consequences.

CD40L downregulated *Spic* expression in splenic murine B cells both alone and in combination with other cytokines. Interestingly, of all the combinations assessed, *Spic* was downregulated to the greatest extent in cells cultured in CD40L + IL-4 + IL-5. Compared to freshly isolated B cells, expression of *Spic* was reduced by only 5-fold in B cells cultured with only CD40L, but over 20-fold in B cells cultured with CD40L + IL-4 + IL-5. This combination of cytokines is a well-established model of T cell-dependent B cell activation and ASC generation, and *Spic* is known to be highly expressed in PBs and PCs (Hasbold et al., 2004; Laramée et al., 2020; Nutt et al., 2015). The initial downregulation of *Spic* observed is likely due to the early 24-hour time point, when differentiation into ASCs has not yet occurred. In general, experiments examining *Spic* expression over time must consider the possibility of a diverse population of activated and differentiated B cells arising over a 72-hour period. For example, in experiments involving CD40L + IL-4 + IL-5, the combination of cytokines first initiates B cell activation and proliferation. It is not until 3-5 days in culture that high frequencies of $CD138^+$ ASCs are generated (Laramée et al., 2020). This is also corroborated by the downregulation of *Spic* in actively cycling GC B cells, as well as the requirement for several rounds of B cell division prior to commitment to the ASC fate (Hasbold et al., 2004; Laramée et al., 2020). Therefore, loss of *Spic* expression in a time of abundant proliferation is plausible, and upregulation may not occur until later in differentiation when PBs begin to lose their proliferative capacity as they mature into terminally-differentiated PCs (Nutt et al., 2015). In fact, downregulation of *Spic* to allow for rounds of cell division may be required for initiation of the early ASC-generation pathway.

One noteworthy finding was the opposing effects of LPS on *Spic* expression in B cells and BMDMs. Of all signals assessed, LPS downregulated *Spic* expression to the greatest extent – over 200-fold – whereas LPS treatment upregulated *Spic* by approximately 5-fold in BMDMs. It is known that the NF-κB pathway becomes activated in both B cells and macrophages following TLR4 engagement (Sakai et al., 2017; Sharif et al., 2007). However, one main 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 pro-inflammatory cytokines and nitric oxide (Sharif et al., 2007; Vadiveloo et al., 2001). Even in the presence of M-CSF, proliferation is inhibited due to repression of cyclins (Lotze & Hamilton, 2003). In contrast, B cells activated by LPS initiate a response

characterized by robust proliferation and differentiation (Xu et al., 2008). Therefore, while 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.

Of the SPI family factors, *Spic* was upregulated to the greatest extent in unstimulated B cells. This finding, in combination with evidence that *Spic* expression is increased in response to drugs that block cell division, led to the conclusion that *Spic* is induced in quiescent B cells. It should be noted that Cytochalasin D does not block cell cycle entry specifically, and rather inhibits actin polymerization, which may have global consequences for cellular signaling cascades. Therefore, experiments using the drug should be interpreted carefully. Previous findings show that Spi-C is highly expressed in transitional B cells, PBs, and PCs – all of which have a fairly low capacity for proliferation and respond abnormally to BCR engagement (Laramée et al., 2020; Zhu et al., 2008). As well, Spi-C was found to directly inhibit proliferation of B cells during the pre-B cell stage of development (Bednarski et al., 2016; Soodgupta et al., 2019).

Given the abundance of evidence that Spi-C is active in quiescent cells and turned off during proliferation, we propose that Spi-C is a cell cycle-responsive gene. This may be mediated in a similar fashion as seen in the regulation of RAG 1/2, which are well-established examples of cell cycle-dependent regulation (Lin & Desiderio, 1994; Ochodnicka-Mackovicova et al., 2016; Schlissel et al., 1993). Taken together, we believe it is likely that Spi-C both represses cell cycle entry and is regulated by cell cycle-regulated genes to become active during periods of quiescence and repressed during proliferation.

We found evidence that NF-κB activates transcription of *Spic* through interactions with its promoter. This is corroborated by work done by Alam et al. that identifies a role for Spi-C in modulating inflammatory responses in macrophages (Alam et al., 2020). They showed that LPS signaling activates expression of Spi-C in an NF-κB-dependent manner, pushing macrophages to an anti-inflammatory phenotype to control and/or resolve the inflammatory environment. In the B cell lineage, activation of the non-canonical NF-κB pathway was found to be crucial for activation of Spi-C expression during B cell development (Bednarski et al., 2016; Soodgupta et al., 2019). Previous observations from our group also identified *Nfkb1* as

a target of the SPI family, with Spi-C and Spi-B repressing and enhancing its transcription, respectively (Li., et al., 2015a; Li, et al., 2015b). Taken together, we propose that Spi-C exists as a key factor in a regulatory loop involving NF-κB-family members across the myeloid and lymphoid lineages, with its expressing controlling cell cycle entry during development, inflammation, and differentiation.

We also noted greater activity of the *Spic* promoter when transfected into WEHI-279 lymphoma cells than 38B9 pro-B cells. This difference in activity may be explained by the signaling pathways constitutively active in each cell line. 38B9 pro-B cells are a leukemic cell line dependent on the Abl kinase pathway for survival and proliferation (Kawano et al., 2021; Muljo & Schlissel, 2003). Alternatively, constitutive expression of the NF-κB pathway is a hallmark of many lymphomas and is directly linked to the dysregulation of cell cycle entry (Davis et al., 2001; Nagel et al., 2014). Electrophoretic mobility shift assays performed by another group using WEHI-279 nuclear extracts indeed showed high constitutive activation of the NF-κB pathway (Fields et al., 2000). Therefore, WEHI-279 B cells may express substantially higher levels of NF-κB proteins localized to nuclei than 38B9 pro-B cells, which could enhance transcription of the luciferase reporter through interaction with the *Spic* promoter. Overall, this finding emphasizes the need for careful interpretation of cell line work and the need for validation in more than one *in vitro* model.

Opposing regulation of *Bach2* by Spi-B and Spi-C provides insight into the mechanism by which Spi-C promotes ASC differentiation. As described previously, Bach2 is important in the generation of MBCs and must be downregulated to allow for PC differentiation (Kometani et al., 2013; Muto et al., 2010). We propose the following model of regulation in B cells: Spi-B may upregulate expression of *Bach2* in activated B cells to promote GC formation and/or MBC differentiation. Downregulation of *Spib* would in turn decrease expression of *Bach2*, allowing for the de-repression of *Spic*. Alternatively, Spi-C expression may be upregulated through a heme-dependent mechanism in response to infection. As Spi-C expression increases, it would enforce continued repression of *Bach2* to promote the generation of ASCs. This model highlights the importance of Spi-B and Spi-C in B cell fate decisions during immune responses.

4.2 Future Directions

This work characterized the regulation of the lineage-instructive transcription factor Spi-C in response to external signals in B cells. While the downstream effects of Spi-C in B cell development and differentiation have been described to some extent, this is to our knowledge the first study to examine its upstream regulation in mature B cells.

Research on Spi-C has in part been limited by the lack of reliable antibodies for the protein. This thesis has reported changes in *Spic* at the mRNA transcript level in response to a variety of stimuli, but questions remain regarding whether these findings can be observed at the protein level. As demand for anti-Spi-C antibodies grows, we hope to be able to repeat key experiments of interest, with a focus on investigating changes in protein expression. Lack of antibodies has also prevented genome-wide identification of binding sites, as has been done for other members of the SPI subfamily (Solomon et al., 2015). As a result, no detailed studies have been performed to correlate Spi-C with histone modifications, chromatin structure, or genomic organization using techniques including chromatin immunoprecipitation-sequencing, Assay for Transposase-Accessible Chromatin using Sequencing or Chromosome Conformation Capture-sequencing technologies such as Hi-C. Overall, the development of higher quality antibodies for Spi-C will allow for further study of the transcription factor.

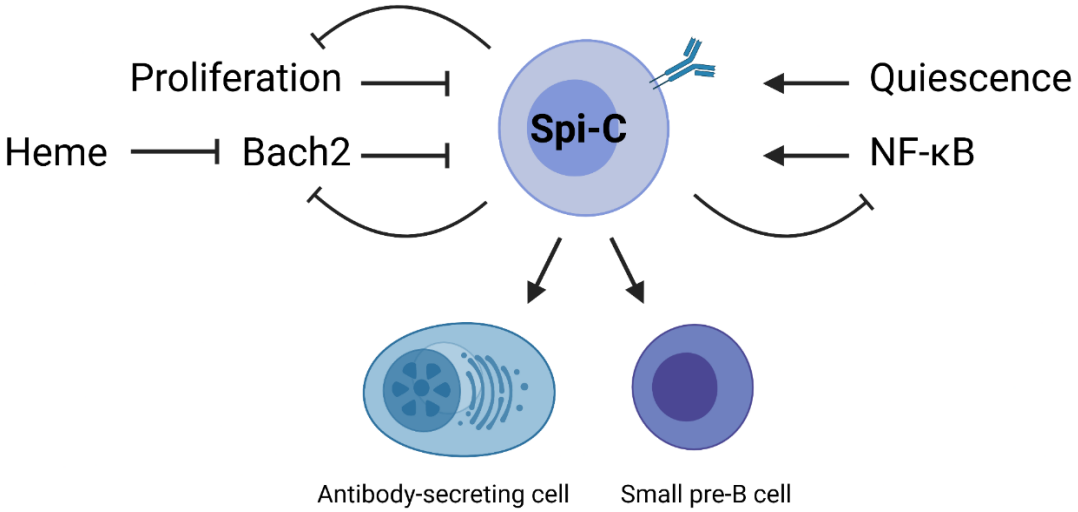
While numerous roles for Spi-C in B cell development and differentiation have emerged, we aim to continue to explore its contributions to B cell fate decisions. We have previously made use of *Spib*$^{-/-}$ *Spic*$^{+/-}$ and transgenic Eμ-Spic mice to investigate its roles in B cell responses. However, use of *Spic*$^{-/-}$ mice has been limited to studies on its roles in macrophages, with minimal characterization of changes in the B cell compartment. We are in the process of generating *Spic*$^{-/-}$ mice, which will be used to extensively investigate the frequency and function of numerous B cell subsets and responses to antigen. As well, we aim to perform single-cell RNA sequencing (scRNA-seq) with a focus on trajectory inference to determine how the loss of Spi-C impacts the polarizing decision to pursue a memory or antibody-secreting B cell fate. Software such as Slingshot will allow for the positioning of single cells as a function of pseudotime along the differentiation pathway (Street et al., 2018). This will allow for the determination of the frequency of cells at different stages of differentiation between genotypes of interest. Additionally, this technology will allow for the examination of global gene

expression in mice with modified Spi-C expression, leading to the discovery of novel Spi-C target genes in B cells.

One exciting frontier in Spi-C knowledge is to identify drugs that can target this transcription factor. Although transcription factors have been considered "undruggable" for many years, several breakthrough studies have begun to alter this perception (Bushweller, 2019). Immunomodulatory drugs such as lenalidomide have been shown to induce downregulation of PU.1 and Spi-B through alteration of ubiquitin-mediated degradation pathways (Pal et al., 2010). More direct inhibition of PU.1 has been achieved with small heterocyclic diamidine molecules that allosterically interfere with PU.1 chromatin binding by disruption of the interaction of the DNA binding domain with the DNA minor groove that flanks PU.1 binding motifs (Antony-Debré et al., 2017). Other small molecules called YK-4-279 and TK-216 have been shown to inhibit Spi-B in lymphoma cells by blocking protein–protein interaction with RNA helicases, as well as by other mechanisms of action that are not fully understood (Spriano et al., 2019). In summary, there are strong prospects for using small molecule inhibitors to alter Spi-C activity in disease.

Finally, the development of a disease model which incorporates *in vivo* activation of Spi-C in B cells would allow for examination of its possible translational relevance. Diseases marked by abnormally high levels of free heme such as β-thalassemia or hemolytic anemia may cause aberrant activation of Spi-C expression in macrophages and B cells (Kayama et al., 2018; Martins & Knapp, 2018). Since administration of heme is known to push B cells towards premature commitment to the ASC fate, it would be valuable to examine if this is occurring in a Spi-C-dependent manner in mouse models and human patients (Watanabe-Matsui et al., 2011). These types of studies can further elaborate on the role of Spi-C *in vivo*, while also introducing novel clinical considerations.

Figure 4–1. Proposed model for Spi-C regulation during B cell development and differentiation. Spi-C expression can be induced in NF-κB- or heme-dependent pathways, or by quiescence. Spi-C represses NF-κB protein expression. Spi-C is readily downregulated in response to proliferation and/or Bach2, and Spi-C in turn represses Bach2 expression and proliferation. Depending on when Spi-C expression is induced in the life of a B cell, it may promote differentiation into antibody-secreting cells or maturation into small pre-B cells.



B cell differentiation, development

References

Aich, A., Freundlich, M., & Vekilov, P. G. (2015). The free heme concentration in healthy human erythrocytes. *Blood Cells, Molecules, and Diseases*, *55*(4), 402–409. https://doi.org/10.1016/j.bcmd.2015.09.003

Akilesh, H. M., Buechler, M. B., Duggan, J. M., Hahn, W. O., Matta, B., Sun, X., Gessay, G., Whalen, E., Mason, M., Presnell, S. R., Elkon, K. B., Lacy-Hulbert, A., Barnes, B. J., Pepper, M., & Hamerman, J. A. (2019). Chronic TLR7 and TLR9 signaling drives anemia via differentiation of specialized hemophagocytes. *Science*, *363*(6423). https://doi.org/10.1126/science.aao5213

Alam, M. Z., Devalaraja, S., & Haldar, M. (2017). The heme connection: Linking erythrocytes and macrophage biology. In *Frontiers in Immunology* (Vol. 8, Issue JAN). Frontiers Research Foundation. https://doi.org/10.3389/fimmu.2017.00033

Alam, Z., Devalaraja, S., Li, M., To, T. K. J., Folkert, I. W., Mitchell-Velasquez, E., Dang, M. T., Young, P., Wilbur, C. J., Silverman, M. A., Li, X., Chen, Y. H., Hernandez, P. T., Bhattacharyya, A., Bhattacharya, M., Levine, M. H., & Haldar, M. (2020). Counter Regulation of Spic by NF-κB and STAT Signaling Controls Inflammation and Iron Metabolism in Macrophages. *Cell Reports*, *31*(13). https://doi.org/10.1016/j.celrep.2020.107825

Allman, D., & Pillai, S. (2008). Peripheral B cell subsets. In *Current Opinion in Immunology* (Vol. 20, Issue 2, pp. 149–157). NIH Public Access. https://doi.org/10.1016/j.coi.2008.03.014

Antony-Debré, I., Paul, A., Leite, J., Mitchell, K., Kim, H. M., Carvajal, L. A., Todorova, T. I., Huang, K., Kumar, A., Farahat, A. A., Bartholdy, B., Narayanagari, S. R., Chen, J., Ambesi-Impiombato, A., Ferrando, A. A., Mantzaris, I., Gavathiotis, E., Verma, A., Will, B., … Steidl, U. (2017). Pharmacological inhibition of the transcription factor PU.1 in leukemia. *Journal of Clinical Investigation*, *127*(12), 4297–4313. https://doi.org/10.1172/JCI92504

Arinobu, Y., Mizuno, S. ichi, Chong, Y., Shigematsu, H., Iino, T., Iwasaki, H., Graf, T., Mayfield, R., Chan, S., Kastner, P., & Akashi, K. (2007). Reciprocal Activation of GATA-1 and PU.1 Marks Initial Specification of Hematopoietic Stem Cells into Myeloerythroid and Myelolymphoid Lineages. *Cell Stem Cell*, *1*(4), 416–427. https://doi.org/10.1016/j.stem.2007.07.004

Batista, C. R., & DeKoter, R. P. (2018). No Title. In I. S. Choi (Ed.), *Encyclopedia of signaling molecules* (2nd ed.). Springer International Publishing.

Bednarski, J. J., Pandey, R., Schulte, E., White, L. S., Chen, B. R., Sandoval, G. J., Kohyama, M.,

Haldar, M., Nickless, A., Trott, A., Cheng, G., Murphy, K. M., Bassing, C. H., Payton, J. E., & Sleckman, B. P. (2016). RAG-mediated DNA double-strand breaks activate a cell type-specific checkpoint to inhibit pre-B cell receptor signals. *Journal of Experimental Medicine*, *213*(2), 209–223. https://doi.org/10.1084/jem.20151048

Bemark, M., Mårtensson, A., Liberg, D., & Leanderson, T. (1999). Spi-C, a novel Ets protein that is temporally regulated during B lymphocyte development. *Journal of Biological Chemistry*, *274*(15), 10259–10267. https://doi.org/10.1074/jbc.274.15.10259

Bennett, L. F., Liao, C., Quickel, M. D., Yeoh, B. S., Vijay-Kumar, M., Hankey-Giblin, P., Prabhu, K. S., & Paulson, R. F. (2019). Inflammation induces stress erythropoiesis through heme-dependent activation of SPI-C. *Science Signaling*, *12*(598). https://doi.org/10.1126/scisignal.aap7336

Boyer, L. A., Tong, I. L., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., & Young, R. A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*, *122*(6), 947–956. https://doi.org/10.1016/j.cell.2005.08.020

Brass, A. L., Zhu, A. Q., & Singh, H. (1999). Assembly requirements of PU.1-Pip (IRF-4) activator complexes: Inhibiting function in vivo using fused dimers. *EMBO Journal*, *18*(4), 977–991. https://doi.org/10.1093/emboj/18.4.977

Burda, P., Laslo, P., & Stopka, T. (2010). The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. In *Leukemia* (Vol. 24, Issue 7, pp. 1249–1257). Nature Publishing Group. https://doi.org/10.1038/leu.2010.104

Bushweller, J. H. (2019). Targeting transcription factors in cancer — from undruggable to reality. In *Nature Reviews Cancer* (Vol. 19, Issue 11, pp. 611–624). Nature Publishing Group. https://doi.org/10.1038/s41568-019-0196-7

Carlsson, R., Hjalmarsson, A., Liberg, D., Persson, C., & Leanderson, T. (2002). Genomic structure of mouse SPI-C and genomic structure and expression pattern of human SPI-C. *Gene*, *299*(1–2), 271–278. https://doi.org/10.1016/S0378-1119(02)01078-8

Carlsson, R., Persson, C., & Leanderson, T. (2003). SPI-C, a PU-box binding ETS protein expressed temporarily during B-cell development and in macrophages, contains an acidic transactivation domain located to the N-terminus. *Molecular Immunology*, *39*(16), 1035–1043. https://doi.org/10.1016/S0161-5890(03)00032-4

Cerutti, A., Cols, M., & Puga, I. (2013). Marginal zone B cells: Virtues of innate-like antibody-producing lymphocytes. In *Nature Reviews Immunology* (Vol. 13, Issue 2, pp. 118–132). NIH Public Access. https://doi.org/10.1038/nri3383

Chaplin, D. D. (2010). Overview of the immune response. *Journal of Allergy and Clinical Immunology*, *125*(2 SUPPL. 2), S3. https://doi.org/10.1016/j.jaci.2009.12.980

Chopin, M., Lun, A. T., Zhan, Y., Schreuder, J., Coughlan, H., D'Amico, A., Mielke, L. A., Almeida, F. F., Kueh, A. J., Dickins, R. A., Belz, G. T., Naik, S. H., Lew, A. M., Bouillet, P., Herold, M. J., Smyth, G. K., Corcoran, L. M., & Nutt, S. L. (2019). Transcription Factor PU.1 Promotes Conventional Dendritic Cell Identity and Function via Induction of Transcriptional Regulator DC-SCRIPT. *Immunity*, *50*(1), 77-90.e5. https://doi.org/10.1016/j.immuni.2018.11.010

Chung, J. B., Silverman, M., & Monroe, J. G. (2003). Transitional B cells: Step by step towards immune competence. In *Trends in Immunology* (Vol. 24, Issue 6, pp. 342–348). Elsevier Ltd. https://doi.org/10.1016/S1471-4906(03)00119-4

Cole And, M. F., & Young, R. A. (2008). Mapping key features of transcriptional regulatory circuitry in embryonic stem cells. *Cold Spring Harbor Symposia on Quantitative Biology*, *73*, 183–193. https://doi.org/10.1101/sqb.2008.73.027

Crayne, C. B., Albeituni, S., Nichols, K. E., & Cron, R. Q. (2019). The immunology of macrophage activation syndrome. In *Frontiers in Immunology* (Vol. 10, Issue FEB, p. 119). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2019.00119

Cyster, J. G., & Allen, C. D. C. (2019). B Cell Responses: Cell Interaction Dynamics and Decisions. In *Cell* (Vol. 177, Issue 3, pp. 524–540). Cell Press. https://doi.org/10.1016/j.cell.2019.03.016

Davis, T. L., & Rebay, I. (2017). Master regulators in development: Views from the Drosophila retinal determination and mammalian pluripotency gene networks. In *Developmental Biology* (Vol. 421, Issue 2, pp. 93–107). Academic Press Inc. https://doi.org/10.1016/j.ydbio.2016.12.005

De Silva, N. S., & Klein, U. (2015). Dynamics of B cells in germinal centres. *Nature Reviews Immunology*, *15*(3), 137–148. https://doi.org/10.1038/nri3804

Debnath, I., Roundy, K. M., Dunn, D. M., Weiss, R. B., Weis, J. J., & Weis, J. H. (2008). Defining a transcriptional fingerprint of murine splenic B-cell development. *Genes and Immunity*, *9*(8), 706–720. https://doi.org/10.1038/gene.2008.70

DeKoter, R. P., Geadah, M., Khoosal, S., Xu, L. S., Thillainadesan, G., Torchia, J., Chin, S. S., &

Garrett-Sinha, L. A. (2010). Regulation of Follicular B Cell Differentiation by the Related E26 Transformation-Specific Transcription Factors PU.1, Spi-B, and Spi-C. *The Journal of Immunology*, *185*(12), 7374–7384. https://doi.org/10.4049/jimmunol.1001413

DeKoter, R. P., & Singh, H. (2000). Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science*, *288*(5470), 1439–1442. https://doi.org/10.1126/science.288.5470.1439

Dutra, F. F., & Bozza, M. T. (2014). Heme on innate immunity and inflammation. In *Frontiers in Pharmacology: Vol. 5 MAY*. Frontiers Research Foundation. https://doi.org/10.3389/fphar.2014.00115

Eissa, N., Hussein, H., Wang, H., Rabbi, M. F., Bernstein, C. N., & Ghia, J. E. (2016). Stability of reference genes for messenger RNA quantification by real-time PCR in mouse dextran sodium sulfate experimental colitis. *PLoS ONE*, *11*(5), e0156289. https://doi.org/10.1371/journal.pone.0156289

Eric Davis, R., Brown, K. D., Siebenlist, U., & Staudt, L. M. (2001). Constitutive nuclear factor κB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *Journal of Experimental Medicine*, *194*(12), 1861–1874. https://doi.org/10.1084/jem.194.12.1861

Ferreira, R., Ohneda, K., Yamamoto, M., & Philipsen, S. (2005). GATA1 Function, a Paradigm for Transcription Factors in Hematopoiesis. *Molecular and Cellular Biology*, *25*(4), 1215–1227. https://doi.org/10.1128/mcb.25.4.1215-1227.2005

Fields, E. R., Seufzer, B. J., Oltz, E. M., & Miyamoto, S. (2000). A Switch in Distinct IκBα Degradation Mechanisms Mediates Constitutive NF-κB Activation in Mature B Cells. *The Journal of Immunology*, *164*(9), 4762–4767. https://doi.org/10.4049/jimmunol.164.9.4762

Figueiredo, R. T., Fernandez, P. L., Mourao-Sa, D. S., Porto, B. N., Dutra, F. F., Alves, L. S., Oliveira, M. F., Oliveira, P. L., Graça-Souza, A. V., & Bozza, M. T. (2007). Characterization of heme as activator of toll-like receptor 4. *Journal of Biological Chemistry*, *282*(28), 20221–20229. https://doi.org/10.1074/jbc.M610737200

Franke, F., Kirchenbaum, G. A., Kuerten, S., & Lehmann, P. V. (2020). IL-21 in Conjunction with Anti-CD40 and IL-4 Constitutes a Potent Polyclonal B Cell Stimulator for Monitoring Antigen-Specific Memory B Cells. *Cells*, *9*(2). https://doi.org/10.3390/cells9020433

Fulkerson, P. C., Fischetti, C. A., McBride, M. L., Hassman, L. M., Hogan, S. P., & Rothenberg, M.

E. (2006). A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(44), 16418–16423. https://doi.org/10.1073/pnas.0607863103

Ganz, T. (2012). Macrophages and systemic iron homeostasis. In *Journal of Innate Immunity* (Vol. 4, Issues 5–6, pp. 446–453). https://doi.org/10.1159/000336423

Garrett-Sinha, L. A., Su, G. H., Rao, S., Kabak, S., Hao, Z., Clark, M. R., & Simon, M. C. (1999). PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction. *Immunity*, *10*(4), 399–408. https://doi.org/10.1016/S1074-7613(00)80040-0

Gearing, L. J., Cumming, H. E., Chapman, R., Finkel, A. M., Woodhouse, I. B., Luu, K., Gould, J. A., Forster, S. C., & Hertzog, P. J. (2019). CiIIder: A tool for predicting and analysing transcription factor binding sites. *PLoS ONE*, *14*(9), e0215495. https://doi.org/10.1371/journal.pone.0215495

Gilbert, S. F. (2000). *Developmental Biology* (6th ed.). Sinauer Associates. https://www.ncbi.nlm.nih.gov/books/NBK9983/

Goldmacher, V. S., Tinnel, N. L., & Nelson, B. C. (1986). Evidence that pinocytosis in lymphoid cells has a low capacity. *Journal of Cell Biology*, *102*(4), 1312–1319. https://doi.org/10.1083/jcb.102.4.1312

Haldar, M., Kohyama, M., So, A. Y. L., Kc, W., Wu, X., Briseño, C. G., Satpathy, A. T., Kretzer, N. M., Arase, H., Rajasekaran, N. S., Wang, L., Egawa, T., Igarashi, K., Baltimore, D., Murphy, T. L., & Murphy, K. M. (2014). Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. *Cell*, *156*(6), 1223–1234. https://doi.org/10.1016/j.cell.2014.01.069

Hao, S., & August, A. (2005). Actin depolymerization transduces the strength of B-cell receptor stimulation. *Molecular Biology of the Cell*, *16*(5), 2275–2284. https://doi.org/10.1091/mbc.E04-10-0881

Hardy, R. R., & Hayakawa, K. (2001). B cell development pathways. In *Annual Review of Immunology* (Vol. 19, pp. 595–621). Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA . https://doi.org/10.1146/annurev.immunol.19.1.595

Hardy, Richard R., Carmack, C. E., Shinton, S. A., Kemp, J. D., & Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *Journal of Experimental Medicine*, *173*(5), 1213–1225. https://doi.org/10.1084/jem.173.5.1213

Hardy, Richard R., Kincade, P. W., & Dorshkind, K. (2007). The Protean Nature of Cells in the B Lymphocyte Lineage. In *Immunity* (Vol. 26, Issue 6, pp. 703–714). Cell Press. https://doi.org/10.1016/j.immuni.2007.05.013

Harusato, A., Naito, Y., Takagi, T., Uchiyama, K., Mizushima, K., Hirai, Y., Higashimura, Y., Katada, K., Handa, O., Ishikawa, T., Yagi, N., Kokura, S., Ichikawa, H., Muto, A., Igarashi, K., & Yoshikawa, T. (2013). BTB and CNC homolog 1 (Bach1) deficiency ameliorates TNBS colitis in mice: Role of M2 macrophages and heme oxygenase-1. *Inflammatory Bowel Diseases*, *19*(4), 740–753. https://doi.org/10.1097/MIB.0b013e3182802968

Hasbold, J., Corcoran, L. M., Tarlinton, D. M., Tangye, S. G., & Hodgkin, P. D. (2004). Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nature Immunology*, *5*(1), 55–63. https://doi.org/10.1038/ni1016

Hashimoto, S. I., Nishizumi, H., Hayashi, R., Tsubol, A., Nagawa, F., Takemori, T., & Sakano, H. (1999). Prf, a novel Ets family protein that binds to the PU.1 binding motif, is specifically expressed in restricted stages of B cell development. *International Immunology*, *11*(9), 1423–1429. https://doi.org/10.1093/intimm/11.9.1423

Henderson, L. A., & Cron, R. Q. (2020). Macrophage Activation Syndrome and Secondary Hemophagocytic Lymphohistiocytosis in Childhood Inflammatory Disorders: Diagnosis and Management. In *Pediatric Drugs* (Vol. 22, Issue 1, pp. 29–44). Adis. https://doi.org/10.1007/s40272-019-00367-1

Heng, T. S. P., Painter, M. W., Elpek, K., Lukacs-Kornek, V., Mauermann, N., Turley, S. J., Koller, D., Kim, F. S., Wagers, A. J., Asinovski, N., Davis, S., Fassett, M., Feuerer, M., Gray, D. H. D., Haxhinasto, S., Hill, J. A., Hyatt, G., Laplace, C., Leatherbee, K., … Kang, J. (2008). The immunological genome project: Networks of gene expression in immune cells. In *Nature Immunology* (Vol. 9, Issue 10, pp. 1091–1094). https://doi.org/10.1038/ni1008-1091

Herzog, S., Reth, M., & Jumaa, H. (2009). Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. In *Nature Reviews Immunology* (Vol. 9, Issue 3, pp. 195–205). https://doi.org/10.1038/nri2491

Hoebe, K., Janssen, E., & Beutler, B. (2004). The interface between innate and adaptive immunity. In *Nature Immunology* (Vol. 5, Issue 10, pp. 971–974). Nature Publishing Group. https://doi.org/10.1038/ni1004-971

Hollenhorst, P. C., McIntosh, L. P., & Graves, B. J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. *Annual Review of Biochemistry*, *80*, 437–471.

https://doi.org/10.1146/annurev.biochem.79.081507.103945

Horikawa, K., & Takatsu, K. (2006). Interleukin-5 regulates genes involved in B-cell terminal maturation. *Immunology*, *118*(4), 497–508. https://doi.org/10.1111/j.1365-2567.2006.02382.x

Houston, I. B., Kamath, M. B., Schweitzer, B. L., Chlon, T. M., & DeKoter, R. P. (2007). Reduction in PU.1 activity results in a block to B-cell development, abnormal myeloid proliferation, and neonatal lethality. *Experimental Hematology*, *35*(7), 1056–1068. https://doi.org/10.1016/j.exphem.2007.04.005

Howard, M., Kesslert, S., Chusedt, T., & Paul, W. E. (1981). *Long-term culture of normal mouse B lymphocytes (B-cell activation/growth factors/proliferation/B-cell lines)* (Vol. 78, Issue 9).

Igarashi, K., & Itoh-Nakadai, A. (2016). Orchestration of B lymphoid cells and their inner myeloid by Bach. In *Current Opinion in Immunology* (Vol. 39, pp. 136–142). Elsevier Ltd. https://doi.org/10.1016/j.coi.2016.01.012

Igarashi, K., Kurosaki, T., & Roychoudhuri, R. (2017). BACH transcription factors in innate and adaptive immunity. In *Nature Reviews Immunology* (Vol. 17, Issue 7, pp. 437–450). Nature Publishing Group. https://doi.org/10.1038/nri.2017.26

Inoue, K., Oikawa, M., Kamimura, S., Ogonuki, N., Nakamura, T., Nakano, T., Abe, K., & Ogura, A. (2015). Trichostatin A specifically improves the aberrant expression of transcription factor genes in embryos produced by somatic cell nuclear transfer. *Scientific Reports*, *5*. https://doi.org/10.1038/srep10127

Itoh-Nakadai, A., Hikota, R., Muto, A., Kometani, K., Watanabe-Matsui, M., Sato, Y., Kobayashi, M., Nakamura, A., Miura, Y., Yano, Y., Tashiro, S., Sun, J., Ikawa, T., Ochiai, K., Kurosaki, T., & Igarashi, K. (2014). The transcription repressors Bach2 and Bach1 promote B cell development by repressing the myeloid program. *Nature Immunology*, *15*(12), 1171–1180. https://doi.org/10.1038/ni.3024

Itoh-Nakadai, A., Matsumoto, M., Kato, H., Sasaki, J., Uehara, Y., Sato, Y., Ebina-Shibuya, R., Morooka, M., Funayama, R., Nakayama, K., Ochiai, K., Muto, A., & Igarashi, K. (2017). A Bach2-Cebp Gene Regulatory Network for the Commitment of Multipotent Hematopoietic Progenitors. *Cell Reports*, *18*(10), 2401–2414. https://doi.org/10.1016/j.celrep.2017.02.029

Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E. A., Iwasaki-Arai, J., Mizuno, S. I., Arinobu, Y., Geary, K., Zhang, P., Dayaram, T., Fenyus, M. L., Elf, S., Chan, S., Kastner, P., Huettner, C. S., Murray, R., Tenen, D. G., & Akashi, K. (2005). Distinctive and indispensable roles of PU.1 in

maintenance of hematopoietic stem cells and their differentiation. *Blood*, *106*(5), 1590–1600. https://doi.org/10.1182/blood-2005-03-0860

Kageyama, S. ichiro, Liu, H., Nagata, M., & Aoki, F. (2006). The role of ETS transcription factors in transcription and development of mouse preimplantation embryos. *Biochemical and Biophysical Research Communications*, *344*(2), 675–679. https://doi.org/10.1016/j.bbrc.2006.03.192

Kawano, Y., Yoshikawa, S., & Minegishi, Y. (2021). *Repertoire by Delivering Differential Signals IgH Chains and Tunes the Pre-B Cell Pre-B Cell Receptor Assesses the Quality of and Hajime Karasuyama*. https://doi.org/10.4049/jimmunol.177.4.2242

Kayama, H., Kohyama, M., Okuzaki, D., Motooka, D., Barman, S., Okumura, R., Muneta, M., Hoshino, K., Sasaki, I., Ise, W., Matsuno, H., Nishimura, J., Kurosaki, T., Nakamura, S., Arase, H., Kaisho, T., & Takeda, K. (2018). Heme ameliorates dextran sodium sulfate-induced colitis through providing intestinal macrophages with noninflammatory profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(33), 8418–8423. https://doi.org/10.1073/pnas.1808426115

Kerfoot, S. M., Yaari, G., Patel, J. R., Johnson, K. L., Gonzalez, D. G., Kleinstein, S. H., & Haberman, A. M. (2011). Germinal Center B Cell and T Follicular Helper Cell Development Initiates in the Interfollicular Zone. *Immunity*, *34*(6), 947–960. https://doi.org/10.1016/j.immuni.2011.03.024

Kin Chan, S. S. (2013). What is a Master Regulator? *Journal of Stem Cell Research & Therapy*, *03*(02). https://doi.org/10.4172/2157-7633.1000e114

Kiraz, Y., Adan, A., Kartal Yandim, M., & Baran, Y. (2016). Major apoptotic mechanisms and genes involved in apoptosis. In *Tumor Biology* (Vol. 37, Issue 7, pp. 8471–8486). Springer Netherlands. https://doi.org/10.1007/s13277-016-5035-9

Kohyama, M., Ise, W., Edelson, B. T., Wilker, P. R., Hildner, K., Mejia, C., Frazier, W. A., Murphy, T. L., & Murphy, K. M. (2009). Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature*, *457*(7227), 318–321. https://doi.org/10.1038/nature07472

Kometani, K., Nakagawa, R., Shinnakasu, R., Kaji, T., Rybouchkin, A., Moriyama, S., Furukawa, K., Koseki, H., Takemori, T., & Kurosaki, T. (2013). Repression of the Transcription Factor Bach2 Contributes to Predisposition of IgG1 Memory B Cells toward Plasma Cell Differentiation. *Immunity*, *39*(1), 136–147. https://doi.org/10.1016/j.immuni.2013.06.011

Kreft, L., Soete, A., Hulpiau, P., Botzki, A., Saeys, Y., & De Bleser, P. (2017). ConTra v3: A tool to

identify transcription factor binding sites across species, update 2017. *Nucleic Acids Research*, *45*(W1), W490–W494. https://doi.org/10.1093/nar/gkx376

Kurosaki, T., Kometani, K., & Ise, W. (2015). Memory B cells. *Nature Reviews Immunology*, *15*(3), 149–159. https://doi.org/10.1038/nri3802

Laramée, A. S., Raczkowski, H., Shao, P., Batista, C., Shukla, D., Xu, L., Haeryfar, S. M. M., Tesfagiorgis, Y., Kerfoot, S., & DeKoter, R. (2020). Opposing Roles for the Related ETS-Family Transcription Factors Spi-B and Spi-C in Regulating B Cell Differentiation and Function. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.00841

Lebien, T. W., Thomas, *, & Tedder, F. (2008). B lymphocytes: how they develop and function. *Blood*, *112*(5), 1570–1580. https://doi.org/10.1182/blood

Li, J., Ghiani, C. A., Jin, Y. K., Liu, A., Sandoval, J., DeVellis, J., & Casaccia-Bonnefil, P. (2008). Inhibition of p53 transcriptional activity: A potential target for future development of therapeutic strategies for primary demyelination. *Journal of Neuroscience*, *28*(24), 6118–6127. https://doi.org/10.1523/JNEUROSCI.0184-08.2008

Li, S. K. H., Abbas, A. K., Solomon, L. A., Groux, G. M. N., & DeKoter, R. P. (2015a). Nfkb1 Activation by the E26 Transformation-Specific Transcription Factors PU.1 and Spi-B Promotes Toll-Like Receptor-Mediated Splenic B Cell Proliferation . *Molecular and Cellular Biology*, *35*(9), 1619–1632. https://doi.org/10.1128/mcb.00117-15

Li, S. K. H., Solomon, L. A., Fulkerson, P. C., & DeKoter, R. P. (2015b). Identification of a Negative Regulatory Role for Spi-C in the Murine B Cell Lineage. *The Journal of Immunology*, *194*(8), 3798–3807. https://doi.org/10.4049/jimmunol.1402432

Lian, X., Yan, C., Qin, Y., Knox, L., Li, T., & Du, H. (2005). Neutral lipids and peroxisome proliterator-activated receptor-γ control pulmonary gene expression and inflammation-triggered pathogenesis in lysosomal acid lipase knockout mice. *American Journal of Pathology*, *167*(3), 813–821. https://doi.org/10.1016/S0002-9440(10)62053-6

Lin, W. C., & Desiderio, S. (1994). Cell cycle regulation of V(D)J recombination-activating protein RAG-2. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(7), 2733–2737. https://doi.org/10.1073/pnas.91.7.2733

Lotze, M. T., & Hamilton, J. A. (2003). Macrophage Colony Stimulating Factor [CSF-1]. In *The Cytokine Handbook* (pp. 927–958). Elsevier Inc. https://doi.org/10.1016/B978-012689663-3/50044-2

Lu, Y., Basatemur, G., Scott, I. C., Chiarugi, D., Clement, M., Harrison, J., Jugdaohsingh, R., Yu, X., Newland, S. A., Jolin, H. E., Li, X., Chen, X., Szymanska, M., Haraldsen, G., Palmer, G., Fallon, P. G., Cohen, E. S., McKenzie, A. N. J., & Mallat, Z. (2020). Interleukin-33 Signaling Controls the Development of Iron-Recycling Macrophages. *Immunity*, *52*(5), 782-793.e5. https://doi.org/10.1016/j.immuni.2020.03.006

MacLennan, I. C. M., Toellner, K. M., Cunningham, A. F., Serre, K., Sze, D. M. Y., Zúñiga, E., Cook, M. C., & Vinuesa, C. G. (2003). Extrafollicular antibody responses. In *Immunological Reviews* (Vol. 194, Issue 1, pp. 8–18). John Wiley & Sons, Ltd. https://doi.org/10.1034/j.1600-065X.2003.00058.x

Martin, F., & Kearney, J. F. (2002). Marginal-zone B cells. In *Nature Reviews Immunology* (Vol. 2, Issue 5, pp. 323–335). Nat Rev Immunol. https://doi.org/10.1038/nri799

Martínez-Riaño, A., Bovolenta, E. R., Mendoza, P., Oeste, C. L., Martín-Bermejo, M. J., Bovolenta, P., Turner, M., Martínez-Martín, N., & Alarcón, B. (2018). Antigen phagocytosis by B cells is required for a potent humoral response. *EMBO Reports*, *19*(9), e46016. https://doi.org/10.15252/EMBR.201846016

Martins, R., & Knapp, S. (2018). Heme and hemolysis in innate immunity: adding insult to injury. In *Current Opinion in Immunology* (Vol. 50, pp. 14–20). Elsevier Ltd. https://doi.org/10.1016/j.coi.2017.10.005

Matzinger, P. (1994). Tolerance, danger, and the extended family. In *Annual Review of Immunology* (Vol. 12, pp. 991–1045). Annual Reviews Inc. https://doi.org/10.1146/annurev.iy.12.040194.005015

McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., Klemsz, M., Feeney, A. J., Wu, G. E., Paige, C. J., & Maki, R. A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO Journal*, *15*(20), 5647–5658. https://doi.org/10.1002/j.1460-2075.1996.tb00949.x

Melchers, F. (2015). Checkpoints that control B cell development. *Journal of Clinical Investigation*, *125*(6), 2203–2210. https://doi.org/10.1172/JCI78083

Minderjahn, J., Schmidt, A., Fuchs, A., Schill, R., Raithel, J., Babina, M., Schmidl, C., Gebhard, C., Schmidhofer, S., Mendes, K., Ratermann, A., Glatz, D., Nützel, M., Edinger, M., Hoffman, P., Spang, R., Längst, G., Imhof, A., & Rehli, M. (2020). Mechanisms governing the pioneering and redistribution capabilities of the non-classical pioneer PU.1. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-019-13960-2

Miura, Y., Morooka, M., Sax, N., Roychoudhuri, R., Itoh-Nakadai, A., Brydun, A., Funayama, R., Nakayama, K., Satomi, S., Matsumoto, M., Igarashi, K., & Muto, A. (2018). Bach2 Promotes B Cell Receptor–Induced Proliferation of B Lymphocytes and Represses Cyclin-Dependent Kinase Inhibitors. *The Journal of Immunology*, *200*(8), 2882–2893. https://doi.org/10.4049/jimmunol.1601863

Monticelli, S., & Natoli, G. (2017). Transcriptional determination and functional specificity of myeloid cells: Making sense of diversity. In *Nature Reviews Immunology* (Vol. 17, Issue 10, pp. 595–607). Nature Publishing Group. https://doi.org/10.1038/nri.2017.51

Muljo, S. A., & Schlissel, M. S. (2003). A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines. In *Nature Immunology* (Vol. 4, Issue 1, pp. 31–37). Nat Immunol. https://doi.org/10.1038/ni870

Muto, A., Ochiai, K., Kimura, Y., Itoh-Nakadai, A., Calame, K. L., Ikebe, D., Tashiro, S., & Igarashi, K. (2010). Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch. *EMBO Journal*, *29*(23), 4048–4061. https://doi.org/10.1038/emboj.2010.257

Muto, A., Tashiro, S., Nakajima, O., Hoshino, H., Takahashi, S., Sakoda, E., Ikebe, D., Yamamoto, M., & Igarashi, K. (2004). The transcriptional programme of antibody class switching involves the repressor Bach2. *Nature*, *429*(6991), 566–571. https://doi.org/10.1038/nature02596

Nagel, D., Vincendeau, M., Eitelhuber, A. C., & Krappmann, D. (2014). Mechanisms and consequences of constitutive NF-κB activation in B-cell lymphoid malignancies. In *Oncogene* (Vol. 33, Issue 50, pp. 5655–5665). Nature Publishing Group. https://doi.org/10.1038/onc.2013.565

Nerlov, C., Querfurth, E., Kulessa, H., & Graf, T. (2000). GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood*, *95*(8), 2543–2551.

Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M., & Corcoran, L. M. (2015). The generation of antibody-secreting plasma cells. *Nature Reviews Immunology*, *15*(3), 160–171. https://doi.org/10.1038/nri3795

Ochodnicka-Mackovicova, K., Bahjat, M., Maas, C., van der Veen, A., Bloedjes, T. A., de Bruin, A. M., van Andel, H., Schrader, C. E., Hendriks, R. W., Verhoeyen, E., Bende, R. J., van Noesel, C. J. M., & Guikema, J. E. J. (2016). The DNA Damage Response Regulates RAG1/2 Expression in Pre–B Cells through ATM-FOXO1 Signaling. *The Journal of Immunology*, *197*(7), 2918–2929. https://doi.org/10.4049/jimmunol.1501989

Ohno, S. (1979). *Major Sex-Determining Genes* (Vol. 11). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-81261-3

Oikawa, T., & Yamada, T. (2003). Molecular biology of the Ets family of transcription factors. In *Gene* (Vol. 303, Issues 1–2, pp. 11–34). Elsevier. https://doi.org/10.1016/S0378-1119(02)01156-3

Oliver, A. M., Martin, F., & Kearney, J. F. (1999). IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *Journal of Immunology (Baltimore, Md. : 1950), 162*(12), 7198–7207.

Orf, K., & Cunnington, A. J. (2015). Infection-related hemolysis and susceptibility to Gram-negative bacterial co-infection. In *Frontiers in Microbiology* (Vol. 6, Issue JUN, p. 666). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2015.00666

Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., & Igarashi, K. (1996). Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Molecular and Cellular Biology*, *16*(11), 6083–6095. https://doi.org/10.1128/mcb.16.11.6083

Pal, R., Monaghan, S. A., Hassett, A. C., Mapara, M. Y., Schafer, P., Roodman, G. D., Ragni, M. V., Moscinski, L., List, A., & Lentzsch, S. (2010). Immunomodulatory derivatives induce PU.1 down-regulation, myeloid maturation arrest, and neutropenia. *Blood*, *115*(3), 605–614. https://doi.org/10.1182/blood-2009-05-221077

Palm, A. K. E., & Henry, C. (2019). Remembrance of Things Past: Long-Term B Cell Memory After Infection and Vaccination. In *Frontiers in immunology* (Vol. 10, p. 1787). NLM (Medline). https://doi.org/10.3389/fimmu.2019.01787

Pelanda, R., & Torres, R. M. (2012). Central B-Cell tolerance: Where selection begins. *Cold Spring Harbor Perspectives in Biology*, *4*(4). https://doi.org/10.1101/cshperspect.a007146

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H., & Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, *349*(6306), 257–260. https://doi.org/10.1038/349257a0

Pfaffl, M. W. (2004). *Quantification strategies in real-time PCR.*

Pillai, S., & Cariappa, A. (2009). The follicular versus marginal zone B lymphocyte cell fate decision. In *Nature Reviews Immunology* (Vol. 9, Issue 11, pp. 767–777). Nature Publishing Group.

https://doi.org/10.1038/nri2656

Polli, M., Dakic, A., Light, A., Wu, L., Tarlinton, D. M., & Nutt, S. L. (2005). The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood*, *106*(6), 2083–2090. https://doi.org/10.1182/blood-2005-01-0283

Pradeu, T., & Cooper, E. L. (2012). The danger theory: 20 years later. *Frontiers in Immunology*, *3*(SEP). https://doi.org/10.3389/fimmu.2012.00287

Raczkowski, H. L., & DeKoter, R. P. (2021). Lineage-instructive functions of the E26 - transformation-specific-family transcription factor Spi-C in immune cell development and disease . *WIREs Mechanisms of Disease*. https://doi.org/10.1002/wsbm.1519

Rekhtman, N., Radparvar, F., Evans, T., & Skoultchi, A. I. (1999). Direct interaction of hematopoietic transcription factors PU.1 and GATA- 1: Functional antagonism in erythroid cells. *Genes and Development*, *13*(11), 1398–1411. https://doi.org/10.1101/gad.13.11.1398

Rhee, J., Solomon, L. A., & DeKoter, R. P. (2019). A role for ATP Citrate Lyase in cell cycle regulation during myeloid differentiation. *Blood Cells, Molecules, and Diseases*, *76*, 82–90. https://doi.org/10.1016/j.bcmd.2019.02.006

Roco, J. A., Mesin, L., Binder, S. C., Meyer-Hermann, M., Victora, G. D., Correspondence, C. G. V., Nefzger, C., Gonzalez-Figueroa, P., Canete, P. F., Ellyard, J., Shen, Q., Robert, P. A., Cappello, J., Vohra, H., Zhang, Y., Nowosad, C. R., Schiepers, A., Corcoran, L. M., Toellner, K.-M., … Vinuesa, C. G. (2019). Class-Switch Recombination Occurs Infrequently in Germinal Centers Article Class-Switch Recombination Occurs Infrequently in Germinal Centers. *Immunity*, *51*, 337-350.e7. https://doi.org/10.1016/j.immuni.2019.07.001

Sakai, J., Cammarota, E., Wright, J. A., Cicuta, P., Gottschalk, R. A., Li, N., Fraser, I. D. C., & Bryant, C. E. (2017). Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-01600-y

Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M., & Peng, A. (1993). Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'- phosphorylated, RAG-dependent, and cell cycle regulated. *Genes and Development*, *7*(12 B), 2520–2532. https://doi.org/10.1101/gad.7.12b.2520

Schweitzer, B. L., Huang, K. J., Kamath, M. B., Emelyanov, A. V., Birshtein, B. K., & DeKoter, R. P. (2006). Spi-C Has Opposing Effects to PU.1 on Gene Expression in Progenitor B Cells. *The*

Journal of Immunology, *177*(4), 2195–2207. https://doi.org/10.4049/jimmunol.177.4.2195

Scott, E. W., Simon, M. C., Anastasi, J., & Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, *265*(5178), 1573–1577. https://doi.org/10.1126/science.8079170

Sharif, O., Bolshakov, V. N., Raines, S., Newham, P., & Perkins, N. D. (2007). Transcriptional profiling of the LPS induced NF-κB response in macrophages. *BMC Immunology*, *8*(1), 1–17. https://doi.org/10.1186/1471-2172-8-1

Sharrocks, A. D. (2001). The ETS-domain transcription factor family. *Nature Reviews Molecular Cell Biology*, *2*(11), 827–837. https://doi.org/10.1038/35099076

Shi, W., Liao, Y., Willis, S. N., Taubenheim, N., Inouye, M., Tarlinton, D. M., Smyth, G. K., Hodgkin, P. D., Nutt, S. L., & Corcoran, L. M. (2015). Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nature Immunology*, *16*(6), 663–673. https://doi.org/10.1038/ni.3154

Shinnakasu, R., Inoue, T., Kometani, K., Moriyama, S., Adachi, Y., Nakayama, M., Takahashi, Y., Fukuyama, H., Okada, T., & Kurosaki, T. (2016). Regulated selection of germinal-center cells into the memory B cell compartment. *Nature Immunology*, *17*(7), 861–869. https://doi.org/10.1038/ni.3460

Shinnakasu, R., & Kurosaki, T. (2017). Regulation of memory B and plasma cell differentiation. In *Current Opinion in Immunology* (Vol. 45, pp. 126–131). Elsevier Ltd. https://doi.org/10.1016/j.coi.2017.03.003

Sims, G. P., Ettinger, R., Shirota, Y., Yarboro, C. H., Illei, G. G., & Lipsky, P. E. (2005). Identification and characterization of circulating human transitional B cells. *Blood*, *105*(11), 4390–4398. https://doi.org/10.1182/blood-2004-11-4284

Smulski, C. R., & Eibel, H. (2018). BAFF and BAFF-receptor in B cell selection and survival. In *Frontiers in Immunology* (Vol. 9, Issue OCT, p. 2285). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.02285

Solomon, L. A., Li, S. K. H., Piskorz, J., Xu, L. S., & DeKoter, R. P. (2015). Genome-wide comparison of PU.1 and Spi-B binding sites in a mouse B lymphoma cell line. *BMC Genomics*, *16*(1). https://doi.org/10.1186/s12864-015-1303-0

Soodgupta, D., White, L. S., Yang, W., Johnston, R., Andrews, J. M., Kohyama, M., Murphy, K. M., Mosammaparast, N., Payton, J. E., & Bednarski, J. J. (2019). RAG-Mediated DNA Breaks

Attenuate PU.1 Activity in Early B Cells through Activation of a SPIC-BCLAF1 Complex. *Cell Reports*, *29*(4), 829-843.e5. https://doi.org/10.1016/j.celrep.2019.09.026

Spriano, F., Chung, E. Y. L., Gaudio, E., Tarantelli, C., Cascione, L., Napoli, S., Jessen, K., Carrassa, L., Priebe, V., Sartori, G., Graham, G., Selvanathan, S. P., Cavalli, A., Rinaldi, A., Kwee, I., Testoni, M., Genini, D., Ye, B. H., Zucca, E., … Bertoni, F. (2019). The ETS inhibitors YK-4-279 and TK-216 are novel antilymphoma agents. *Clinical Cancer Research*, *25*(16), 5167–5176. https://doi.org/10.1158/1078-0432.CCR-18-2718

Street, K., Risso, D., Fletcher, R. B., Das, D., Ngai, J., Yosef, N., Purdom, E., & Dudoit, S. (2018). Slingshot: cell lineage and pseudotime inference for single-cell transcriptomics. *BMC Genomics*, *19*(1), 477. https://doi.org/10.1186/s12864-018-4772-0

Swaminathan, S., Huang, C., Geng, H., Chen, Z., Harvey, R., Kang, H., Ng, C., Titz, B., Hurtz, C., Sadiyah, M. F., Nowak, D., Thoennissen, G. B., Rand, V., Graeber, T. G., Koeffler, H. P., Carroll, W. L., Willman, C. L., Hall, A. G., Igarashi, K., … Müschen, M. (2013). BACH2 mediates negative selection and p53-dependent tumor suppression at the pre-B cell receptor checkpoint. *Nature Medicine*, *19*(8), 1014–1022. https://doi.org/10.1038/nm.3247

Takemori, T., Kaji, T., Takahashi, Y., Shimoda, M., & Rajewsky, K. (2014). Generation of memory B cells inside and outside germinal centers. In *European Journal of Immunology* (Vol. 44, Issue 5, pp. 1258–1264). Wiley-VCH Verlag. https://doi.org/10.1002/eji.201343716

Turkistany, S. A., & Dekoter, R. P. (2011). The transcription factor PU.1 is a critical regulator of cellular communication in the immune system. In *Archivum Immunologiae et Therapiae Experimentalis* (Vol. 59, Issue 6, pp. 431–440). https://doi.org/10.1007/s00005-011-0147-9

Vadiveloo, P. K., Keramidaris, E., Morrison, W. A., & Stewart, A. G. (2001). Lipopolysaccharide-induced cell cycle arrest in macrophages occurs independently of nitric oxide synthase II induction. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1539*(1–2), 140–146. https://doi.org/10.1016/S0167-4889(01)00102-1

Voehringer, D., van Rooijen, N., & Locksley, R. M. (2007). Eosinophils develop in distinct stages and are recruited to peripheral sites by alternatively activated macrophages. *Journal of Leukocyte Biology*, *81*(6), 1434–1444. https://doi.org/10.1189/jlb.1106686

Wang, A., Pope, S. D., Weinstein, J. S., Yu, S., Zhang, C., Booth, C. J., & Medzhitov, R. (2019). Specific sequences of infectious challenge lead to secondary hemophagocytic lymphohistiocytosis-like disease in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(6), 2200–2209. https://doi.org/10.1073/pnas.1820704116

Watanabe-Matsui, M., Matsumoto, T., Matsui, T., Ikeda-Saito, M., Muto, A., Murayama, K., & Igarashi, K. (2015). Heme binds to an intrinsically disordered region of Bach2 and alters its conformation. *Archives of Biochemistry and Biophysics*, *565*, 25–31. https://doi.org/10.1016/j.abb.2014.11.005

Watanabe-Matsui, M., Muto, A., Matsui, T., Itoh-Nakadai, A., Nakajima, O., Murayama, K., Yamamoto, M., Ikeda-Saito, M., & Igarashi, K. (2011). Heme regulates B-cell differentiation, antibody class switch, and heme oxygenase-1 expression in B cells as a ligand of Bach2. *Blood*, *117*(20), 5438–5448. https://doi.org/10.1182/blood-2010-07-296483

Wontakal, S. N., Guo, X., Smith, C., MacCarthy, T., Bresnick, E. H., Bergman, A., Snyder, M. P., Weissman, S. M., Zheng, D., & Skoultchi, A. I. (2012). A core erythroid transcriptional network is repressed by a master regulator of myelo-lymphoid differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(10), 3832–3837. https://doi.org/10.1073/pnas.1121019109

Wortis, H. H., Teutsch, M., Higer, M., Zheng, J., & Parker, D. C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(8), 3348–3352. https://doi.org/10.1073/pnas.92.8.3348

Xu, H., Liew, L. N., Kuo, I. C., Huang, C. H., Goh, D. L. M., & Chua, K. Y. (2008). The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology*, *125*(2), 218–228. https://doi.org/10.1111/j.1365-2567.2008.02832.x

Ye, M., Ermakova, O., & Graf, T. (2005). PU.1 is not strictly required for B cell development and its absence induces a B-2 to B-1 cell switch. *Journal of Experimental Medicine*, *202*(10), 1411–1422. https://doi.org/10.1084/jem.20051089

Zaret, K. S., Watts, J., Xu, J., Wandzioch, E., Smale, S. T., & Sekiya, T. (2008). Pioneer factors, genetic competence, and inductive signaling: Programming liver and pancreas progenitors from the endoderm. *Cold Spring Harbor Symposia on Quantitative Biology*, *73*, 119–126. https://doi.org/10.1101/sqb.2008.73.040

Zhang, P., Zhang, X., Iwama, A., Yu, C., Smith, K. A., Mueller, B. U., Narravula, S., Torbett, B. E., Orkin, S. H., & Tenen, D. G. (2000). PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood*, *96*(8), 2641–2648. https://doi.org/10.1182/blood.V96.8.2641

Zhou, Y., Zhang, Y., Han, J., Yang, M., Zhu, J., & Jin, T. (2020). Transitional B cells involved in

autoimmunity and their impact on neuroimmunological diseases. In *Journal of Translational Medicine* (Vol. 18, Issue 1, p. 131). BioMed Central Ltd. https://doi.org/10.1186/s12967-020-02289-w

Zhu, X., Schweitzer, B. L., Romer, E. J., Sulentic, C. E. W., & DeKoter, R. P. (2008). Transgenic expression of Spi-C impairs B-cell development and function by affecting genes associated with BCR signaling. *European Journal of Immunology*, *38*(9), 2587–2599. https://doi.org/10.1002/eji.200838323

Curriculum Vitae – Hannah Lily Raczkowski

EDUCATION

Master of Science (MSc) in Microbiology and Immunology 2019-2021
- Collaborative Specialization in Developmental Biology
- Western University, London, Ontario, Canada
- Thesis Supervisor: Dr. Rodney P. DeKoter

Honors Bachelor of Medical Sciences (BMSc) 2015-2019
- Honors Specialization in Microbiology and Immunology
- Western University, London, Ontario, Canada

TEACHING EXPERIENCE

Graduate Teaching Assistant, MICROIMM 3620G "Immunology Laboratory" 2020, 2021
Western University, London, ON

PUBLICATIONS

Raczkowski, H.L., DeKoter, R.P. Lineage-instructive functions of the E26-transformation-specific-family transcription factor Spi-C in immune cell development and disease. WIREs Mech Dis. 2021;e1519.

Laramée, A. S., Raczkowski, H.L, Shao, P., Batista, C., Shukla, D., Xu, L., Haeryfar, S., Tesfagiorgis, Y., Kerfoot, S., & DeKoter, R. (2020). Opposing Roles for the Related ETS-Family Transcription Factors Spi-B and Spi-C in Regulating B Cell Differentiation and Function. Frontiers in immunology, 11, 841.

HONOURS AND AWARDS

- Silver Tier Poster Award, Canadian Society for Immunology Annual Conference ($300) 2021
- Second Place Basic Science Talk Award, Child Health Research Day 2020 ($100) 2021
- Dr. Frederick W. Luney Travel Award in Microbiology & Immunology ($80) 2021
- First Place Basic Science Poster Award, Child Health Research Day 2020 ($200) 2020
- Dr. Frederick W. Luney Graduate Entrance Scholarship in Microbiology & Immunology, Western University ($2,000) 2019
- Dean's Honours List, Western University 2017-2019
- Continuing Admission Scholarship, Western University ($10,000) 2015

CONFERENCE PRESENTATIONS

2021 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Oral and Poster Presentations. Canadian Society for Immunology Annual Conference. Victoria, BC (Virtual).

2021 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Oral Presentation. Child Health Research Day. London, ON (Virtual).

2021 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Poster Presentation. London Health Research Day. London, ON (Virtual).

2021 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Oral Presentation. Beyond Sciences Initiative Annual Conference. Toronto, ON (Virtual).

2020 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Oral Presentation. Infection and Immunity Research Forum. London, ON (Virtual).

2020 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Poster Presentation. Child Health Research Day. London, ON (Virtual).

2020 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Poster Presentation. London Health Research Day. London, ON. Conference Canceled due to COVID-19 pandemic.

2020 Hannah L. Raczkowski & Rodney P. DeKoter. *Spi-C is dynamically regulated by external signals in B cells*. Poster Presentation. Western Research Forum. London, ON. Conference Canceled due to COVID-19 pandemic.

2019 Hannah L. Raczkowski, Anne-Sophie Laramée, Li S. Xu, & Rodney P. DeKoter. *Opposing roles of transcription factors Spi-B and Spi-C in B cell differentiation*. Poster Presentation. Infection and Immunity Research Forum. London, ON.

2019 Hannah L. Raczkowski, Anne-Sophie Laramée, Li S. Xu, & Rodney P. DeKoter. *Role of Spi-B and Spi-C in B Cell Differentiation*. Oral Presentation. Ontario-Quebec Undergraduate Immunology Conference. Toronto, ON.