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MicroRNA regulation of B cell receptor signaling

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Summary

B lymphocytes play a central role in host immune defense. B cell receptor (BCR) signaling regulates survival, proliferation and differentiation of B lymphocytes. Signaling through the BCR signalosome is a multi-component cascade that is tightly regulated and is important in the coordination of B cell differentiation and function. At different stages of development, B cells that have BCRs recognizing self are eliminated to prevent autoimmunity. microRNAs (miRNAs) are small single-stranded non-coding RNAs that contribute to post-transcriptional regulation of gene expression and have been shown to orchestrate cell fate decisions through the regulation of lineage specific transcriptional profiles. Studies have identified miRNAs to be crucial for B cell development in the bone marrow and their subsequent population of the peripheral immune system. In this review, we focus on the role of miRNAs in the regulation of BCR signaling as it pertains to B lymphocyte development and function. In particular, we discuss the most recent studies describing the role of miRNAs in the regulation of both early B cell development and peripheral B cell responses and examine the ways by which miRNAs regulate signals downstream of B cell antigen receptor to prevent aberrant activation and autoimmunity.

Keywords

miRNA; BCR; PI3K; AKT; MAPK; NF-κB

Section 1: Introduction

microRNAs

microRNAs (miRNAs) are evolutionarily conserved, small non-coding RNAs (ncRNAs) that are crucial in the regulation of a myriad of cellular functions via RNA interference (RNAi). In hematopoietic cell lines, multiple studies describe the impact of miRNAs on lineage determination and maturation.[1,2] Likewise, because miRNAs regulate many important biological pathways in lymphocytes, their deregulation has been implicated in malignant disease and autoimmunity.[3] Transcribed in the nucleus by RNA polymerase II, miRNAs undergo processing by the microprocessor (Drosha and DGCR8) to cleave the double-stranded hairpin primary miRNA into shorter stem-loop precursor miRNAs (pre-miRNAs). In the cytoplasm, Dicer, an RNaseIII enzyme that cleaves double-stranded

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RNA, further cuts the pre-miRNAs into 20–23 nucleotide duplexes. Mature miRNAs are incorporated into the RNA induced silencing complex and guide the translational repression or degradation of mRNA target genes via miRNA-dependent recognition of 3'untranslated regions (3'UTRs).[1,2,4,5]

B cell development

B lymphocyte development and maturation is a tightly orchestrated process during which progenitor cells generate unique antigen receptors following rearrangement of immunoglobulin heavy chain (IgH) and light chain (IgL) loci.[6] After the induction of critical transcription factors (E2A, EBF, and Pax5), the bone marrow-resident common lymphoid progenitors (CLP) commit to the B cell lineage and enter the pro-B cell stage.[7] Here, the enzymatic activity of recombination activating genes 1 and 2 (RAG1 and RAG2) induce V(D)J recombination at the IgH locus.[8,9] Successful rearrangement of the IgH locus allows for the progression of the B cells to the large pre-B cell stage where the IgH pairs with a surrogate light chain and is presented on the cell surface as a pre-B cell receptor (pre-BCR).[8] Proper signaling through the pre-BCR ensures that the recombinatorial events result in a functional IgH which then leads to the downregulation of RAG1/2 and the initiation of rapid proliferation. The pre-B cell exits the cell cycle as a small pre-B cell and a second wave of RAG1/2 expression promotes VJ recombination at the IgL loci.[8,10,11] Two molecules of the IgL associate with two molecules of the IgH to form the B cell receptor (BCR) on the cell surface of immature B lymphocytes. It is the combination of the heavy and light chains that confers BCR specificity for a cognate antigen. This process also has the potential to generate BCRs with self-reactive specificities. Immature B cells with newly minted BCRs undergo negative selection to eliminate self-reactive lymphocytes. If the membrane-bound BCR reacts strongly to a self-antigen, the immature B cell either internalizes the BCR, decreasing signaling and increasing RAG1/2 mediated light chain editing, or undergoes clonal deletion.[11,12] Basal tonic signaling through the BCR of the immature B cell restricts RAG1/2 expression and is critical for the maintenance of immature B lymphocytes as they exit the bone marrow to mature in the periphery.[13] Throughout this carefully coordinated developmental process in the bone marrow, signaling through the pre-BCR and, in later stages, mature BCR governs the survival and proliferation of B lymphocytes and the B cell antigen receptor continues to play a central role in peripheral B cells.

Peripheral immature B cells, often referred to as transitional B cells, undergo further negative selection wherein cells that receive a strong signal via the BCR in the absence of T cell help or cytokine signals undergo apoptosis or become anergic (enter a state of hypo-responsiveness).[14,15] Amplitude of BCR signaling, cytokine stimulation via B cell activating factor (BAFF), and engagement of NOTCH2 and BTK influence which compartment of maturity the B cell will enter: marginal zone (MZ) or follicular (FO) B cells.[16–19] FO B cells, which constitute the majority of mature B lymphocytes, reside in follicles within secondary lymphoid organs adjacent to T cell zones and are quickly engaged in response to T cell-dependent antigens. MZ B cells are largely in the white pulp of the spleen and have intrinsic properties that ensure rapid response to blood borne pathogens in a T-independent manner – leading to the generation of plasma cells that secrete natural, polyreactive IgM antibodies (MZ and FO B cell fate is reviewed in Pillai et al. 2009).[18] FO B cells form

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from precursors with stronger BCR signaling and they are dependent on the BCR-dependent induction of BTK.[18,20] Conversely, weaker signaling through the BCR and the induction of NOTCH2 favors the development of MZ B cells.[20]

Upon engagement of its B cell antigen receptor, FO B cells either differentiate into antibody-producing plasma cells or enter specialized microstructures, called germinal centers (GCs) – a fate decision thought to be primarily governed by the strength of BCR signal in response to cognate antigen. Naïve, mature FO B cells that initially respond strongly to their cognate antigen differentiate into plasma cells.[21] Weaker BCR signaling promotes GC entry where the Ig loci that encode the antibody undergo somatic hypermutation to optimize the binding affinity of the BCR and where class switch recombination (CSR) that leads to a change in the antibody isotype takes place.[21,22] Within GCs, B lymphocytes undergo rapid proliferation and a subsequent step of Darwinian-like selection which is driven by competition for limited antigen.[23] Both of these processes require activation-induced cytidine deaminase (AID).[21] GC B cells that outcompete other B cell clones through stronger recognition of the cognate antigen receive proliferative and differentiation signals from T follicular helper cells (T_{FH}) cells. This triggers the induction of transcriptional programs which determine whether the B cell will differentiate into a plasma cell or become a memory B cell.[24] As a plasma cell, the BCR heavy chain is alternatively spliced and secreted as an antibody.[25] Because so many cell fate decisions throughout B cell development are governed by BCR signal strength, it is critical to understand how miRNAs fine-tune this signaling cascade.

Components of the BCR signalosome

The BCR is a type-I transmembrane immunoglobulin that has two heavy chains and two light chains. Associated with the immunoglobulin is an Igα/Igβ heterodimer that, along with co-receptors, is essential for the intracellular signaling of the BCR.[8] After binding of the antigen to the BCR, the cytoplasmic tail of the Igα/Igβ heterodimer and co-receptors are phosphorylated by Lyn kinase.[26–28] These events lead to the recruitment of multiple kinases and scaffolding proteins to the inner side of the plasma membrane and form the BCR signalosome.[29] Syk is the principle kinase that activates many of the proteins recruited to the inner membrane through the actions of Lyn.[30] CD19 co-receptor and BTK promote the aggregation of BCR molecules into microclusters on the plasma membrane, which further amplify B cell activation.[31] Signaling through the BCR is critical for the maintenance of mature B cells, and stage specific ablation of surface Ig or Igα/Igβ signaling subunit results in apoptosis of B cells.[16,32] Downstream of the BCR signalosome there are three key signaling pathways that activate following BCR engagement: the NF-κB pathway, the RAS-MAPK pathway, and the PI3K-AKT pathway (Figure 1). The cross talk between these three signaling pathways adds to the complexity of BCR signaling. The brief overview of signal transduction through each of these three signaling pathways is critical for appreciating the myriad of nodes where miRNAs exert control over BCR signaling.

NF-κB: Activation of the canonical NF-κB pathway is an important survival signal downstream of BCR engagement. Prior to activation, NF-κB is initially bound to IκB in the cytoplasm which prevents its translocation into the nucleus. The kinases BTK and Syk,

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which are activated upon BCR stimulation, phosphorylate and activate Phospholipase C-γ2 (PLC-γ2) leading to the hydrolyzation of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to diaglycerol (DAG) and inositol triphosphate (IP3).[29,33] IP3 increases intracellular Ca^{2+} which, in combination with DAG, activates protein kinase-C (PKC). The activation of PKC and BTK in concert with other recruited factors phosphorylate and activate the inhibitor of IκB kinase (IKK) complex. The activation of IKK then phosphorylates IκB, leading to ubiquitin-mediated degradation of IκB. The unbound NF-κB then translocates to the nucleus to regulate genes responsible for cell proliferation and survival critical to B cell activation.[33,34]

ERK-MAPK: The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) is activated downstream of BCR signaling and is important for B cell proliferation and survival during development and differentiation. The ERK-MAPK pathway is primarily regulated by the G protein RAS. The inactive form of RAS has GDP bound, and guanine nucleotide exchange factors (GEFs) swap GDP for GTP to activate RAS and GTPase-activating proteins (GAPs) deactivate RAS.[35] RAS-GTP activates Raf-1/B-Raf which activates MEK1/2 and ERK which translocate to the nucleus, resulting in the activation of a number of transcription factors (Fos, Jun, Ets) which are involved in cell proliferation and survival.[29,33]

PI3K: The phosphoinositide 3-kinase (PI3K) signaling cascade is essential for orchestration of B cell development and BCR signaling transduction.[36–40] PI3K is recruited to the plasma membrane by the BCR co-receptor, CD19, where it is phosphorylated by LYN, leading to the conversion of phosphatidylinositol (4,5)-biphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3).[33,41,42] AKT and phosphoinositide-dependent protein kinase1 (PDK1) are recruited to the plasma membrane by PIP_3 where AKT is phosphorylated by PDK1 and mTOR. The phosphorylation of AKT in turn, leads to a variety of critical pro-survival, growth, proliferation, and anabolic pathways including the regulation of FOXO1/3, c-Myc, NF-kB, and NFAT.[29,33,43–46] In addition to the fact that antigen engagement of the BCR leads to robust PI3K activation, low amplitude PI3K “tonic” signal is essential for the survival of B lymphocytes – blunting of this tonic PI3K signal results in apoptosis of B cells.[23,47]

Tight regulation of these three pathways is necessary for proper development, as inappropriate BCR signaling can result in malignant transformation and the development of auto-reactive B cells.[48] Among the most potent regulators of BCR signaling are those modulators that prevent the formation of the signalosome. The factors FcγRIIb and SHIP work synergistically to prevent the recruitment of PLC-γ2, BTK and AKT.[49] Lyn, an important kinase in the propagation of BCR signaling, also has a negative regulatory role in B cell activation through its activation of both SHIP and FcγRIIb. In addition to these negative BCR modulators, each of the three pathways discussed above have additional regulatory molecules involved in mitigating signal strength. As discussed below, miRNA-dependent post-transcriptional regulation of the components of these signaling networks is critical to the proper attenuation of BCR signaling.

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In this review, we will focus on miRNA-dependent regulation of BCR signaling as the B cell progresses through various stages of B cell development and discuss its implications for the development of autoimmunity. There are a number of miRNAs that have been implicated in B cell lineage commitment and B cell malignant transformation – these studies are beyond the scope of this review and have been previously reviewed.[12,50–55]

Section 2: miRNAs in early BCR development and signaling

Early BCR Development

Analysis of a murine model where *Dicer1* is selectively ablated in B lymphocytes via *mb1*-cre, highlights the importance of ncRNAs in early stages of B cell development. Analysis of B cell hematopoeisis in these mice reveals a block in the pro- to pre-B cell transition marked by a significant increase in apoptosis of Dicer-deficient pre-B cells and a near-complete loss of peripheral B lymphocytes in secondary lymphoid organs.[56] *Mb1*-cre mediated deletion of *Dgcr8* or *Drosha* in B cells recapitulates these findings indicating that miRNAs are essential for coordination of early B cell development.[57,58] In all three mutant mouse models, the proapoptotic protein BIM was significantly upregulated.[56–58] Indeed, *Bim* has conserved miRNA binding sites in its 3'UTR implicating miRNAs in supressing its expression.[56,59] Intriguingly, overexpression of the anti-apoptotic Bcl-2 transgene in miRNA-deficient B cells resulted in only partial rescue (~12%) of B cell development, suggesting that miRNAs contribute to regulation of other critical pathways during B cell differentiation.[56,58]

A number of these early studies which looked at global miRNA ablation in the hematopoeitic lineage, and later work using conditional ablation of individual miRNA clusters, implicate miRNAs in the coordination of pro- to pre-B cell transition. Differential gene expression analysis in immortalized pro-B cells in which *Dicer* is inducibly ablated reveals upregulation of gene transcripts with 3'UTRs that have a miR-17~92 binding site.[56] Furthermore, conditional deletion of miR-17~92 in B cells leads to a similar phenotype as described above, including a block in pro- to pre-B lymphocyte transition with a concominant increase in apoptosis in the setting of dysregulated BIM and PI3K signaling (discussed in more detail below).[59,60] The authors attributed this block in the pro- to pre-B cell transition to miR-17, because *in vitro* expression of miR-17 alone in HSCs from these mice was sufficient to rescue B cell development.[60] Interestingly, ablation of miR-17~92 cluster along with the two paralogue clusters, miR-106a~363 and miR-16b~25, recapitulates the pro- to pre-B cell block even in the absence of BIM dysregulation. This observation suggests that BIM is not the target responsible for the block in B cell development in mice lacking these miRNA clusters and that *Bim* may not be a physiologically relevant target of miR-17~92 in B cell progenitors. Moreover, a recent study utilizing a hematopoietic lineage-specific cre to mutate the miR-17~92 binding sites within the 3'UTR of *Bim* only showed a modest increase in BIM protein, with minimal impact on B cell development.[61] Furthermore, this developmental block in mice where miR-17~92 was ablated in B cells could not be rescued by a Bcl2-transgene (Tg).[60] Collectively, these findings suggest that miRNA likely contribute to the regulation of additional targets and molecular pathways apart from *Bim* during B cell development that remain to be elucidated.

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Inappropriate overexpression of miRNAs can also be detrimental to B cell development. miR-150 and miR-34 are both maintained at low levels throughout early B cell development with miR-150 increasing as the B cell matures.[62] Overexpression of miR-150 results in a downregulation of transcription factor *c-Myb*,[63,64] which regulates early B cell development.[65] Indeed, through a series of loss-of and gain-of-function experiments Xiao et al showed that upregulation of *c-Myb* was responsible for the observed failure in appropriate B cell differentiation upon miR-150 overexpression.[63] Similarly, gene targeting experiments have demonstrated that constitutitve expression of miR-34, a miRNA normally induced by p53, leads to ihibition of Foxp1 and a subsequent block in B cell development.[66] These findings highlight the importance of appropriate temporal expression of individual miRNAs and the presented rescue experiments support the fact that it is often a few crtical targets of any individual miRNA that are responsible for regulatory function of these ncRNAs in any given cellular context.

As previously discussed, successful rearrangement of heavy and light chains, at pro- and pre-B cell stages respectively, is essential for assembly of the BCR which supports B cell survival and differentiation. While the basic mechanisms of V(D)J recombination did not appear to be perturbed in miRNA-deficient B cells, repertoire analyses have revealed that Dicer-dependent ncRNAs do influence BCR repertoire diversity and that while Drosha/DGCR8 miRNAs did not appear to have impact on Ig repertoire, loss of miRNAs were shown to impact the expression level of antigen receptor on B cells.[56,58] Amplifying the IgH loci of *Dicer*-deficient B cells revealed a change in D_H element usage compared with controls.[56] Additionally, sequence analysis of light chain rearrangements have revealed an increase in the frequency of non-template N nucleotides that corresponded with a prolonged expression of the enzyme TdT throughout the maturation of Dicer KO B cells.[56] Consistent with this, a study by Ventura et al. (2008) observed less efficient V_H-to-DJ_H rearrangement in miR-17~92 null pro-B cells compared with controls, however the degree of dysregulation was not reported and further analysis is needed to elucidate a mechanism.[59] Furthermore, ablation of either *Dicer, Dgcr8* or *Drosha* in B cells has been found to reduce levels of BCR expression and analysis of mature B cells deficient in miRNAs revealed ongoing BCR editing even in peripheral lymphocytes as a consequence of dysregulated PI3K signaling (discussed in more detail below).[57,58] Strikingly, while a transgene encoding a fully rearranged IgH and IgL (IgHEL) led to only partial (~5%) rescue of B cell development, a combination of IgHEL-Tg and Bcl2-Tg was sufficient to fully rescue B cell development in the absence of miRNAs.[56,58]

Taken together, these findings suggest a central role for miRNAs in choreographing early B cell development, and regulation of signaling pathways critical for B cell survival. In particular, while the review below is on the role of miRNAs in the regulation of signal transcduction downstream of the BCR, ncRNAs also appear to contribute to the assembly of a functional repertoire of B lymphocytes.

PI3K-AKT Pathway

PI3K signaling regulates B cell development and survival and is critical for the coordination of rerrangement events in the bone marrow.[36] Pre-BCR and BCR induced PI3K signaling

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during B cell development provides survival signals and contributes to downregulation of RAG1/2 enzymes leading to the termination of further V(D)J rearrangement.[8,10,11,36] In addition to the regulation of survival and rearrangmeent, PI3K-dependent signaling is also required for cell cycle progression and proliferation of developing B lymphocytes.[10,11]

The PI3K-AKT pathway has been studied in various miRNA-deficient mouse models. An analysis miRNA-deficient B cells that were resuced with a Bcl2-Tg from animals in which *Dicer*, *Drosha*, or *Dgcr8* were selectively deleted revealed that developing B cells have an increase in the protein levels of the PI3K negative regulator, phosphatase and tensin homolog (PTEN).[56,58] The 3' UTR of PTEN contains numerous miRNA binding sites and PTEN dysregulation in these miRNA-deficient B cells leads to impaired PI3K signaling, a reduction in AKT activation and a corresponding increase in FOXO1 and RAG1/2 expression. Compromised PI3K signaling was also found to persist in peripheral lymhocytes devoid of microprocessor enzymes, such that ongoing light chain editing continued in the peripheral B cells from secondary lymphoid organs of these animals.[58]

As previously described, developing Dicer and microprocessor deficient B cells exhibited an increase in apoptosis that was initially attributed to direct miRNA dependent dysregulation of BIM protein. However, as BIM is negatively regulated by the PI3K pathway and the ablation of miR-17~92:Bim interactions led to only a modest increase in BIM protein without dysregulation of B cell development, its upregulation in the setting of increased PTEN expression offers an alternative explanation.[61] B cells in which miR-17~92 was genetically ablated have also been reported to have an increase in BIM protein in the setting of the upregulation of two PI3K negative regulators, PTEN and Phlpp2.[60] However, while PTEN has been confirmed as a direct target of the miR-17~92 family, overexpression of these miRNAs fails to reduce the *Phlpp2* reporter activity in renilla-luciferase assays.[60] Furthermore, *ex vivo* DRAQ5 analysis of miRNA-null pre-B cells reveals a decrease in proliferation in the setting of increased PTEN expression, suggesting that PI3K-dependent proliferation of B lymphocytes is also impacted upon upregulation of this negative regulator of the pathway. Collectively, these studies highlight the importance of miRNA regulation of the PTEN-PI3K pathway throughout B cell development.

Section 3: miRNAs in central tolerance and autoimmunity

It is estimated that 50 to 75% of B lymphocytes produced following V(D)J recombination in the bone marrow are autoreactive, but only 3 to 8% of the population develop evident autoimmune disease.[67,68] As briefly discussed above, through negative selection, tightly regulated checkpoints ensure that most autoreactive immature B cells are prevented from joining the mature B cell compartment by receptor editing, apoptosis, or anergy.[69] Newly minted BCRs in the bone marrow are tested for autoreactivity at the immature B cell stage during a selection and editing process called central tolerance. If a BCR engages with a self-antigen, leading to the activation of signaling pathways above a certain threshold, the immature B cell will either undergo clonal deletion or halt maturation, internalize the BCR and undergo additional light chain editing.[70–72]

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RAS-MAPK Pathway

The role of MAPK signaling in central tolerance remains to be fully elucidated. Increased MAPK signaling has been described in B cells from both lupus patients and mouse models.[73–75] Furthermore, constitutively activated RAS signaling has been found to promote the differentiation of autoreactive immature B cells and lead to inceased autoantibody titers.[76,77] However, a recent study found that the direct activation of the MAPK pathway alone was insufficient to break central B cell tolerance and hypothesized that its activation in conjunction with other pathways are required.[78]

Using a functional retroviral screen to overexpress batches of miRNAs in transferred HSPCs, Gonzalez-Martin et al. (2016) identified 7 miRNAs that were enriched in B cells that evaded central tolerance in the IgM^b-macroself mouse model.[79] This mouse model ubiquitously expresses a membrane-bound superantigen that binds the IgM heavy chain constant region on the surface of immature B lymphocytes, thus mimicing self-recognition by B cells, leading to clonal deletion.[80] Although five of the seven miRNAs significantly broke IgM^b-macroself central tolerance in a transfer model upon transduction of HSPCs with each of these these miRNAs, overexpression of miR-148a resulted in the most significant break of central tolerance.[79] While this study focused specifically on miR-148a, a miRNA shown to compromise B cell tolerance and one that has been implicated in lupus pathogenesis, future studies are needed to fully investigate the importance of the other candidate miRNAs involved in the regulation of B cell central tolerance in this study (miR-26a, miR-26b, miR-342, miR 423 and miR-182).[79]

Indeed, miR-148a has been shown to be upregulated in a number of autoimmune diseases including lupus, multiple sclerosis, and asthma.[9,81,82] Furthermore, miR-148a was found to be highly expressed in pro- and pre-B cells and downregulated at the immature B cell stage where central tolerance occurs.[79] miR-148a was also found to be upregulated in human germinal center B cells and plasma cells, suggesting that expression of this miRNA is induced in response to B cell activation.[83] The autoimmune suppressor, Gadd45a, a target of p38/JNK pathway induced by DNA-damage response and environmental stress, was found to be significantly upregulated in miR-148a transduced WEHI-231 cell lines compared to controls via RNAseq and qPCR validation.[79] Dual luciferase assays validated the 3'UTR of *Gadd45a* as a direct target of miR-148a. To determine if the break in central tolerance observed upon overexpression of miR-148a was linked to the regulation of Gadd45a, the authors reconstituted IgM^b-macroself mice with bone marrow from *Gadd45a* $^{-/-}$ mice. These mice exhibited a very significant break in central tolerance with a higher percentage of peripheral B lymphocytes than was previously shown with miR-148a overexpression.[79] *Gadd45a* $^{+/-}$ B lymphocytes also had evidence of compromised central tolerance, albeit with lower frequency compared to B cells transduced with miR-148a, suggesting that precise regulation of *Gadd45a* is essential for proper selection of B cells. Together, these findings suggest that a miR-148a-Gadd45a axis plays an important role in the regulation of central tolerance.[79]

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PI3K-AKT Pathway

A number of studies have established a role for the PI3K cascade in central tolerance. PI3K gain-of-function mutations have been shown to contribute to a predisposition to a wide-range of autoimmune/inflammatory disease. A third of individuals in a well-characterized cohort harboring germline PI3Kδ mutations were found to develop autoimmune and inflammatory manifestations.[84] Mice bearing similar mutations recapitulate many of these phenotypes including increased high-avidity autoreactive B cells persisting in the periphery and increased autoantibody production.[85–88] Furthermore, deletion of PI3K negative regulators, including PTEN, has been shown to promote the survival of autoreactive B cells in various transgenic mouse models.[60,79,89,90]

The same group that has described the role of miR-148a in the regulation of MAPK pathway in B cells has also demonstrated miR-148a-dependent regulation of the PI3K pathway and its implications for central tolerance.[79] These investigators overexpressed miR-148a in an immature B cell line (WEHI-231) and reported a significant decrease in transcript and protein levels of PTEN and BIM. Dual luciferase assays using 3' UTRs from these transcripts suggested that both BIM and PTEN are direct targets of miR-148a. Furthermore, when WEHI-231 cells transduced with miR-148a are stimulated with anti-IgM, Annexin V and caspase 3 staining reveal reduced apoptosis in these cells.[79] These *in vitro* findings were consistent with the break in central tolerance observed in miR-148a transduced B cells *in vivo*. The authors also report a decrease in *Bim* and *Pten* transcript and protein levels following transduction of WEHI-231 cells with a miR-26a-Tg, but further studies are needed to evaluate the role of miR-26a in maintenance of B lymphocyte central tolerance.[79] Bone marrow reconstitution experiments using IgM^b-macroself mice as hosts revealed that BIM and PTEN play a critical role in maintenance of central tolerance, as B cells deficient for these proteins are able to escape negative selection. Together, these studies suggest that appropriate regulation of *Gadd45a*, *Pten* and *Bcl2l11* (the transcript that encodes BIM) by miR-148a is instrumental in B cell central tolerance, highlighting how a single miRNA can play a central role in regulation of multiple pathways during a complex biological process. The functional consequences of miR-148a-mediated regulation of the target genes that regulate BCR signaling and B cell survival was further highlighted by the investigators when they overexpressed the miRNA in MRL-lpr mice and observed greatly accelerated lethal autoimmune disease in this mouse model of lupus.[79]

The miR-17–92 cluster has been associated with the manifestation of multiple autoimmune conditions.[3,91,92] Utilizing a transgene to overexpress the miR-17~92 cluster in the IgM^b-macroself mouse model (introduced above), Lai et al. (2016) described the importance of this miRNA family in controlling central tolerance through PI3K regulation. The authors observed a complete restoration of peripheral B lymphocytes in animals in which this miRNA cluster is selectively expressed in B cells, suggesting that overexpression of miR-17~92 is sufficient to potentiate escape of negative selection.[60] These results were recapitulated in a second mouse model used to study central tolerance, IgHEL;mHEL.[60,93,94] The authors then dissected the contribution of individual miRNAs from miR-17~92 family to B cell development. Using a lentivirus vector to transduce HSCs, which were then used to reconstitute IgM^b-macroself hosts, miR-19 was identified

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as the specific miRNA from the miR-17~92 cluster that was responsible for the escape of central tolerance at the immature B cell stage.[60] As described above, the members of the miR-17~92 family of miRNAs, including miR-19, were shown to directly target *Pten*. Furthermore, *Pten* haploinsufficiency was sufficient to break central tolerance. In an effort to determine whether miR-19 controls central tolerance through PTEN dysregulation, the authors co-expressed miR-19 and *Pten* via lentiviruses in HSCs that were transferred into irradiated IgM^b-macroself hosts. The percentage of B cells that escaped central tolerance through the overexpression of both PTEN and miR-19 was comparable to the percentage of B lymphocytes that escaped central tolerance in the IgM^b-macroself mouse model controls.[60] These findings cement the importance of miR-19 control of central tolerance through PTEN regulation.

Section 4: miRNAs in peripheral BCR signaling and autoimmunity

Introduction to miRNAs in the periphery

As discussed with respect to B cell development, the BCR, composed of heavy and light chains, forms a signaling complex with the Igα and Igβ heterodimers on the B lymphocyte surface. The Igα/Igβ heterodimer plays a critical role in cytoplasmic signaling through the BCR and is responsible for activation of protein tyrosine kinases.[34] Following BCR stimulation and phosphorylation of costimulatory molecule CD19, multiple signaling pathways are engaged leading to B cell activation and internalization of the bound antigen.[33,95] The nature of BCR engagement and signaling for developing and peripheral B lymphocytes is largely the same, but the context of peripheral B cell antigen receptor signaling is critical for coordinating terminal differentiation of the lymphocytes, antibody-dependent responses, and affinity maturation. BCR antigen affinity and avidity, dictated by the corresponding binding sites on the IgH/IgL chains, establishes the strength of B cell activation.[28,96] As previously mentioned, well defined activation pathways downstream of BCR activation include the PI3K-AKT, RAS-MAPK, and NF-κB pathways.[33,95,97] Figure 1 details these pathways, and specific miRNAs associated with their regulation that will be discussed in detail below.

B cell activation plays a critical role in host response to infection, however, BCR recognition of self can lead to autoimmunity.[33,98,99] For that reason, B cell activation through the antigen receptor is a tightly regulated process. In this section, we will highlight ways in which regulatory miRNAs regulate PI3K-AKT, and MAPK/NF-kB signaling following BCR engagement in peripheral B lymphocytes.

As described earlier, miRNAs are required for B cell development within the bone marrow compartment.[51,56–58] In addition to reduced overall B cell numbers, ablation of *Dicer1* conditional allele using a *CD19*-cre driver impairs FO development, and increases transitional and MZ B cells.[100] In another study in which *Dicer1* was ablated under *CD19*-cre, the deletion results in a skewed BCR repertoire and features of autoimmunity, with female mice developing systemic lupus erythematous (SLE), Sjogren's, or scleroderma-like phenotypes.[100] These early studies of global miRNA ablation in the B cell lineages suggest that overall loss of miRNA regulatory potential leads to inappropriate selection and/or activation of peripheral B lymphocytes.

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A mouse model that contains a conditional deletion of *Dicer1* under *Aicda*-cre, led to ablation of the conditional *Dicer* allele at a later stage of B cell development than in the *mb1-cre and CD19-cre* models that were discussed earlier. B cells from *Dicer*$^{fl/fl}$ *Aicda*-cre mice have a lower amount of CSR, a reduction in antibody affinity maturation, and compromised plasma cell differentiation when mice are immunized. These mice also display a reduction in germinal center formation, both in the presence and absence of immunization.[101] Analysis of B cells from *Dicer*$^{fl/fl}$ *Aicda*-cre animals highlights that ablation of miRNA biogenesis using a cre specific to late stage B cell development and activated peripheral B cells impacts: B cell differentiation, germinal center reaction processes, and antibody production—all processes intimately dependent on appropriately tuned BCR signaling.

Analysis of B cells from SLE patients, a disease where pathology is well-established to be dependent on antibody mediated inflammation,[102] also reveals that autoantibody production in this disease is linked to loss of regulatory control by miRNAs. In a study that examined miRNA landscape using TaqMan global miRNA arrays in patient biospecimens stratified for being sera-positive for anti-nuclear, anti-double stranded-(ds)-DNA antibodies or both, the results indicate distinct miRNA profiles and transcriptional signatures withing each "sub-type" of SLE.[103] This study highlights the critical regulatory role of miRNAs in maintenance of proper B cell selection and activation and the possible failure in this type of post-transcriptional control to autoimmune pathogenesis. Beyond the global impact of miRNAs on fine tuning B cell activation and autoimmune susceptibility, we will highlight specific studies that have identified miRNAs involved in post-transcriptional regulation of the BCR signalosome.

PI3K-AKT Pathway

The PI3K-AKT pathway is activated downstream of the BCR and maintains B cell transcriptional programming. We will discuss how the miRNAs miR-29, miR-1246, and miR-148a target aspects of the PI3K-AKT pathway, and what implications this has on B cell signaling (Figure 1).

A recent study revealed the miR-29 family of miRNAs as a regulator of PI3K signaling in mature B lymphocytes in the periphery. The conditional ablation of *miR-29ab1* cluster coupled with the germline deletion of *miR-29b2c* (*mb1*-cre *miR-29ab1*$^{fl/fl}$ miR29b2c$^{-/-}$) in mice results in an increase in PTEN protein levels with a corresponding dampening of PI3K signaling in mature B cells as evidenced by a decrease in active AKT. Since the PI3K signaling cascade is so vital for maintenance of mature B cells, it is not surprising that these mice were found to have a significant reduction in splenic B cell numbers with a corresponding increase in apoptosis. Furthermore, the surviving FO B cells were found to have low plasma cell differentiation and an increase in CSR. Renilla-luciferase assays confirmed *Pten* as a target of miR-29. These phenotypes were rescued with a conditional deletion of one copy of *Pten (mb1*-cre *miR-29ab1*$^{fl/fl}$ *miR29b2c*$^{-/-}$ *PTEN*$^{fl/+}$).[104]

Another study that examined the role of miR-29 in B cell function, evaluated the impact of miR-29 loss on induction of collagen induced arthritis.[105] The authors showed that global loss of miR-29ab1 locus results in a global loss of splenic and thymic cellularity and has

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a profound impact on T cell differentiation. This likely contributed to the observed defect in germinal center formation. The authors also observed that deletion of miR-29a *in vitro* is associated with a reduction in B cell proliferation and antibody production, enabling them to make conclusions about the role of miR-29a in these cell-intrinsic processes.[105] In all, despite the findings that miR-29 loss was associated with protection against collagen induced arthritis, the study[105] is consistent with our conclusions[104] that miR-29 is a positive regulator of PI3K signaling (via repression of *Pten*).

SLE is an autoimmune disease driven by the dysregulation of multiple immune cell types that impacts multiple organ systems.[106] Multiple miRNAs have been shown to be differentially expressed in SLE patients.[106–108] A miRNA microarray performed on MACS enriched B cells from blood of SLE patients and healthy controls identified six miRNAs with significantly different levels of expression: miR-4286, miR-451, and miR-424 were upregulated and miR-1246, miR-122, and miR-877 were downregulated in B cells from SLE patients. The downregulation of miR-1246 was validated by RT-PCR, and bioinformatic analysis identified the transcription factor Early B cell factor 1 (EBF1) as a predicted target of miR-1246. Inhibition of miR-1246 in primary human B cells, allowed authors to assess EBF1 protein levels and confirm that this putative miRNA target was elevated relative to the control transfection group. Furthermore, flow cytometric analysis revealed that B cells upregulated activation markers CD40, CD80 and CD86 in the presence of the miR-1246 inhibitor. When primary human B cells were transfected with a miR-1246 mimic, there was a reduction in EBF1 and p-AKT as measured by western blot. In addition, the expression of activation markers CD40, CD80 and CD86 was blunted in cells transfected with the miR mimic.[107] As miR-1246 is not found in mice, mechanistic studies on the role of this regulatory RNA are, so far, lacking. The study by Lou et al. (2015) implicates low expression of miR-1246 in the dysregulation of B cells in SLE patients, and based on the results of the miR-1246 transfection experiments, it is likely that this miRNA contributes to the regulation of PI3K-AKT pathway, thereby fine-tunning B cell activation and proliferation. Evaluation of miR-1246 expression and function in other B-cell mediated autoimmune diseases may reveal whether the miR-1246-PI3K axis is a consistent player in the regulation of B cell responses.

Another miRNA that has been found to be elevated in a number of autoimmune diseases, including SLE, multiple sclerosis and rheumatoid arthritis, is miR-148 (previously discussed for its role in central tolerance).[103,109,110] In a screen of murine B cell subsets from spleen and bone marrow, and human blood-derived B cells, plasmablasts, and plasma cells, miR-148a was identified to have higher expression in plasmablasts and plasma cells and make up 30% of all miRNAs in these cell types.[111] miR-148 is also one of the most abundant and specific miRNAs in GC B cells.[112] When murine B cells are stimulated *in vitro* with LPS or anti-IgM, miR-148a is upregulated in a time dependent manner when measurements are made by northern blot and qPCR. miR-148a was found to target the 3’ UTR of *Pten*, and the transcription factors *Bach2* and *Mitf* which subsequently induce expression of *Blimp1* and *Irf4* to promote plasma cell differentiation and survival.[111] Utilizing a conditional knockout mouse in which the miR-148a allele is flanked with loxP sites, ablation was achieved by either *CD19*-cre/$^{+}$ or tamoxifen-inducible Rosa26CreERT2$^{+/-}$. The deletion of miR-148a results in a reduction of splenic plasma

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cells and plasmablasts and serum immunoglobulins (IgG, IgA, IgM). The study team also observed a reduction of $CD93^{+}$ long-lived plasma cells in the bone marrow of mice in which miR-148 was deleted selectively in B cells (assessed by RNA-Seq and confirmed using flow cytometry). Transcriptomics and metabolomics experiments revealed miR-148a deletion led to reduced oxidative phosphorylation, impaired glucose uptake, and altered chemokine expression. As noted by the authors, miR-148 expression coincides with upregulation of *Blimp-1* and helps reinforce plasma cell differentiation via repression of GC transcription factors *Bach2* and *Mitf*, along with reducing expression of proapoptotic proteins BIM and PTEN.[111,113,114]

NZB/WF1 strain is a murine lupus model in which females are more biased to disease, mimicking the known sex bias in lupus patients.[102] To explore sex-driven differences in miRNA expression, a TaqMan miRNA assay system was employed to compare miRNA expression between male and female NZB/WF1 splenocytes. miR-148a is observed to be highly expressed in splenocytes from female NZB/WF1 mice when compared to males, and is estrogen responsive, suggesting that expression of this miRNA in lymphocytes contributes to the observed sex-associated differences in lupus incidence in this murine model.[115] Along with the finding that miR-148 also promotes lupus-like disease in the MRL-lpr mice,[79] these data suggest that the miR-148-PI3K regulatory axis in critical in the regulation of BCR signaling and terminal B cell differentiation. These observations further indicate that miR-148 regulation of this PI3K-AKT signal cascade can contribute to autoimmunity in murine models.

Observations that miR-29, miR-1246, and miR-148a are found to be inappropriately expressed in autoimmune diseases and/or mouse models of lupus are consistent with the critical role these miRNAs play in the regulation of PTEN/PI3K and maintenance of proper B cell selection and differentiation.

NF-kB and RAS-MAPK Pathways

Upon BCR engagement, NF-κB and RAS-MAPK signaling pathways regulate B cell survival and proliferation. The microRNAs −185, −30, −29, −146a, and −155 have been shown to regulate these pathways downstream of BCR signaling.

BTK, described earlier, is a kinase downstream of BCR activation, that has been identified as a target of miR-185.[100] Ablation of *Dicer1* under *CD19*-cre, as previously discussed, was found to alter B cell differentiation and favor transitional and MZ differentiation versus FO B cells. A microarray analysis was performed on FO and MZ B cells from *CD19*-cre *Dicer1*$^{fl/+}$, and FO B cells from *Dicer1*$^{fl/fl}$ mice to identify miRNAs responsible for governing FO cell fate. Using microarray data, 31 miRNAs were found to be upregulated in FO B cells relative to MZ B cells and were validated by RT-PCR.[100] When multiple miRNAs are identified in microarray studies, *in silico* analysis is employed to predict potential miRNA target transcripts so that candidate miRNAs can be selected for downstream validation.[116] Using computational predictive binding approaches, Belver et al. (2010) identified the 3'UTR of *Btk* as a target of miR-185 for further study, but the role of the other 30 differentially expressed miRNAs identified in the course of this study that potentially regulate BCR signaling with respect to B cell differentiation remain to be

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explored.[100] To validate the role of miR-185 in BCR signaling, MACS sorted splenic B cells were transduced with retroviral vectors to overexpress miR-185. The overexpression of miR-185 results in dampened BCR signaling and a reduction in BTK expression. When splenic B cells are transduced to overexpress *Btk*, it mimics the phenotype of *Dicer1* ablation that skews differentiation towards transitional and MZ B cells supporting the role of miR-185 regulation of BTK levels.[100]

Primary human B cells were isolated from healthy donor PBMCs and stimulated in culture with LPS, anti-IgM, or IL-4 and the investigators were able to show that EphB2, an ephrin receptor tyrosine kinase, was upregulated during B cell activation and played an important role in B cell activation downstream of the BCR.[117] When EphB2 expression was reduced in human B cells using siRNA, stimulated B cells had an appreciable reduction in activation.[117] Further work to elucidate the precise mechanisms by which EphB2 regulates BCR signaling is still required. Current data indicate that the EphB2 induces Src-p65 and Notch1 signaling, and that this can impact NF-κB expression.[117,118] Using the miRWalk database miR-185, miR-661, miR-515–5p, miR-593, miR-204, and miR-211 were identified to have predictive binging sites to the *Ephb2* 3'UTR. After comparing miRNA expression between stimulated (LPS-treated) and unstimulated human B cells by RT-PCR, miR-185, miR-204 and miR-515p was found to have significantly reduced expression in activated cells. miR-185 had the most significant reduction and was subjected to downstream analysis, while miR-204 and miR-515p were not validated further.[117] Other investigators were able to show that miR-204 regulates EphB2 expression in murine hippocampal neurons, so despite not being subject to further experimental validation in B cells, it may, nonetheless, play a role in regulating a target relevant to BCR signaling cascades.[119] When Yu et al. (2014) transfected primary human B cells to overexpress miR-185, there was lower B cell activation indicated by lower proliferation and IgG production; and reduced EphB2 and NF-κB expression, determined by western blot. Further, when a *Renilla* luciferase vector containing the *Ephb2* 3'UTR was transfected alongside a miR-185 mimic into 293T cells, miR-185 reduced Luciferase reporter activity indicating that this miRNA can directly target the *Ephb2* 3'UTR.[117] Taken together, these studies identify miR-185 as a negative regulator of two tyrosine kinases critical in modulation of the NF-κB pathway downstream of BCR signaling.

A number of studies in humans have associated miR-185 with autoimmunity.[120,121] Global miRNA landscape profiling via TaqMan array in the serum of Myasthenia gravis (MG) patients from different disease subgroups and healthy controls implicated loss of miR-185 in disease pathogenesis.[120] Extracellular miRNAs were discovered and confirmed to be stable in various biofluids including serum. Profiling human serum samples permits the identification of miRNAs as potential biomarkers for disease and prognosis.[122] Although this study was limited by sample size, the results identified 32 differentially expressed miRNAs in MG patients and these targets were validated in a larger cohort using targeted qPCR. This validation confirmed the downregulation of miR-185 in MG patients compared to healthy controls.[120] A second study profiled miR-185 expression in circulating B cells of patients with dilated cardiomyopathy (DCM). B cells were targeted as they contribute to DCM by generating anti-heart autoantibodies (anti-adenine nucleotide translocator, anti-β1 adrenergic receptor, anti-myosin heavy chain, and anti-L-type calcium channel

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antibodies) that lead to heart fibrosis and injury. Measuring miR-185 transcripts using qPCR revealed that DCM patients have higher miR-185 expression than healthy controls, and that DCM patients could be stratified into miR-185 high and low groups, with miR-185-high group having better prognosis and survival.[121] Although an interesting association that is consistent with the role of miR-185 dampening BCR signaling, this study did not profile the contributions of other miRNAs and did not identify any specific mechanisms by which miR-185 impacts B cell signaling or activation in DCM patients.

Another miRNA, miR-30a was identified to regulate expression of the gene *Lyn*. LYN, a member of the SRC family tyrosine kinases that is engaged following B cell activation, is found to have reduced protein and mRNA expression by western blot and PCR in B cells enriched from the PBMCs of SLE patients.[123] Mice deficient for LYN have lower peripheral B cells, and with age, develop an autoimmune phenotype including high autoantibody titers, enlarged spleen and lymph nodes, and evident kidney inflammation.[124,125] Following a bioinformatic screen, five members of the miR-30 family (a-e) were identified to potentially target the 3'UTR of *Lyn*. Transfection of miR-30 family mimics into two human B cell lines, enabled investigators to identify miR-30a as the miRNA capable to reducing the expression of LYN protein by western and *Lyn* mRNA by RT-PCR. When a luciferase reporter construct containing the *Lyn* 3'UTR was transfected along with miR-30a mimic and compared to a control miRNA, miR-30a was shown to reduce luciferase activity, indicating it could effectively bind the 3'UTR of *Lyn*. B cells isolated from SLE patient blood were shown to have reduced LYN expression and elevated miR-30a expression with a negative correlation between miR-30a gene expression and LYN protein expression measured by RT-PCR and western blot respectively.[126] Altogether, these studies suggest that miR-30a regulation of LYN, a tyrosine kinase linked to MAPK and NF-κB signal transduction, is an important axis in normalization of BCR signaling and loss of this regulatory function contributes to autoimmunity.

Data from human B cells has shown that *Aicda* has miR-29b binding site in the 3'UTR that is not conserved in mice. Over expression of miR-29b in B cells from human tonsils results in reduced *Aicda* transcripts and reduced AID protein expression. This overexpression of miR-29b in human B cells also results in reduced *Myc* gene expression and provides an association in human cells for the miR-29 family regulating the MAPK pathway.[127] The role of miR-29 in immune regulation, and particularly in autoimmune disease progression and malignant transformation of B cell lymphomas is reviewed here.[128] Given that a number of studies implicate miR-29 in regulation of AKT, MAPK and MYC in human disease, along with mechanistic studies on the regulation of PI3K signaling by miR-29, a picture of the pleotropic role of this miRNA cluster in lymphocyte regulation emerges.

When miRNA, mRNA, and ChiP-seq were utilized contemporaneously to define miRNA expression in FACS sorted mouse and human tonsil hematopoietic cells, miR-146a was found to be upregulated in human GC B cells.[112] Additional microarray and gene expression studies of human patients with autoimmune disease such as rheumatoid arthritis, multiple sclerosis, Sjögren's syndrome, and psoriasis revealed that PBMCs, synovial fibroblasts and tissue, salivary glands, and skin samples from autoimmune patients had upregulated miR-146a expression relative to healthy controls.[129–132] To explore the role

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of miR-146 overexpression in autoimmune disease pathogenesis, a transgenic mouse that carries a miR-146a allele was generated. Young mice with this allele develop spontaneous autoimmunity reminiscent of human autoimmune lymphoproliferative syndrome (ALPS) in which individuals have abnormal regulation of lymphocyte homeostasis.[83] miR-146a transgenic animals had greatly enlarged lymph nodes and spleens, along with elevated serum IgG, and increased frequency of GC B cells. Downregulation of FAS on B cells in response to sustained expression of miR-146a was likely responsible for the observed dysregulation of B cells and analysis of the 3' UTR and luciferase assays suggest that *Fas* is a direct target of miR-146. Microarray profiling on sorted B cells was used to assess gene expression of miR-146a targets, and several members of the NF-κB pathway were identified among miR-146a targets (as was AP1 and STAT signaling pathways). Furthermore, B cells from miR-146a transgenic mice proliferate to a lesser extent than those from wild-type animals when stimulated with LPS in culture.[83] LPS stimulation initiates a BCR independent activation of B cells via Toll like receptor 4 (TLR4) that nonetheless engages both the NF-κb and Ras-MAPK pathways independent of PI3K signaling. *In vitro* stimulation of B cells via TLR4 is often used to engage signaling networks relevant to antigen receptor signaling.[133] The reduced proliferative capacity of B cells overexpressing miR-146a is consistent with a reduction in NF-κB signaling.[83] While interpretation of inflammatory phenotypes from these transgenic mice is hampered by the fact that miR-146a is globally overexpressed, the study implicates miR-146a is regulation of BCR signaling of mature B cells. Moreover, it is clear that miR-146a is upregulated in autoimmune conditions, and it is possible that dysregulation of the NF-κB signaling pathway results in altered proliferation capacity downstream of BCR signaling based on the *in vitro* LPS stimulation conditions. Dysregulation of the NF-κB pathway may also lead to irregular lymphocyte division and frequencies resulting in ALPS like conditions in the transgenic mouse model. Additional work will be required to further elucidate all the mechanisms and specific targets by which miR-146a regulates B cells and BCR signaling.

miR-155, a miRNA that has been previously implicated in a number of B cell malignancies, has been shown to play a critical role in GC B cells and in regulation of terminal differentiation of B cells into plasmablasts.[134–137] Using IgHEL transgenic B cell adaptive transfer system, with cells either wild-type for the *Bic*/miR-155 locus or lacking the miRNA, the authors were able to show that miR-155 deficient plasmablasts displayed reduced proliferation and increased apoptosis. Transcriptome analysis revealed miR-155 regulated DNA replication, and that miR-155−/− B cells had reduced expression of E2F1, E2F2, and Myc.[134] Myc is a downstream target of the MAPK pathway. Profiling of miRNAs from lymphocytes of murine lupus models, including MRL-lpr; B6-lpr; NZB/WF1 identified miR-155 as one of the miRNAs highly upregulated in splenic B cells in these autoimmune mouse models.[81,115] Additional studies found that miR-155 is also upregulated in splenic B cells following stimulation with LPS and IL-4. Predicative algorithms identified a binding site for miR-155 in the 3'UTR of *Aicda* and *in vitro* class-switch recombination assays and transfections with luciferase reporter constructs containing the *Aicda* 3'UTR further demonstrate that miR-155 contributes to the regulation of AID expression. Using an AID-GFP transgenic mouse model with impaired miR-155 binding in the 3'UTR of *Aicda*, the loss of the binding site within *Aicda* leads to increased CSR *in vitro* after stimulation

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and a rapid increase in *Aicda-Gfp* transcripts and GFP fluorescence when measured by flow cytometry.[136] Transgenic *Aicda-Gfp* mice and *Aicda-Gfp* mice with a mutated *Aicda* 3'UTR were immunized with NP-CGG; 21 days post immunization the mutant Aicda-Gfp mice had increased GFP expression in GC B cell populations.[6,136] This increased AID-GFP expression persisted in the mutant transgenic mice, but not in the control mice that contained the intact *Aicda* 3'UTR, indicating that miR-155 directly regulates *Aicda* in post-GC B cells. The *Aicda-Gfp* mutant mice also display impaired affinity maturation after NP immunization, demonstrating physiological relevance of this miRNA-target interaction.[136] Collectively, these data from several mouse models identify roles for miR-155 in the regulation of Myc, DNA replication, and AID upon B cell activation.

All of the aforementioned microRNAs, miR-185, miR-30, miR-29, miR-146a, and miR-155, have data to support their role in fine tuning BCR signaling via different mechanisms in which they reduce the expression of genes critical to B cell activation and proliferation. Further, these miRNAs work in concert with those that regulate the PI3K-AKT signaling pathway to ensure appropriate BCR downstream activation (Figure 1). Due to these critical roles in regulating components of the BCR signal cascade, any kind of alteration in miRNA expression levels can lead to inappropriate BCR signaling that permits the development of autoimmunity and self-reactive B cells.

Section 5: Future directions and outlook for miRNAs and the BCR

Mouse models have enabled mechanistic studies into the role that miRNAs play in orchestrating B cell development in bone marrow and regulating peripheral B cell differentiation and responses.[56–58,101] Much of B cell development, differentiation and function is intimately linked to BCR signaling, and we have discussed multiple miRNAs in this review that target components of the BCR signal cascade in early B cell development, central tolerance, and peripheral B cell differentiation. Despite the results of many studies, gaps remain in our knowledge of the precise targets of miRNA regulation and how these specific targets impact signaling downstream of the antigen receptors, with respect to individual pathways involved.

Gene-targeted mouse models in which miRNAs are either deleted or over-expressed, particularly those models that enable manipulation of miRNAs in a cell-type specific manner, are extremely informative for identification of physiologically relevant miRNA-target interaction. A major limitation of these models is that they fail to take into account the pleiotropic nature of miRNA regulatory pathways, even within a given cell type. While we have highlighted some dominant miRNA-target interactions that can be pivotal in the regulation of BCR signaling, it is just as likely that loss of a given miRNA will contribute to dysregulation of multiple pathways, obfuscating results and interpretations. Overexpression models or utilization of miRNA mimics either *in vitro* or *in vivo* have provided additional insights into miRNA regulation of the BCR signalosome. But again, these models have limitations as they often exceed physiological parameters of miRNA expression, and the impact of overexpression is often dependent on the abundance of binding sites, genomic context and accessibility within the 3' UTRs and availability of targeted transcripts. Large miRNA arrays and next-generation sequencing technologies have

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proved valuable for identifying miRNAs that are differentially expressed, particularly when comparing control and disease states. However, downstream of this analysis, miRNAs of interest are often selected based solely on predicative binding. Incorporation of crosslinking-immunoprecipitation methods such as PAR-CLIP, Ago HITS-CLIP and Halo-Ago2 pull down, allows for characterization of miRNA target relevance *in vivo,* enabling identification of the most promising miRNA-target interactions.[138–140] Distinguishing the most critical miRNA-target relationships in sorted B cells will provide a strong foundation for elucidating the mechanisms of miRNA gene regulation in B cells and BCR signaling.

Future work, as it pertains to BCR signaling, should consider miRNA targets in the context of the signalosome network and validate transcript and protein expression down and upstream of suspected targets. Utilizing systems in which miRNA binding sites within 3'UTRs have been targeted to prevent specific miRNA binding will provide confident assessment of miRNA-target relationships. With improved gene editing tools such as CRISPR, generation of precisely edited animal models with only minimal alterations solely in the miRNA binding motifs is now more feasible than ever. In parallel, next generation transcriptomic and proteomic platforms enable assessment of global effects of individual miRNAs in cell lines and gene-targeted animal models – allowing investigators to appreciate how altering these regulatory networks impacts cellular programs. Integration of precise genetic editing with approaches like transcriptomics and proteomics will continue to advance our understanding of the role of individual miRNAs in B cell biology.

As we have considered in the context of this review, post-transcriptional regulation of BCR signaling pathways by miRNAs plays an important role in autoimmune disease pathogenesis and progression. While the contribution of individual miRNAs in disease etiology remains to be elucidated, given the stability of these short non-coding RNAs, they have inherent potential in clinical applications.[122] Dysregulated miRNA expression could have diagnostic or prognostic value, thereby permitting early intervention using traditional therapies.[141]

Improving our understanding of miRNA contributions to autoimmune disease risk and pathogenesis will further advance translation of laboratory findings to the clinic as therapeutic approaches that dampen expression of individual miRNAs become available. A number of companies are testing miRNA-focused therapeutics in the context of cancer and inflammatory disease (SantarisPharma, MiRagen, etc.), with several in clinical trials.[142] In the cancer field, miRNAs are currently being explored to alter tumor gene expression to make drug-resistant tumor cells sensitive to chemotherapeutics.[143,144] Targeted delivery of miRNAs to alter tumor gene expression to render the cancer susceptible to the delivered chemotherapeutic represents an opportunity to exploit miRNAs to enhance clinical outcomes.[143] The applications of miRNAs being employed to regulate B cell gene transcription to prevent inappropriate activation in the case of autoimmunity holds promise. Although delivery of miRNAs to exploit their therapeutic potential represents a challenge, numerous methods are being tested in current miRNA-focused therapeutics at both the developmental and clinical trial stages (reviewed by Chakraborty et al. 2021).[142] Conversely, the impact of endogenous miRNA regulation of BCR signaling could be altered by inhibiting miRNAs or using approaches to target their degradation by exploiting cellular processes such as target RNA-directed miRNA degradation (TRMD).[145] As implementation

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of these miRNA-targeted therapeutics becomes a reality, fundamental understanding of the many nodes in BCR signaling that are targeted by miRNAs will be critical to fully harness the potential for these approaches.

The studies highlighted in our review reveal a diverse array of mechanisms by which individual miRNAs contribute to the regulation of BCR signaling during early B cell development and in the course of peripheral differentiation. Given the central role of B cell antigen receptor in the survival, proliferation and indeed in the very identity of the B cell lymphocytes, it is not surprising that regulation of BCR signaling by miRNAs plays a critical role in the modulation of B cell responses and in the genesis of autoimmune diseases. Given the clinically relevant tools being developed for deployment of miRNA-based therapeutics, and the increased accessibility of genetic engineering in murine models and human cell lines, advancement of our understanding of precisely how miRNAs regulate BCR signaling can have tremendous implications for future therapeutic approaches.

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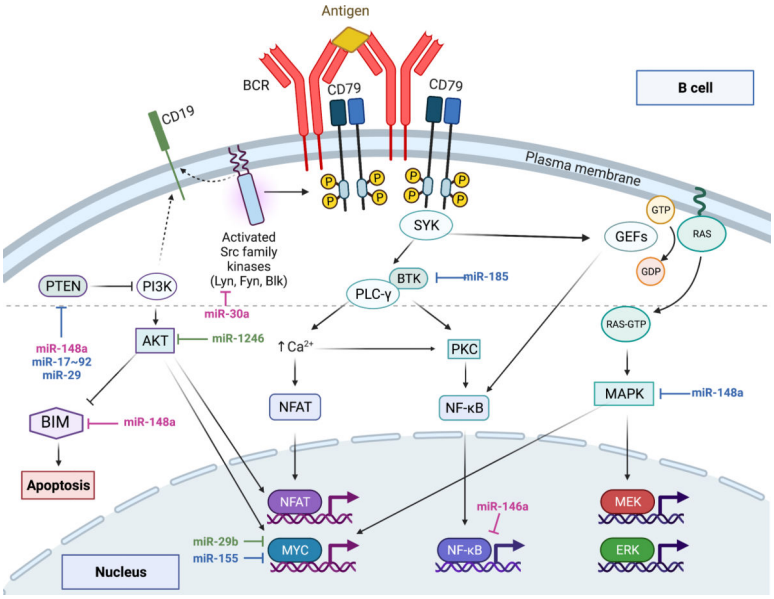

Figure 1: Regulation of B cell receptor signaling by microRNAs.

The PI3K-AKT, NF-κB, and RAS-MAPK signaling pathways downstream of the BCR are depicted.

MiRNAs discussed in this review are highlighted in association with relevant targets within the signaling networks. miRNAs in blue were those identified in murine studies, miRNAs in green were identified in human studies, and those in pink have data from both murine and human studies that highlight the role of these miRNA in the regulation of BCR signaling, B cell development, or autoimmunity.